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Evaluating the role of the bovine vaginal microbiome in neonatal and maternal health outcomes

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Evaluating the role of the bovine vaginal microbiome in neonatal and maternal health outcomes

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The dam vaginal microbiota is the first major microbial inoculating community within the neonate. The composition of the dam vaginal microbiota has implications in calf commensal microbiota development. Alterations of the dam microbial community prior to parturition could alter inoculating communities and immune responses in both the dam and calf. Thus, authors aimed to elucidate the microbial community composition of the bovine dam vaginal and calf nasal microbiota post-partum after utilizing betadine lavages (BL). The dam vaginal and calf nasal microbial communities and immune responses were evaluated at 0-, 15-, 30- and 60-day post-partum. Microbiota composition of the dam haircoat, udder, and IgG in the colostrum/calf sera were also evaluated at day 0. Serial BLG prior to parturition did not alter the alpha diversity of the dam-vaginal microbiota but did alter the calf-nasal microbiota at parturition (P = 0.03). Dams receiving BLG prior to calving had increased colostrum IgG concentrations compared to CON dams (P =0.04). These results suggest physiological insults (BLG) prior to parturition led to an increased immune response which altering dam colostrum IgG. Thus, neonatal colostrum consumption could drive immune responses against inoculating bacteria resulting in differing nasal microbial communities between treatment groups. The beta diversity of the calf nasal

microbiota was significantly different at day 0 compared to all other timepoints ($P = 0.006$). The calf nasal beta diversity at day 15 was similar to day 30 ($P = 0.38$) but significantly different compared to day 60 ($P = 0.006$). There was no effect of time on altering the alpha ($P = 0.60$) or beta ($P = 0.06$) diversity of the dam vaginal microbiota. The calf nasal microbiota was different from the dam vaginal microbiota at all timepoints post-partum, regardless of treatment. At day 15, the alpha and beta diversity of calves was altered compared to day 0, suggestive of a reinoculation timepoint between 0 and 14 days of age. Together, this data contributes to the paucity within beef cattle dam-calf post-partum microbiota literature and provides directionality for future research objectives within this field.

DEDICATION

I would like to dedicate this dissertation to all the young girls who, like myself, have a passion for animals and a love for science. May you find a path in life that is as fulfilling as the one I have found.

&

"To the stars that listen, and the dreams that are answered."

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CHAPTER I

BOVINE NEONATAL MICROBIOME ORIGINS: A REVIEW OF PROPOSED MICROBIAL COMMUNITY PRESENCE FROM CONCEPTION TO COLOSTRUM

This chapter of the dissertation has been accepted for publication in Translational Animal Science in May 2023: Messman, R.D. and C.O. Lemley. 2023. Bovine Neonatal Microbiome Origins: A review of proposed microbial community presence from conception to colostrum. Trans. Anim. Sci. 7(1) doi: 10.1093/tas/txad057

Introduction

Within the last decade, there has been a massive influx of bovine reproductive microbiome research introduced to the literature. The complex nature of biome data, combined with differing interpretations, has led to numerous questions regarding the role and relevance of the bovine reproductive tract microbiome. Authors agree the potential for microbial communities to modulate fertility within the dam exists, with recently published articles focus on eliciting mechanisms by which these fertility modulations occur (Srinivasan et al., 2021; Adanane & Chapwanya, 2022). However, literature exploring how the pre-existing proposed inoculation times affect the developing conceptus is minimal. Thus, this review will focus on literature proposing microbial communities and their roles during the stages of conceptus development. Additionally, the physiological insult of parturition on both neonatal and maternal microbiomes will be explored to address paucities within the literature and to discuss how passive transfer through colostrum and environmental factors can contribute to the establishment of microbial

communities. The objective of this review is to holistically approach the current literature to identify limitations and key connections that could provide direction for future research within the field of bovine reproduction.

Authors would like to acknowledge that this area of research is fairly novel in livestock. Thus, the studies incorporated in this review are pioneering livestock microbiome collection methods, analysis, and interpretations. In human literature, a uterine (Garcia-Grau et al., 2019), placental (de Goffau et al., 2019), or amniotic (Lim et al., 2018) microbiota in healthy women has still not been confirmed. This review acknowledges the limitations of the livestock current literature but reiterates the importance to highlight current results to drive future research.

Conception

The Maternal Microbiome

The female reproductive tract is the site of copulation, sperm deposition, fertilization, embryogenesis, and gestation within the bovine. Thus, commensal microbiota must be considered when evaluating inoculation of the growing conceptus. Researchers have been working to associate microbial composition within the female reproductive tract to fertility (Ault et al., 2019; Deng et al., 2019; Messman et al., 2020). These characterization studies have produced data representative of a dynamic healthy reproductive tract microbiota, and shown clear markers for dysbiosis, such as decreased diversity and loss of heterogeneity (Galvao et al., 2019). Recent characterization research has primarily focused on the vaginal and uterine microbiota; thus, these biomes will be the focus for potential female microbial contributions during conception.

Swartz et al. (2014) drove further research in the field by comparing human vaginal microbiota to bovine and revealing a stark contrast. Humans have a *Lactobacillus* spp.

dominated reproductive tract microbiome; the lactic acid produced creates an acidic environment (pH < 4.5) is effective in preventing pathogen colonization (Stout et al., 2020). However, the bovine vaginal environment has a near neutral pH (7.3), creating an environment where the phyla Bacteroidetes, Fusobacteria, and Proteobacteria dominate (Swartz et al., 2014). Additional studies in non-pregnant *Bos taurus* cows also found that the phyla Tenericutes and Firmicutes present within the vaginal tract (Messman et al., 2020).

Due to the vulva lying directly ventral to the anus, contamination within the vaginal tract with fecal material is highly likely. The cattle fecal microbiota is dominated by Firmicutes, Bacteroidetes, and Proteobacteria, respectively (Minseok & Wells, 2015). This clear overlap in shared phyla demonstrates the role of fecal contamination in the establishment of a residential vaginal microbiome, but the consistent presence of Fusobacteria and Tenericutes at varying ratios is noteworthy.

Fusobacteria spp. are gram negative, non-spore forming, obligately anaerobic (survive in low O^2 environments > 8%) bacilli that are ubiquitous in the oral cavities of humans and animals (Brennan & Garrett, 2019). *Fusobacteria* spp. are mutualists within oral biofilms playing a role in structural support and binding secondary colonizers (Kolenbrander et al., 2010). Thus, the *Fusobacteria spp.* present within the vaginal tract could play similar role in biofilm formation, but an interesting linkage between *Fusobacteria nucleatum* and pre-term delivery, still birth, and late term abortions has been recently proposed within human literature.

There is an association between periodontal disease in pregnant women and preterm delivery (Offenbacher et al., 1996). The proposed theory suggests transient bacteriemia, due to chronic periodontal disease, combined with an increase in blood flow to the uterus during pregnancy drives hematogenous spread of bacteria to the placenta resulting in fetal inoculation

during late gestation (Meschia, 1983; Han et al., 2004). *F. nucleatum* is a causative agent of periodontal disease in humans and the bacteria has been identified in amniotic fluid of 10-30% of women preterm labor (Hill et al., 1998). To strengthen this association, Han et al. (2004) intravenously injected pregnant mice during late gestation with *F. nucleatum* to evaluate fetal outcomes. The injection resulted in preterm birth (entire litter stillborn) or full-term delivery with live (non-viable) and dead fetuses. *F. nucleatum* was isolated from injected mice's placentas, amniotic fluids, and fetuses after delivery (Han et al., 2004). This study is notable because it introduces potential pathogenic roles of *Fusobacteria spp.* can cause during gestation. A Fusobacteria and Bacteroidetes dominated reproductive tract microbiota were associated with cows that eventually developed reproductive disease; these phyla synergistically cause reproductive disease via virulence and growth factor expression (Ong et al., 2021). Deeper metagenomic sequencing evaluating the species presence and virulence factors expressed is needed to determine Fusobacteria's exact role within the bovine vaginal microbiota, but potential for uterine contamination as an opportunistic pathogen and subsequent negative consequences to fertility should be considered.

Tenericutes are considered commensal bacteria within the lower reproductive and urogenital tract, but their presence within the uterine environment can lead to adverse reproductive outcomes (Santos et al., 2011; Ong et al., 2021; Adanane & Chapwanya, 2022). The genera of *Ureaplasma* and *Mycoplasma* spp. are of particular interest as they lack a cell wall and typically exist within mammalian biomes as opportunistic pathogens (Santos Jr. et al., 2021). Moreover, these genera can be sexually transmitted between animals via secretions, semen, seminal plasma, and preputial and vaginal mucus (Miller et al., 1994). Thus, bacteria transfer is of concern when using assisted reproductive technologies (Crane & Hughes, 2018).

Ureaplasma diversum has been shown to cause granular vulvovaginitis syndrome in female cattle. This infection causes acute inflammation within the reproductive tract, tissue damage, and decreased fertility (endometritis, spontaneous abortion, early embryonic death) (Nascimento-Rocha et al., 2017; Santos Jr. et al., 2021). *U. diversum* is commonly isolated within placental tissue, lungs, and abomasal fluid of late abortion fetuses and neonates post-mortem (Anderson, 2007; Gagea et al., 2006). Thus, the presence of Tenericutes at high ratios within the vaginal microbiome of cows should be considered a risk-factor for subsequent infection within the dam or fetus. However, no physiological mechanism by which *Ureaplasma* or *Mycoplasma* spp. proliferate within a eubiotic system has been elicited.

Previous research hypothesized Tenericutes ascend from the vaginal tract to uterine body during estrus, or artificial insemination, when the cervix is dilated (Santos Jr. et al., 2021). *U. diversum* is an obligate intra-cellular pathogen capable of infecting endometrial cells and altering prostaglandin production by increasing PGF2a production and decreasing PGE2 (Kim et al., 1994). These studies provide evidence that Tenericutes have the potential to affect fertility via both virulence and alterations of hormone concentrations with the uterine environment. To conclude, the presence of these opportunistic pathogens within vaginal biofilms should be further explored, especially regarding infertility or persistent infections within female cattle. Figure 1 shows relevant bacteria within bovine reproduction that could be attributed to both negative and positive outcomes.

The Paternal Microbiome

The paternal microbiome is often overlooked in female reproductive microbiome research. However, the spread of bacteria from the male to female through coitus or within the ejaculate is well reported (Givens, 2008). Thus, implications of introduction of the native male

biome to the female reproductive tract should be further explored regarding colonization, immune response, and fertility.

Wickware et al. (2020) characterized the microbiome of the epithelial surface microbiome of the penis and prepuce in 92 healthy post-pubertal bulls via 16S rRNA sequencing. The dominant phyla within the bull penis/prepuce included Firmicutes, Fusobacteria, Bacteroidetes, Proteobacteria, and Actinobacteria. These phyla overlap with dominant phyla within both the cow vaginal tract and fecal matter (Wickware et al., 2020; Minseok & Wells, 2015; Swartz et al., 2014). Authors concluded the soil, feces, urine, and the cow's vagina likely contribute to the external epithelial surface biome of the penis and prepuce in the bull (Wickware et al., 2020). These conclusions further implicate the role of the environment and nutrition (feces produced) in colonization of the bovine reproductive tract.

Moreover, the microbial composition of bull ejaculates is of interest considering the site of semen deposition is the uterine body in cattle artificial insemination (AI). Due to the introduction of AI, semen collection and processing techniques introduce a secondary source of ejaculate contamination (Sannat et al., 2015). To compensate for bacterial contamination, antibiotics are commonly added to cryopreserved ejaculate, but bacteria can still be isolated from thawed bull semen samples (Zampieri et al., 2013). The presence of bacteria within an ejaculate does not equate infection or decrease sperm quality (Baud et al., 2019), but the effects of bacteria within the uterine environment of the female is unknown. Cojkic et al. (2021) evaluated ejaculates from healthy Holstein bulls (n =18); ejaculates were collected, extended, and frozen in liquid nitrogen until DNA extraction for 16S rRNA analysis. The most common genera found in the ejaculates included *Porphyromonas*, *Fusobacterium*, and *Ruminococcaceae*; these findings agree with the Wickware et al. (2020) showing overlap in phyla presence within the

penis/prepuce and the ejaculates of bulls (Cojkic et al., 2021). Despite an increase in research focused on associating male fertility with the paternal microbiome, there is limited literature evaluating effects of the paternal microbiome colonization in the maternal reproductive can impact the conceptus during gestation.

The Embryonic Microbiome

In summation, the maternal and paternal reproductive microbiomes likely contribute to the uterine environment pre- and post-conception. The roles of specific microbes in modulating fertility are not well established, but bacteria, such as *U. diversum*, is capable of attaching to sperm and endometrial cells (Lingwood et al., 1990). Thus, the likelihood of microbes within the ampullary-isthmus junction during conception is likely. To the authors' knowledge, microbial characterization research within the bovine oviduct has not been performed to date.

Exposure to microbes during the embryonic stage is not well defined in bovine research. In cryopreserved embryos, both bacterial and fungal isolates were found, but the relevance of these findings to in-vivo conception is negligible (Bielanski et al., 2003). Moreover, contamination of in-vitro fertilization culture media is associated with negative outcomes for the embryo (Borges & Vireque, 2020). However, the central dogma of a sterile environment within the reproductive tract is controversial due to the development of advanced culture independent methodologies, such as 16S rRNA sequencing, shotgun sequencing, and metagenomic sequencing (Wang et al., 2022; Perez-Muñoz et al., 2017). An interesting hypothesis within recent human literature is that microbial populations within the oviduct could have epigenetic effects on the embryo (El Hajj & Haff, 2013). Specifically, bacterial pathogens can be considered epimutagens that can reshape genomes to cause lasting effects within the embryo (Borges & Vireque, 2020; Bierne et al., 2012). Thus, the susceptibility of embryos and the

maternal environment to microbial modulations poses an interesting hypothesis, that the microbial environment is integral in conceptus programming starting at fertilization.

Gestation

Historically, the mammalian conceptus was regarded as sterile with the placenta serving as a physiological barrier preventing microbial colonization (Funkhouser & Bordenson, 2013; Escherich, 1886). However, through decades of research in reproductive physiology, the likelihood of a completely sterile uterine environment during the mammalian pregnancy is low. Pregnancy modulates the maternal circulatory system, specifically there is an increase (30-40%) in blood volume resulting in increased cardiac output (30%) and increased blood flow (16%) to the uterus during late gestation (Rosenfield, 1984). These compensatory mechanisms of the circulatory system are a result of the increased nutrient demand of the growing conceptus as pregnancy progresses (Vonnahme et al., 2013). There is a direct correlation between uterine placental blood flow and placental nutrient uptake; moreover, increased blood flow also increases delivery of circulating hormones, cytokines, metabolites, and microbes to the placental vascular bed (Hsu & Nana, 2014; Rosenfield, 1984). Current literature supports the theory of microbial inoculation of the uterus via hematogenous route in post-partum dairy cattle (Jeon et al., 2017), pregnant mice (Fardini et al., 2010), and humans (Katz, 2009). Thus, sterility in the uterus throughout gestation is improbable, but the question remains of the inoculating microbe's role in conceptus development.

Within human research, the initial microbial colonization of the neonate is considered the most important determinant of future host-microbe interactions that can modulate an individual's risk for non-communicable disease (Collado et al., 2016). Thus, current bovine microbiome research has begun to explore the microbial presence during gestation to investigate the critical

role of microorganisms in fetal development ([1]Amat et al., 2022; Hummel et al., 2022). Throughout gestation, the major physiological changes that are occurring in the dam, growing conceptus, and uterine environment attributes to the potential for conceptus exposure to a dynamic community of microorganisms throughout gestation (Fig 2).

Early Gestation

A recent study by [2]Amat et al. (2022) characterized the relative abundance and microbiome composition in the amniotic fluid (1855 OTU), allantoic fluid (2704 OTU), intestine (1323 OTU), and placental cotyledonary tissue (1347 OTU) in 83-day old calf fetuses collected via ovariohysterectomy. Interestingly, only 55 OTU were shared within these tissue samples, but the overlap represented the dominating phyla of bacteria ([2]Amat et al, 2022); these characterizations indicate an overall dominant microbial community within the reproductive tract, but OTU fluctuations within individual biomes is highly likely. A diverse and relatively unique microbial population was found in all four fetal samples. Within all samples, Proteobacteria (54.8%), Firmicutes (16.3%), and Actinobacteriota (13.7%) were the most abundant ([1]Amat et al., 2022). Interestingly, these microbial populations are consistent with the common phyla found within the vaginal microbiota (Swartz et al., 2014; Ault et al., 2019; Messman et al., 2020). The presence of *Actinobacteriota* was greater in the intestine (5.5%) and placenta (3.2%) compared to the allantoic (0.5%) and amniotic (1.0%) fluids ([2]Amat et al, 2022). Authors hypothesized that there is a unique microbiota between fetal intestine, placenta, and placental fluids due to differences in physiological, biochemical, and immunological properties that vary between sites ([2]Amat et al, 2022). This observation is consistent with general microbial principles that discuss microbial preferences for a certain environment based on species, strain, and function (Keller & Zengler, 2004). The observation of distinct microbial communities this

early in gestation, indicates an even earlier inoculation timepoint. Interestingly, similar microbial populations were identified in calves harvested later in gestation. This leads authors to question if the microbiome is developing with the conceptus instead of inoculating at a specific timepoint.

Mid Gestation

In a study by Guzman et al. (2020), calf tissues were harvested at 5 (n = 4), 6 (n = 4), and 7 (n =4) months of age after slaughter of dam. Briefly, the uterus, with the placenta and fetus, was removed 35-45 minutes post-slaughter; all tissue samples (amniotic fluid, meconium, ruminal fluid, ruminal tissue, cecal fluid, and cecal tissue) were collected within the abattoir utilizing consistent and sterile techniques. Contamination control samples were cross referenced to account for contamination through the collection and analysis process. In total, 559 bacterial exact sequence variants (ESVs) and 1736 archaeal ESVs were identified. Across all samples, Proteobacteria (32%), Firmicutes (31%), and Actinobacteria (26%) were the most dominant bacterial phyla; Euryarcheota (88%), Crenarchaeota (6%), and Kararcheota (5%) were the dominant phyla within the archaeal ESVs (Guzman et al., 2020). Moreover, the dominant phyla in the amniotic fluid were different than the calf gastrointestinal tract tissues; this is consistent with the findings in 83-day old calves ([2]Amat et al, 2022). Guzman et al. (2020) also demonstrated a temporal change from 5 to 7 months of gestation within the abundance of fetal calf microbial communities. Together, these suggest the fetal calf's gastrointestinal microbiome diverges from that of the amnion during gestation, and there are well established and distinct microbial communities within different fetal sites by 5 months of gestation.

This study contributes to the building literature that refutes the sterile womb hypothesis ([2]Amat et al, 2022; Guzman et al., 2020; Husso et al., 2021; Bolte et al., 2022). Interestingly, research is primarily focused on determining the inoculation timepoint, but as this review has

demonstrated microorganisms are present within the reproductive tract prior to conception. Thus, future research should focus on how the physiological shifts in environmental conditions within the reproductive tract during gestation can impact microbial growth. Unfortunately, the current literature most commonly highlights phyla. Taxonomic identification to the phyla level is considered more reliable, but fails to highlight specific characteristics of bacteria living within that environment. Thus, there is likely physiological restraints (pH, oxygen availability, nutrient sources, etc.) that prevent similar species from colonizing within these locations leading to the microbial discrepancies between fetal and placental sites.

Late Gestation

A recent study by Husso et al. (2021) evaluated the amniotic fluid and meconium microbiome in full-term Belgian Blue calves (n = 23) that were delivered via Cesarean section. Interestingly, the amniotic fluid samples had similar (P = 0.17) 16S rRNA gene copies as controls albeit the OTU identified were different; authors commented that it is likely this low microbial biomass has little impact. Moreover, the amniotic and meconium microbial profile was dominated by Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria in agreement with the previous studies in early gestation ([2]Amat et al, 2022; Guzman et al., 2020). Moreover, only 5 of 24 meconium samples were successfully cultured, and all amniotic fluid samples were culture negative. While culture limitations are clearly defined, one could inquire about the physiological status (dead vs. alive) of the bacteria identified using the 16S technique.

Gestational Inoculation vs. Maturation Hypothesis

Upon review of key studies identifying bovine fetal inoculation throughout gestation, there are interesting similarities that warrant further discussion. Firstly, all these studies

throughout gestation were conducted in different countries (United States, Australia, and Belgium) using different collections techniques (ovariohysterectomy, slaughter/harvest, and Cesarean section) and varying levels of contamination controls ([2]Amat et al, 2022; Guzman et al., 2020; Husso et al., 2021). However, despite these differences the microbial populations identified in the amnion and fetal gut (rumen/small intestine tissue) were similar at the phyla level. This suggests a commensal microbial population present in the reproductive tract during gestation. Interestingly, there was a decrease in microbial abundance (OTU/ESV/ASV) throughout bovine gestation with little notable microbial abundance close to parturition (Husso et al., 2021). This leads authors to speculate microbial inoculation throughout gestation may be less likely compared to microbial maturation.

Overlap was identified in dominant phyla (Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria) found within the uterine and vaginal tract of open cows (Swartz et al., 2014; Messman et al., 2020) as well as in the in the epithelial surface microbiota of the bull penis/prepuce and semen (Wickware et al., 2020). Thus, it is likely these bacteria are present in the uterine body prior to conception, embryo migration, and implantation. After conception, the cervix is a formidable barrier with mucosa-lined cartilaginous rings that become tightly convoluted under progesterone influence (Bondurant, 1999). Thus, authors hypothesize microorganisms detected throughout gestation are present prior to conception and those identified are capable of surviving the physiological changes in the uterine environment during gestation.

Likely, the bacteria that fall in the phyla of Bacteroidetes, Proteobacteria, and Firmicutes can survive in harsh conditions with low nutrients and oxygen availability; thus, their presence in the fetal gut (rumen/small intestine tissue) and meconium is due to survival capabilities.

Moreover, the bacteria within the Actinobacteria phyla are identified within the gut early in gestation, but there is an increased prevalence of this phyla within the amniotic fluids as gestation persists ([2]Amat et al., 2022; Guzman et al., 2020; Husso et al., 2021). This is interesting and poses a question about Actinobacteria's ability to survive within these environments. If this phylum lacks attachment capabilities within the fetus, these bacteria (dead or alive) would be shed into the amniotic fluid and then routinely swallowed during the last month of gestation (Gilbert & Brace, 1989). During the movement of Actinobacteria through the gastrointestinal tract of the fetus, other microbes in dominating phyla could be utilizing these dead microbes as a source of protein and energy. Thus, this would explain the decrease in specific phyla abundance throughout gestation. To support this hypothesis further, the only sequencing technique utilized is 16S rRNA gene amplification which does not discriminate between dead or alive bacteria (Li et al., 2017). Thus, while these phyla have been characterized, their metabolic status (dormant, live, dead, non-growing) cannot be determined. Together, these observations indicate microbial findings throughout gestation could be attributed to the maturation and survival of the commensal uterine/vaginal/paternal microbial communities are present at conception due to the closing of the cervix and their inability to leave the system. While inoculation is still probable and has been demonstrated in humans (Bolte et al., 2022), it is less likely all detected microorganisms originated from an inoculation route and more plausible they were within that environment before pregnancy establishment. Future research exploring this hypothesis is needed and could help elucidate some discrepancies within the growing field of research.

Programming Effects of the Gestational Microbiome

Few studies have examined prenatal programming of the microbiome in ruminant species. For example, maternal environmental manipulations are a promising area of research in relation to improving offspring production outputs; however, more controlled studies are needed in this area. Another important area of research is linking the microbiome to host metabolome profiles, which can alter important phenotypic traits. Elolimy et al. (2019) examined maternal rumen-protected methionine supplementation during the last 28 days of gestation on offspring fecal microbiota and metabolome as well as growth performance. For this study, maternal methionine supplementation increased heifer calf size at birth, while measures of beta and alpha diversity of fecal microbial communities were similar at birth. However, shifts in specific bacterial taxa of the hindgut and fecal metabolome were observed. This carried over into the preweaning phase whereby offspring born to methionine supplemented dams had increased *Ruminococcus* and *Fusobacerium*, which have been linked to volatile fatty acid production in the hindgut (Elolimy et al., 2019). Of great interest, this study showed that maternal supplementation during late gestation can shift offspring microbiota and metabolome to a more efficient profile, such as decreasing pathogens and enhancing production of vitamins.

In a recent study with woman carrying twin pregnancies, researchers examined 150 pairs of twin neonates to explore gut microbial communities and their metabolic profiles in relation to indicators of fetal growth restriction (Yang et al., 2022). Interestingly, early neonatal gut microbiota diversity was positively correlated with the severity of fetal growth restriction and an adverse intrauterine environment was associated with neonatal gut microbiota dysbiosis, which was more pronounced in monochorionic-diamniotic twins versus dichorionic-diamniotic twins. Specifically, in monochorionic-diamniotic twins with fetal growth restriction researchers

observed increased *Coprococcus*, *Robinsoniella*, and *Oscillospira* and decreased *Acinetobacter*, *Enterococcus*, and *Actinobacillus*. Similar to changes in gut microbiota, metabolic meconium and fecal profiles were more dissimilar in the monochorionic-diamniotic twins with fetal growth restriction, whereby smaller twins had decreased concentrations of cysteine, methionine, and dipicolinic acid (Yang et al., 2022). These researchers suggested decreased abundance of neonatal *Enterococcus* and *Acinetobacter* may be linked to lowered fecal concentrations of methionine and cysteine. These metabolic changes in methionine are especially interesting as maternal methionine supplementation during late pregnancy shifted microbiota and metabolome profiles during the neonatal period of calves (Elolimy et al., 2019). Interestingly, apart from early immune function and neonatal growth, the maternal gut microbiome in mice has been linked to fetal neurodevelopment (Vuong et al., 2020), which is an area that needs to be further explored in livestock species.

Innovative studies have characterized changes in neonatal gut microbial communities in models of fetal growth restriction or maternal late pregnancy supplementation strategies, however, fewer studies have linked causative roles of the maternal gut microbiome with compromised pregnancies. Researchers have examined contributions of the maternal gut microbiome and targeted metabolomics to preeclampsia, a leading cause of placental dysfunction resulting in greater risk of maternal and perinatal morbidity. Importantly, maternal gut microbial dysbiosis may contribute to the development of preeclampsia, while *Akkermansia muciniphila* was shown to regulate vascular placental remodeling through propionate and butyrate metabolites in preeclamptic rats (Jin et al., 2022). Therefore, maternal microbial and metabolome panels may reveal potential biomarkers for preeclampsia risk.

Perinatal Calf Microbiome

There is a breadth of literature evaluating the post-partum neonatal microbiota in comparison to published literature evaluating the dam reproductive tract microbiota during gestation. Shockingly, despite differences in breed, location, time after birth, and sampling site, there is a consistent gastrointestinal microbiota in calves dominated by Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes (Alipour et al., 2018; Woodruff et al., 2022; Fan et al., 2021; Zhu et al., 2021; Gomez et al., 2017; Barden et al., 2020). These, again, overlap with the early ([2]Amat et al., 2022), mid (Hummel et al., 2022), and late (Guzman et al., 2020) gestation microbiota identified. Moreover, these are the similar biomes to the dam reproductive tract (Swartz et al., 2014) and male reproductive tract (Wickware et al., 2020). This persistent overlap should be noted, but although the dominant phyla remain the same, there is clear shifts within the neonate after parturition.

In a study by Alipour et al. (2018), the feces from calves were evaluated at 0 hr, 24 hr, and 7 d; these samples were also compared to the dam feces, mouth, and vaginal microbiota. Notably, there was a drastic change in fecal microbiota from 0 to 24 hr of age, where there was an increase in *Escherichia Shigella, Clostridium sp,* and *Enterococcus sp.* (Alipour et al., 2018). From 24 hr to 7 d, the calf rectal microbiota began to resemble the dam demonstrating an establishment of residential microbiome was primarily composed of *Faecalibacterium, Bacteroides, Lactobacillus,* and *Butyricicoccus* (Alipour et al., 2018). Authors associated the drastic differences between the 0 hr (meconium) from the 24 hr sample is due to the *in-utero* colonization of the gut, this microbial population is shed in the meconium and environmental colonization of the neonatal gut could occur (Alipour et al., 2018). On day 7, the abundance of bacteria in the rectum increased, but there was a decrease in species richness. This decrease in

diversity is consistent with microbial dysbiosis within the gastrointestinal tract (Wilkins et al., 2019). In studies evaluating diarrhea instances in calves, the fecal microbiome has a decreased richness and abundance compared to healthy animals; moreover, Actinobacteria was decreased in diarrheic calves (Gomez et al., 2017) and *Lactobacillus sp.* were associated with healthy calves (Fan et al., 2021). In the study by Alipour et al. (2018), the dominating genera at 24 hr are consistent with calves that develop diarrhea between 21- 35 days (Fan et al., 2021); thus, it could be hypothesized calves that without the proper gut microbiome development by 7 days of age could be more susceptible to neonatal diarrhea and persistent gastrointestinal dysbiosis.

Colostrum Microbiome

When considering gut recolonization, nutrition should be the first factor evaluated. Thus, the colostrum microbiota could be indicative of neonatal dysbiosis outcomes. Again, colostrum is dominated by the same four phyla Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria (Chen et al., 2021; Lima et al., 2017; Van Hese et al., 2022). In one study, the genera *Actinobacter* comprised 16.2% and *Lactococcus* comprised 4.0% of the colostrum microbiota (Van Hese et al., 2022), these genera fall, respectively, within the Actinobacteria (Gomez et al., 2017) and Lactobacillus (Fan et al., 2021) phyla that have been associated with gastrointestinal health in calves.

Throughout this review, the phyla Actinobacteria has been reported modestly in many articles. Specifically, it is typically the 4th most abundant phyla in healthy animals. During late gestation, Actinobacteria is commonly isolated from the amniotic fluid (Husso et al., 2021; Guzman et al., 2020), and Gomez et al. (2017) demonstrated a positive relationship with Actinobacteria and health in neonatal calves. Notably, genus within this phylum, including *Actinobacter*, has been identified in high quality colostrum samples (Van Hese et al., 2022).

Actinobacteria are gram positive, non-motile, anaerobic, branching rods (Belizario et al., 2015). In humans, the family Bifidobacteria is the most represented in the gut (Binda et al., 2018) and neonates born via Cesarean section had significantly less abundance of Actinobacteria during their first week of life (Dogra et al., 2015), 1 month (Huurre et al., 2008), and 3 months (Kabeerdoss et al., 2013). Interestingly, neonates that were breastfed had a higher abundance of Actinobacteria within the gut than formula fed neonates (Bezirtzoglou et al., 1997). Post-weaning in humans also causes a decrease in the Actinobacteria families (Bifidobacteria) within the gastrointestinal tract (Ley et al., 2006).

The inconsistent presence of Actinobacteria throughout conceptus development is intriguing. In humans, this phylum has been shown to maintain intestinal barrier functions (Ashida et al., 2011), biodegradation of resistant starches (Ryan et al., 2006), decrease inflammatory responses (O'Mahony et al., 2005), and has antidepressant properties due to increase tryptophan production (Desbonnet et al., 2009; Binda et al., 2018). Tryptophan, the sole precursor of serotonin, has been highlighted for its role in neurotransmission, neuroendocrine actions, and intestinal immune responses which subsequentially alter the brain-gut axis (Gao et al., 2020). Together, these provide major implications for this phylum in modulating the bovine neonatal gastrointestinal microbiota and subsequent health outcomes. Evaluation of this phylum in colostrum samples in correlation with neonatal morbidity incidence could be beneficial in elucidating its importance in calf gut colonization.

Post-Natal Microbiome and Metabolome Interactions

In addition to prenatal influences, researchers have examined developmental changes of the rumen microbiome and metabolome from young and sub-adult Tibetan sheep (Li et al., 2020). Using a targeted approach, this study observed stable small chain fatty acid profiles

between developmental stages of lambs; however, microbial community structure and essential amino acid profiles of the rumen varied between young lambs and sub-adult Tibetan sheep (Li et al., 2020). Specifically, rumen microbial diversity increased in sub-adults, with Bacteroidetes being more abundant in sub-adults. This increasing diversity in the rumen microbiome and correlation to metabolites has been proposed to be more stable into adulthood becoming difficult to shift in favor of improved production. This becomes important in animal agriculture where feed costs are a large expense and critical component of increasing producer productivity. Recently researchers observed distinct clustering of ruminal bacterial community structures in Angus heifers divided into high or low residual feed intake determined during the finishing period (Liu et al., 2022). This works appears to show a shift in bacterial communities away from the family *Prevotellaceae* and into *Rikenellaceae*, and *Ruminococcaceae* in heifers with high versus low residual feed intake. Furthermore, rumen microbial communities and rumen metabolites were significantly correlated showing an important link between microbiome-metabolome interactions independent of the host species (Liu et al., 2022). Using a multi-omics approach researchers have also examined the importance of the microbiome and host metabolome on dairy cow performance (Xue et al., 2020). In this study, dairy cows with high milk yield and high protein yield had increased *Prevotella* species in the rumen along with changes in ruminal amino acid pathways which may contribute to host milk protein biosynthesis through increased rumen microbial protein (Xue et al., 2020).

Summary

To summarize, throughout this review it has become evident that the bovine reproductive tract, developing gut, and colostrum are dominated by Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria. Moreover, microbial presence, from both maternal and paternal origins,

within the reproductive tract prior to conception is likely. The presence of microbes can contribute to developmental programming of the conceptus through inoculation and modulations of the uterine environment. While *in-utero* microbial inoculation of the conceptus via hematogenous route is certainly possible, it is more likely microbes are already present and capable of survival. Contributions to the neonatal gut microbiome is multifactorial, with inoculation from *in-utero* exposure, vaginal tract during parturition, dam interactions, external environment, and colostrum are all likely. However, the role of Actinobacteria within this colonization should be further explored due to the numerous beneficial roles that have been demonstrated within human physiology. Lastly, the efforts to identify the microbial origins within a developing conceptus have clearly shown a stable residential microbiome, and future research exploring induced dysbiosis could yield data that lead to a better understanding of these bacteria's roles within the bovids.

Statement of the Problem

Clearly, there is still much to understand regarding the reproductive tract microbiota and its implications in both dam and neonatal health postpartum. Within humans, the vaginal microbiota has been linked to fertility with eubiotic communities improving fertilization, pregnancy establishment, pregnancy maintenance, and fetal/neonatal microbial inoculation (Tomaiuolo et al., 2020). In bovids, research within this field has dramatically increased over the last decade. However, contamination checks, sample collection methods, and outdated sequencing techniques limit the validity of the current literature. Thus, research evaluating the reproductive tract microbiota within the dam and its subsequent effects on neonatal inoculation and dam post-partum fertility is needed. Moreover, this research should utilize current standards

for data sample collection, contamination checks, and use of culture independent methods (16S sequencing/functional analysis).

Currently, the microbiome is a hot topic in both human and animal health, driving the supplement and pharmaceutical industry to capitalize on this trend. The bovine vaginal microbiota is starkly different from humans; in comparison to the Lactobacillus dominated human vaginal microbiota, the bovine vaginal microbiota has a near neutral pH and is dominated by Proteobacteria, Bacteroidetes, and Firmicutes (Swartz et al., 2014). However, the livestock supplement/pharmaceutical industry is promoting intra-vaginal probiotic therapeutics (ProPreg) dominated by lactic acid producing bacteria, mimicking human microbial physiology (Healthy Cow Co., 2023). Therefore, in addition to inconsistencies within methodology, the field of bovine reproductive tract microbiota research is now facing incorrect supplement development and time constraints to fully elucidate the implications of an altered dam vaginal microbiota.

Therefore, this review and the research conducted contributes valuable knowledge to the literature by highlighting similarities within bovine reproductive tract communities and evaluating the effects of these communities on fetal/neonatal development. To the author's knowledge, this is the first research characterizing the dam vaginal and calf nasal microbiota at parturition and post-partum in beef cattle. Understanding how these microbial communities overlap or diverge could explain neonatal and dam post-partum disease. The immune response of dams and calves were also measured to evaluate consistencies between immune status and microbial composition. Together, this data addresses the current paucity within bovine reproductive tract microbiota literature and drives future research objectives to establish a clear bovine eubiotic reproductive tract microbiota composition and understand its implications in reproductive performance and subsequent calf health. Such findings will translate to accurate

information for supplement companies to base therapeutic compositions, economic gains for beef cattle producers, and overall improvement in animal welfare for beef cattle dams and calves.

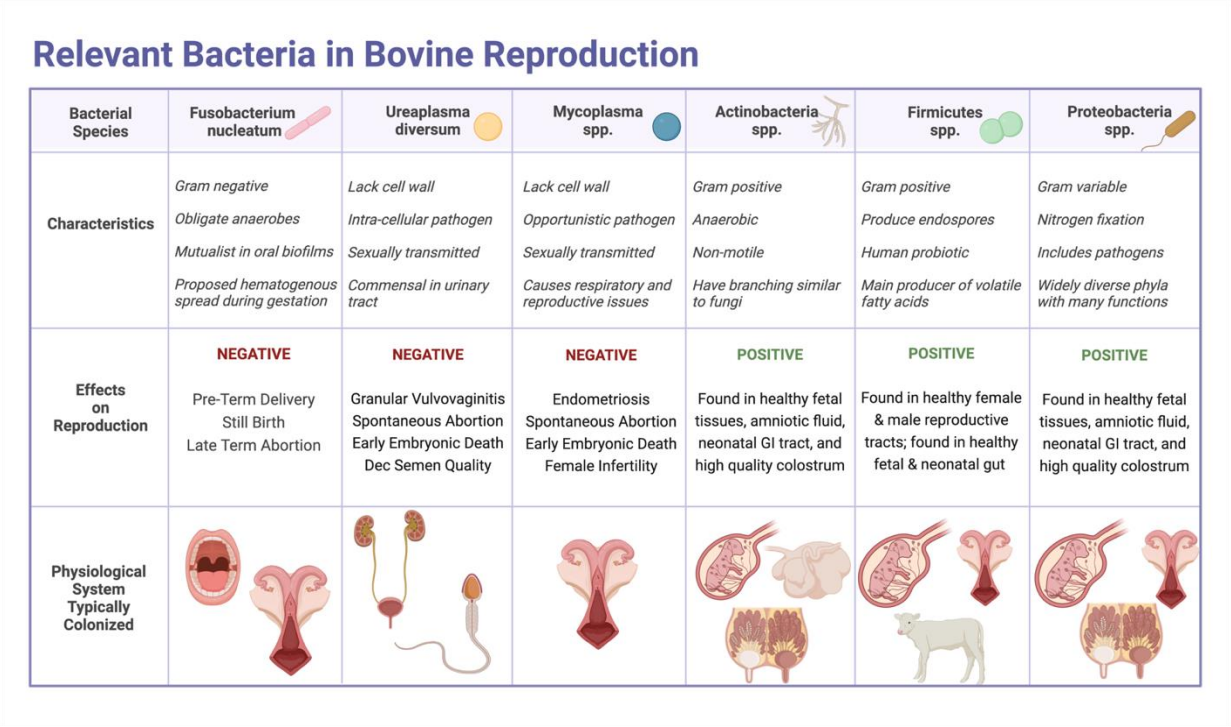

Figure 1 Relevant Bacteria in Bovine Reproduction

Table depicting bacterial species that are relevant within bovine reproductive physiology. This table includes important characteristics, documented effects of the bacteria on reproduction, and the physiological system that is typically colonized within the body. Literature cited can be found within the text.

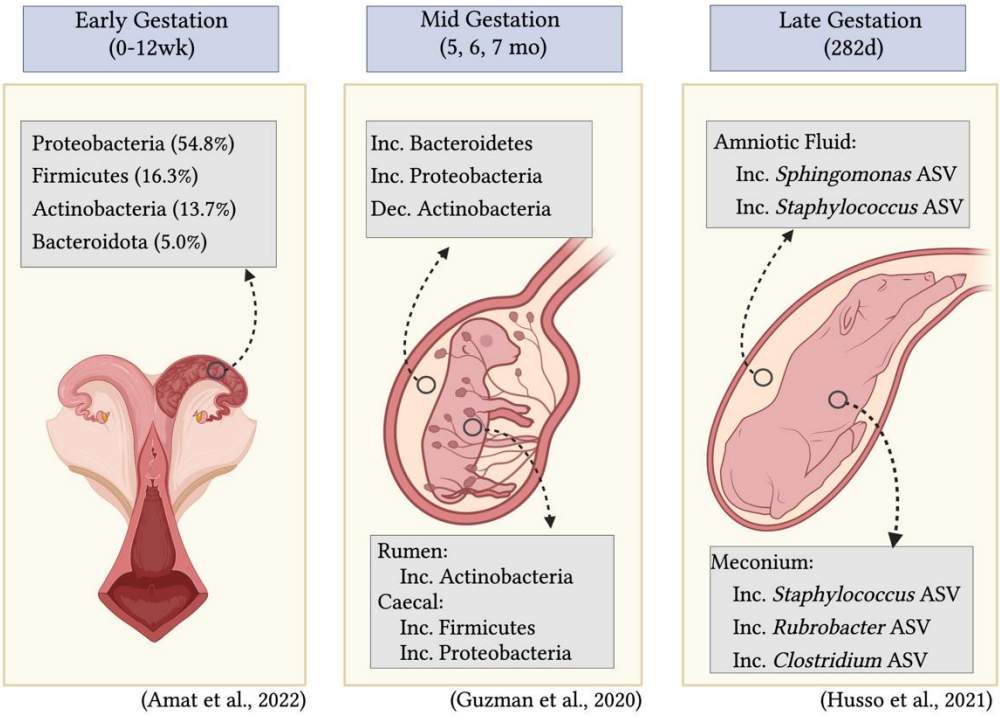

Figure 2 Bovine Gestational Inoculation Timepoints

Diagram of the bovine reproductive tract during early, mid, and late gestation with common bacterial species and where they are located within both the uterus and the fetus.

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CHAPTER II

EVALUATING THE INOCULATING BACTERIAL COMMUNITIES WITHIN THE BOVINE NEONATAL RESPIRATORY TRACT AFTER APPLYING AN ABLATION TECHNIQUE TO THE DAM VAGINAL MICROBIOME PRIOR TO PARTURITION

Abstract

The vaginal microbiota (VM) is the neonate's first contact with microorganisms and can attribute to neonatal health outcomes. The effects of an altered VM at parturition on neonatal microbial inoculation and passive transfer success has not been evaluated. Betadine lavages (BL) are commonly used to mitigate bacterial infections within the reproductive tract by ablating the VM. Ablation of the VM prior to parturition decreases microbial competition during neonatal microbial colonization, leading to increased likelihood for pathogen inoculation. Thus, this study aimed to determine if an altered VM impacts neonatal microbial inoculating bacterial communities and passive transfer status in beef cow-calf pairs. Cows (n = 12) were randomly assigned to either the control group (CON) or BL treatment group (BLG) two weeks prior to calving. Treatment BL bags were infused into the anterior vagina and cows received 1-3 treatments depending on calving date. All samples (dam colostrum, calf sera, dam-vaginal swab, dam-udder swab, and dam-haircoat swab, and calf-nasal swab) were collected within 24 hours of parturition and stored at -80°C. The vaginal bacterial community composition was determined through sequencing of the V3-V4 region of the 16S rRNA gene using the Illumina Miseq

platform. Alpha diversity was compared via two-way ANOVA; beta diversity was compared via PERMANOVA. Taxonomic differences were evaluated using the LEfSe platform. Calf serum and dam colostrum were analyzed for IgG concentration via a commercial ELISA. All IgG and microbiota data were analyzed using the R software package (v. 2023.03+386). Serial BL prior to parturition did not alter the alpha diversity of the dam-vaginal (P = 0.42), dam-udder (P = 0.53), or dam-haircoat (P = 0.21) microbiota. However, serial BL did alter the calf-nasal microbiota at parturition (P = 0.03). Moreover, the beta diversity did not differ within the dam-vaginal (P = 0.66) or dam-udder (P = 0.56) between BLG vs. CON, but there was a trend within both the calf-nasal (P = 0.08) and dam-haircoat (P = 0.09) for BLG pairs to have increased variation compared to CON. Within the calf-nasal microbiota, BLG had increased relative abundance of Actinobacteria and a decreased relative abundance of Proteobacteria compared to CON. There was no difference in passive transfer status between CON and BLG calves, represented by no significant differences in calf serum (P = 0.88). However, BLG dam colostrum had increased IgG concentrations compared to CON dams (P =0.04). Together, these results are indicative of physiological insults (BL) prior to parturition, leading to an increased immune response in BLG dams which altered colostrum IgG. Thus, neonatal colostrum consumption could drive immune responses against inoculating bacteria resulting in differing nasal microbial communities between BLG and CON calves. However, more research is needed to elucidate the intricacies of this relationship.

Key Words: Neonate inoculation, microbiome, bovine

Introduction

At the onset of labor, a natural ascension of the vaginal microbiome into the uterine body to inoculate the neonate has been clearly documented in humans (Aagaard et al., 2014). In fact, infants born via natural labor acquire bacteria that resembles the maternal vaginal microbiome (predominately *Lactobacillus*), whereas infants born via Cesarean section acquire bacteria resembling the skin microbiome (predominantly *Staphylococcus*; Dominguez-Bello et al., 2010). Thus, under natural circumstances the vaginal microbiome should be the first substantial inoculation within the neonate, quickly followed by microbial exposure from the dam and environment. Microbial exposure at birth is thought to attribute to the subsequent maturation of the microbial communities within the neonate, specifically the upper respiratory tract and gut (Tamburini et al., 2016). In dairy cows, Lima et al. (2019) found that the composition of the maternal vaginal microbiota influences the initial colonization of the calf upper respiratory tract, which could have an impact on the health of the calf respiratory tract later in life. This is the first study, that authors are aware of, in bovids suggesting potential perinatal programming effects of the maternal reproductive tract microbiome in the health of offspring. Although important, considerable environmental differences in dairy cattle versus beef cattle production and management means further replication is necessary to fully understand these perinatal programming pathways.

The reproductive tract microbiome within bovids is typically dominated by Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria (Swartz et al., 2014; Messman et al., 2020). Interestingly, known markers of dysbiosis, such as decreased diversity and loss of heterogeneity (Galvao et al., 2019), can also be observed towards the end of gestation in bovids (Messman et al., 2021). Thus, it appears that gestation can alter the vaginal microbiome, but

literature exploring how these compositional changes affect the neonate is limited. Moreover, maternal antigen exposure close to parturition determines the IgG composition within maternal colostrum, due to nearly all serum IgG being transferred into the colostrum during colostrogenesis (Baumrucker & Bruckmaier, 2014). Thus, through passive transfer from the dam, the neonate likely has antigens against bacterial species they obtain from the dam during vaginal delivery. However, rapid alterations of the vaginal microbiome close to parturition could have negative effects due to altering immune responses within the dam, which effects subsequent colostrum quality and neonatal inoculation communities.

Ablation techniques were commonly used in early reproductive physiology literature to better understand endocrinology within the reproductive tract. This technique could prove useful eliciting the role of a eubiotic vaginal microbiome within bovine reproduction and neonatal health outcomes. Povidone iodine (Betadine) is a water-soluble iodine-releasing agent with broad spectrum antimicrobial activity against both Gram-negative and Gram-positive bacteria (Yasuda et al., 1997). Betadine has also been shown to have activity against mature bacterial and fungal biofilms, like those in the vaginal tract (Hoekstra et al., 2017). Moreover, Betadine lavages are commonly used within veterinary theriogenology as a treatment for endometritis in both mares and cows (Olsen et al. 1992; Koujan et al., 1996). Recent studies have shown that utilizing betadine lavages, even in healthy animals, induces transient uterine inflammation promoting regeneration of endometrial epithelial cells resulting in improved fertility (Yoshida et al., 2020). Based on this information, betadine lavages appear to be a logical choice for locally ablating the vaginal microbiome prior to calving without using antibiotics.

Therefore, the objective of this study was to determine if betadine lavages prior to parturition alters the dam vaginal microbiome, microbial upper respiratory tract colonization

within the neonates, colostrum quality, and passive transfer. We hypothesized that dams receiving betadine lavages prior to parturition would have an altered vaginal microbiome composition compared to controls resulting in altered neonatal upper respiratory tract microbiome composition, colostrum quality, and passive transfer success compared to controls.

Materials & Methods

Animal Management and Treatments

Animal care and use was approved by the Mississippi State University Institutional Animal Care and Use Committee (#21-076). Multiparous beef cows (n = 12) bred to separate sires (n = 3) were housed at the H.H. Leveck Animal Research Center (Mississippi State, MS) in a 2-acre pasture during calving and were moved to a 25-acre pasture after calving. Cows were provided ad libitum round bale hay and water throughout the project. Prior to the calving, all cows had a body condition score (BCS) of 6 ± 0.5. Diets were adjusted, 4 weeks prior to rebreeding, to include a concentrate (2.27 kg/hd/d) to address decreasing BCS of cows post-calving. All cows calved within 12 days of the expected calving date.

Three weeks prior to calving, Angus cows were divided into two treatment groups, betadine lavage (BLG; n = 7) or control (CON; n = 5). The lavage bags were composed of 200mL Betadine (5% povidone-iodine) diluted in 800mL of Lactated Ringer's solution for a final dilution of 0.5% povidone-iodine per lavage. Vaginal swabs and vaginal betadine lavages were performed once weekly until calving, cows either received one (n = 2) or two (n = 5) lavages prior to calving. Within 24 hours of calving, swabs were collected from the dam (udder, vaginal, and haircoat) and calf (nasal); blood and colostrum samples were also collected at this time. Angus pair (n = 12) swab samples underwent 16S bacterial community analysis and functional prediction analysis.

Swab Collection

A double guarded equine uterine culture swab (Minitube Ref. 17214/2950) was utilized to sample the anterior vaginal tract, udder, and haircoat of each cow and the nasal tract of each calf within 24 hours after calving. After a sample was collected, the swab unit was broken down by removing the external layer to expose the swab in the sterile tubing. The sterile tube containing the swab was snapped at a pre-determined length, then capped with sterile caps to prevent airborne contamination. All swabs were stored at -80°C until further analysis.

Vaginal Swab Collection

Cows were restrained in a hydraulic chute and the vulva was cleaned by wiping with a paper towel to prevent swab contamination. The double guarded unit containing the swab was removed from sterile packaging and immediately inserted through the vulva into the vaginal tract. The swab was angled upward, over the pelvic shelf, and towards the anterior vagina. Once the swab would not move forward with pressure, the cotton swab was exposed from the sterile guarding to make direct contact with the anterior vagina. The swab was rotated for approximately 30 seconds then retracted back into the sterile guarding. The entire double guarded swab unit was removed from the cow's vaginal tract. The swab was closely examined in the sterile guarding for any urine (yellow staining) or feces. If contamination looked possible, the cow was re-swabbed.

Haircoat Swab Collection

Cows were restrained in a hydraulic chute. The double guarded unit containing the swab was removed from sterile packaging and the cotton swab was exposed from the sterile guarding to make direct contact with the haircoat. Sampling started at the ventral portion fore flank continuing in a straight line to the rear flank to mimic the neonate following pheromone

production when starting to nurse. The swabbing of this area continued for approximately 30 seconds then the swab was retracted back into the sterile guarding.

Udder Swab Collection

Cows were restrained in a hydraulic chute. The double guarded unit containing the swab was removed from sterile packaging and the cotton swab was exposed from the sterile guarding to make direct contact with the udder. All four quadrants, including teats and skin, were sampled. The swabbing of the udder continued for approximately 10 seconds in each quadrant, then the swab was retracted back into the sterile guarding.

Calf Nasal Swab Collection

Calves were restrained manually by two trained personnel. The double guarded unit containing the swab was removed from sterile packaging and placed within the cranial portion of the nasal canal. The cotton swab was exposed from the sterile guarding and advanced into the nasal canal (approximately 8-10 cm) to make direct contact with the nasopharynx. The sampling continued for approximately 30 seconds, then the swab was retracted back into the sterile guarding. This process was then repeated, using the same swab unit in the opposite nasal canal. Thus, each nasal swab sampled both the right and left nasal tracts of the calf.

Blood Collection & ELISA IgG

Whole blood was collected via jugular venipuncture from dams and calves within 24 hours post-partum. Blood was allowed to clot at room temperature and placed on ice until transported to the laboratory for processing. Approximately one hour after collection, blood tubes were centrifuged at 2000xg at 4°C for 10 minutes. Serum was immediately collected and transferred into sterile 2 ml tubes and then stored at -80°C until further analysis. A commercial

sandwich enzyme-linked immunosorbent assay (ELISA; Bethyl Laboratories Inc., Montgomery, TX; Cat No: E11-118) was utilized to determine the IgG concentrations in both dam colostrum and calf sera at 24 hours post-partum. The intra- and inter- assay CV for IgG concentrations were 7.22% and 7.77% respectively.

Bacterial Community Analysis 16S

Samples from Angus dam-calf pairs (n = 12) were selected to undergo 16S bacterial community analysis. The 16S bacterial community analysis was performed by Zymo Research Corporation located in Irvine, CA. Genomic DNA was extracted using the ZymoBIOMICS -96 MagBead DNA Kit (Zymo Research; Irvine, CA) following the manufacturer's protocol. ZymoBIOMICS microbial community standard (Zymo Research; Irvine, CA) was used as a positive control for each DNA extraction. The ZymoBIOMICS microbial community DNA standard (Zymo Research; Irvine, CA) was used as a positive control for each targeted library preparation. Negative controls (blank extraction control, blank library preparation control) were included to assess contamination during the wet-lab process. The DNA samples were prepared for targeted sequencing with the *Quick*-16S Primer Set V3-V4 Plus NGS Library Prep Kit (Zymo Research; Irvine, CA). The sequencing library was prepared using real-time PCR to control cycles and limit PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned up with the Select-a-Size DNA Clean and Concentrator (Zymo Research; Irvine, CA), then quantified with TapeStation (Agilent Technologies, Santa Clara, CA) and Qubit (Thermo Fisher Scientific, Waltham, WA). Sequencing was done with an Illumina MiSeq (Illumina, San Diego, CA, USA) with a v3 reagent kit (600 cycles). The sequencing was performed with 10% PhiX spike-in.

16S sequence data were processed and analyzed using the plugin-based microbiome bioinformatics framework QIIME 2 (Bolyen et al., 2019)(V 2022.8). Cutadapt (v.4.1) (Martin, 2011) was used to remove the primer sequences, from both forward, and reverse reads. DADA2 (Callahan et al., 2016) was used (via the q2-dada2 QIIME 2 plugin) to quality filter the sequence data, removing chimeric, and erroneous reads. Sequences were further trimmed to remove reads where the average quality score dropped below 25 and clustered to ASVs (Amplicon sequence variants) after denoising with DADA2 in QIIME2. Taxonomy was assigned to each sequence variant using q2-feature-classifier plugin (Bokulich et al., 2018) in QIIME 2 with a pre-trained classifier from the SILVA database (Janssen et al., 2018) . The final output table of amplicon sequence variants (ASVs) was used to analyse bacterial community diversity, structure, and composition.

Statistical Analysis

The vaginal bacterial community comparisons of interest were between the pre-calving and 0-d vaginal swab samples and the 0-d vaginal, hair, udder, and nasal samples. A preplanned analysis was carried out to evaluate the effects of treatment within each sample location, focusing primarily on dam vaginal and calf nasal microbiota. The R software program (R Core Team, 2013) was used to conduct the statistical analyses, specifically using the Phyloseq package pipeline (McMurdie & Holmes, 2013). Alpha diversity was calculated using the Shannon index and significance was tested using ANOVA. Beta diversity was computed using the Bray-Curtis dissimilarity and visualized using the principal coordinate analysis (PCoA) plot. Differences in community structure were assessed using the permutational multivariate analysis of variance (PERMANOVA) with 0-d vaginal, hair, udder or nasal as the main fixed factor and using 9,999 permutations for significance testing in R (Adonis function from the Vegan

package). Microbiome Analyst was applied to evaluate taxonomic differences via LEfSe analysis, differences in relative abundance, and functional predictions (Dhariwal et al., 2017). The predetermined p-value cut off was set to ($P < 0.05$) for all Microbiome Analyst statistical analysis. Significance was set to ($P < 0.05$); tendencies were set to ($0.05 < P < 0.01$).

Results

Treatment Effects on Dam & Calf Microbiota at Parturition

A total of 48 swabs were analyzed for 16S sequencing. Vaginal, nasal, udder, and haircoat swabs from BLG cow-calf pairs (n = 7) or CON pairs (n = 5) were analyzed. Both negative and positive controls were utilized throughout laboratory preparation for contamination checks, and contamination was accounted for within the analytic pipeline. A total of 12,589,488 quality filtered reads were obtained with an average of 95,374 quality filtered reads per sample that were assigned to 16,932 ASVs, after quality control analyses and ASV filtering. The four most abundant phyla within all samples were Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes (Fig 10). There were no differences in alpha ($P = 0.12$) or beta diversity ($P= 0.38$) for the interaction between betadine lavage treatment and sample location.

The effect of the betadine lavage treatment was evaluated within the calf nasal and dam vaginal microbiota. There was no difference in alpha ($P = 0.42$; Fig. 3A) or beta ($P = 0.66$; Fig. 4A) diversity within the vaginal microbiota between BLG vs. CON dams. However, the nasal microbiota was significantly different ($P = 0.03$; Fig. 3B) and the beta diversity tended to be different ($P = 0.08$; Fig. 4B) between BLG vs. CON calves. Specifically, the BLG calf nasal microbiota had a decrease in richness/evenness and less bacterial community variation compared to the CON.

There were four phyla that were consistently dominant within all samples, but the ratio of the phyla varied between groups. Within the dam vaginal microbiota ratios varied between BLG vs. CON within Firmicutes (55.89% vs. 46.68%), Proteobacteria (19.11% vs. 28.20%), Actinobacteria (15.25% vs. 9.58%) and Bacteroidetes (11.21% vs. 7.63%), respectively (Suppl Fig. 1). Within the calf nasal microbiota ratios varied between BLG vs. CON within Proteobacteria (39.90% vs. 52.09%), Firmicutes (24.55% vs. 22.38%), Actinobacteria (27.28% vs. 18.77%) and Bacteroidetes (7.80% vs. 2.47%), respectively.

A linear discriminant analysis (LDA) was conducted to evaluate differences at the phyla and genera level between BLG and CON within the calf nasal and dam vaginal microbiota. There were no significantly different phyla or genera with a p-value cut-off of ($P < 0.05$) between the BLG and CON calves' nasal microbiota or dams' vaginal microbiota.

Sampling Loci Differences at Parturition

There was a main effect of sampling location for alpha diversity ($P = 0.002$; Fig. 5). Specifically, the vaginal microbiota had decreased richness & evenness within the bacterial community compared to the nasal, udder, and haircoat microbiota. There was a main effect of sampling location for beta diversity ($P = 0.001$; Fig. 6). Specifically, the dam udder and dam haircoat were similar to each other, demonstrated by close overlap and clustering on the PCoA (Fig. 6). The dam vaginal microbiota was different from all other sampling locations and appeared to correlate more closely with PC1 with increased variation within the microbial community compared to dam udder and haircoat (Fig. 6). The nasal microbiota was also different from all other sampling locations and appeared to correlate more closely to PC2 with increased variation within the microbial community compared to the dam udder and haircoat (Fig. 6).

A linear discriminant analysis (LDA) was conducted on the top 15 differentially abundant (P = 0.05) phyla between the sampling locations (Fig 8). This analysis supports observations within the ratios of the four most abundant phyla differed between the sampling location (Fig 11). The calf nasal microbiota was dominated by Proteobacteria (45.79%). However, Firmicutes dominated the dam vaginal (63%), udder (54.85%), and haircoat (52.14%) microbiota. There was also an increase in Actinobacteria (23.06%) with a decrease in Bacteroidetes (2.69%) within the calf nasal tract compared to the dam vaginal, udder, and nasal microbiota which maintained a close 1:1 ratio of these phyla with averages of 11.3% Actinobacteria and 11.7% Bacteroidetes.

A linear discriminant analysis (LDA) was conducted on the top 10 differentially abundant genera between sampling locations (Fig 9). The dam vaginal microbiota had high amounts of *Streptococcus*, *Alistipes*, and unclassified genera (*5_7N15*). The dam udder microbiota had moderate abundance of *Acinetobacter*, *Streptococcus*, *Ornithinimicrobium*, *Staphylococcus*, *Alistipes*, and *unclassified (5_7N15).* The dam hair microbiota had low relative abundance for all genera except *Acintobacter, Arthrobacter* and *Corynebacterium*. Interestingly, the calf nasal microbiota had seemingly opposite relative abundance of the top 10 genera when compared to the vaginal microbiota. The genera *Ornithinimicrobium, Orthinicoccus, Staphylococcus, Macrococcus, and Corynebacterium* had a high presence within the calf nasal microbiota.

Functional Predictions

Functional predictions were utilized to further understand compositional differences utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) via the MicrobiomeAnalyst platform (Kanehisa et al., 2021; Dhariwal et al., 2017). Following compositional differences, the nasal microbiota between BLG and CON calves were evaluated. There were no significant functional feature differences (KEGG pathways) between in the nasal microbiota of BLG and

CON calves when evaluating using a single factor analysis (Kruskal-Wallis test) or LDA with a p-value cut-off of ($P = 0.05$). This was further iterated with no significant percentage differences in KEGG functional profiling modules within the nasal microbiota between BLG and CON calves (Fig 12). The functional differences within the dam vaginal microbiota were also evaluated. There were no significant functional feature differences (KEGG pathways) between BLG and CON dams when evaluating using a single factor analysis (Kruskal-Wallis test) or LDA with a p-value cut-off of ($P = 0.05$).

Moreover, functional differences were evaluated between location. There were 3120 differentially expressed KEGGs between location ($P = 0.05$). The vaginal microbiota had differentially expressed KEGG functional pathways compared to udder, haircoat, and nasal ($P < 0.05$).

Effects of Treatment on IgG Levels in Dam Colostrum and Calf Sera

There was a significant difference in dam colostrum IgG levels between BLG and CON dams ($P = 0.04$; 47.70 ± 21.8 vs. 22.66 ± 12.27; Fig 7A). However, there was no significant difference in calf sera IgG between BLG and CON calves ($P = 0.88$; 20.9 ± 11.35 vs. 23.05 ± 14.76; Fig 7B).

Discussion

Serial betadine lavages (BL) prior to parturition were unsuccessful in altering the dam vaginal microbiota at parturition. However, BL did alter the calf nasal microbiota and dam colostrum quality at parturition. Together, these results indicate a plasticity within the bovine vaginal microbiota while providing evidence that dam immune responses to a physiological

insult within the vaginal tract likely propagates differences within the neonatal microbial inoculation.

It is well regarded within the literature that the bovine vaginal microbiota is dynamic with significant differences between individuals (Adnane & Chapwanya, 2022). Moreover, within bovine rumen microbiota literature the use of probiotics, antibiotics, feed additives, and other methods of microbiota disruptions are successful in providing acute alterations but no significant persistent changes (Weimer, 2015; Ghorbani et al., 2002). Thus, it is likely the betadine lavages acutely altered the vaginal microbiota within this study, but to discern the acute differences lavages needed to be repeated closer to parturition. This was further iterated by no functional differences shown within the vaginal microbiota between BLG and CON dams. However, the betadine lavages did increase IgG concentrations in BLG dams' colostrum compared to CON.

Immunoglobulins within the dam colostrum is representative of the dam's antigen exposure history and the subsequent immune response (Hurley & Theil, 2011). It is well established that dams can be vaccinated during gestation with the intent of decreasing neonatal morbidity and mortality (Lanza et al., 1995; Moon & Bunn, 1993). Within this study, betadine lavage treatment within the vaginal tract likely led to an immune response. Betadine is bactericidal, fungicidal, mycobactericidal, sporicidal, viricidal, and has limited effects on biofilms (Kavolus et al., 2020); the iodine acts as a potent oxidizer resulting in damaged cell membranes, inactive proteins, and cellular death (Siddiqi et al., 2021). However, the optimal dilution concentration for betadine is not clear in the literature, with some studies reporting damage to host tissue or toxicity regardless of concentration (Blom et al., 2019; Foresman et al., 1993).

In this study, the final concentration of povidone-iodine was 0.5%. Mido et al. (2016) found the ideal lavage concentration to treat endometritis in dairy cattle was 2.0% povidone-iodine; however, this study also utilized 0.5% povidone-iodine and found similar antiseptic outcomes. Thus, authors decided to utilize a less aggressive povidone-iodine concentration to decrease the likelihood of host tissue damage while still achieving optimal antiseptic outcomes. Betadine lavages likely altered the vaginal microbiome and elicited some negative effects to host tissue within the vaginal tract leading to a cell-mediated immune response close to calving.

Colostrogenesis is driven by a decrease in progesterone 2-3 weeks prior to calving, leading to increased IgG recruitment from dam serum to the mammary gland (Smith & Schanbacher, 1973). Thus, the antibodies produced from the dam immune response to betadine lavages would be in circulation to be recruited to the mammary gland during colostrogenesis, explaining the increased IgG concentrations in BLG dams compared to CON. However, within the calves there were no differences in sera IgG concentrations in BLG compared to CON. Moreover, all calves in this study met the threshold level for successful passive transfer, defined at >10mg/mL (Pritchett et al., 1991). Initially, the increase in IgG within BLG colostrum appeared to be a positive side effect of the BL treatment, but the implications of this finding amplified when there were also compositional differences between the nasal microbiota within the BLG vs. CON calves even without compositional differences in their respective dam's vaginal microbiota.

The nasal microbiota within BLG calves had significantly different alpha diversity (P = 0.03) and the beta diversity tended to be different (P = 0.08). Specifically, BLG calves had a decrease in species richness and evenness compared to CON with an increased relative abundance of the phylum Actinobacteria and decreased relative abundance of Proteobacteria.

Previous studies evaluating the upper nasal tract of beef calves also found high levels of Actinobacteria within the upper respiratory tract (McDaneld et al., 2019; McMullen et al., 2018). The variation of this phyla between BLG and CON calves is intriguing; Actinobacteria has been shown to have a positive relationship with health in neonatal calves (Gomez et al., 2017) and been identified in high quality colostrum samples (Van Hese et al., 2022). Within this study, it is not clear the implications of the shifting compositions within calf nasal microbiota. Functional sequencing was utilized, but no significantly different pathways were identified. Thus, while the composition of the calf nasal microbiota is altered, likely by colostrum IgG differences, the functional role of the nasal microbiota remains similar.

This study aimed to evaluate the compositional and functional similarities between the dam vaginal, dam udder, dam haircoat, and calf nasal microbiota at parturition. The vaginal microbiota is the first massive inoculation of microorganisms within a neonate and an important determinant of future host-microbiome interactions (Collado et al., 2016). Within this study, the alpha diversity of the vaginal microbiota was different from the udder, haircoat, and nasal ($P < 0.05$). As expected, the beta diversity of the udder and haircoat were similar. However, the beta diversity of the calf nasal microbiota was different from all dam microbiota (udder, haircoat, and vaginal). This finding was surprising because, in theory, these microbial communities should be the calf's primary contact with microorganisms immediately following parturition.

During vaginal delivery, the neonate contacts the dam vaginal microbiota, after expulsion the neonate contacts the environmental microbiota (soil, air, hay), the dam will lick the calf to remove amniotic fluid, calves should stand within an hour after birth, begin nuzzling and nudging different parts of the dam until finally locating the udder to nurse (Whalin et al., 2021). Within the calf nasal microbiota there is increased Proteobacteria and Actinobacteria compared

to the dam vaginal, udder and haircoat that was dominated by Firmicutes. Further division was seen at the genus level with inconsistencies of genera relative abundance between sampling location (Fig 7). Functional profiling revealed 3120 differentially expressed KEGGs dependent on sampling location. The inconsistencies within these microbiota communities are logical. Each sampling location has a different oxygen exposure, physiological pH, nutrient availability, and epithelial turnover leading to distinctly different bacteria communities that can reside within that location. However, the lack of clear overlap of these communities with the calf nasal microbiota led authors to hypothesize the duration of these contacts with the dam during and immediately after parturition is not enough to result in neonatal colonization.

Summary

Serial betadine lavages were not successful in altering the vaginal microbiome prior to calving. However, the dam immune response coupled with colostrogenesis may explain the differences within the calf nasal microbiota after parturition. Moreover, the lack of overlap of microbiota from anatomical loci on the dam the calf is considered to have high contact with is puzzling. More research to fully understand primary inoculations of the calf and the subsequent transition to a commensal microbial community within the upper respiratory tract is needed. Moreover, it is highly likely that the dam and calf immune system is playing a major regulatory role within microbial colonization; studies focusing on combining immune status with microbiota composition in bovine dams and neonates is essential to further understanding the roles of microbial communities in dam and neonatal health outcomes.

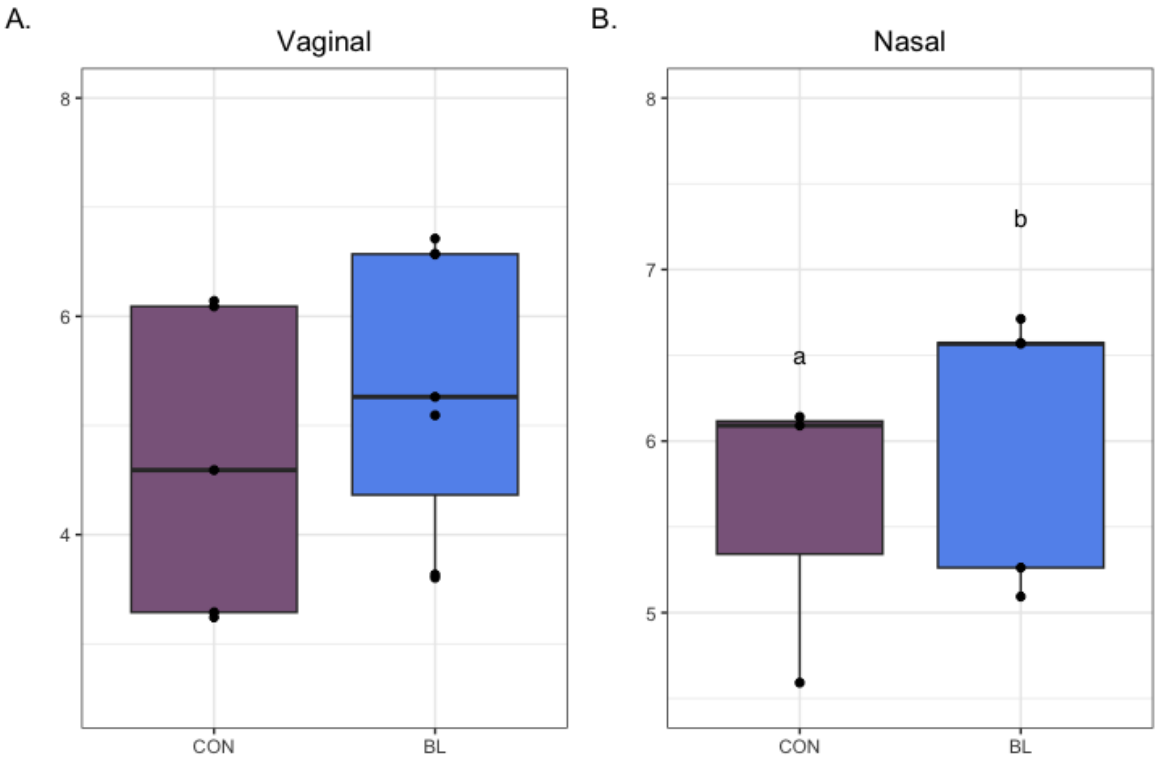

Figure 3 Alpha Diversity Boxplot Between BLG vs. CON Bacterial Communities

Alpha diversity boxplot of the dam vaginal (Panel A) and calf nasal (Panel B) bacterial community between BLG vs. CON Angus cow-calf pairs within 24 hours of parturition (d0) measured by the Shannon diversity index. The left purple box represents the bacterial community within the CON cow-calf pairs (n = 5) and the right blue box represents the bacterial community within the BLG cow- calf pairs (n = 7). Black dots represent values for individual samples. There was no difference in the alpha diversity for the dam vaginal (P = 0.42) bacterial communities between the BLG vs. CON. There was a difference in the nasal microbiota (P = 0.03) between calves from either BLG vs. CON dams.

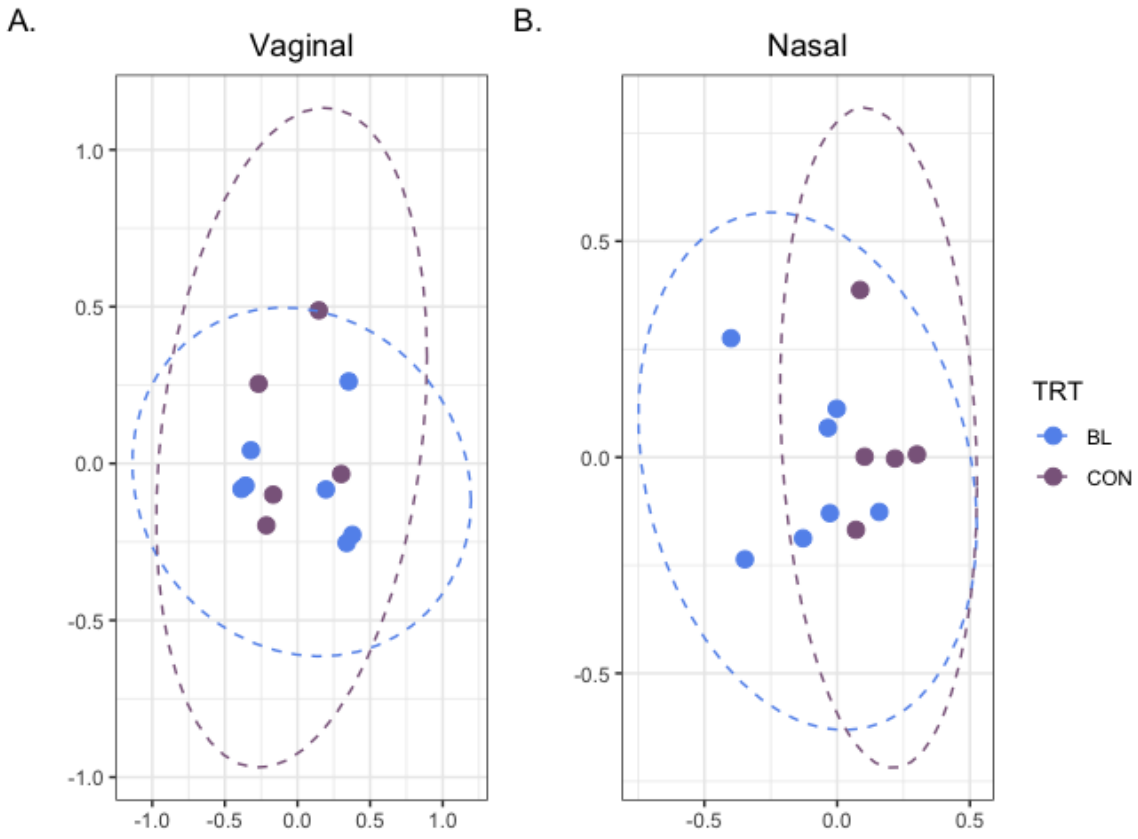

Figure 4 Beta Diversity of BLG vs. CON Bacterial Communities

Principal coordinate analysis (PCoA) depicting Bray-Curtis dissimilarities across samples of the dam vaginal (Panel A) and calf nasal (Panel B) bacterial communities in Angus cow-calf pairs within 24 hours of parturition (d0). The purple dots represent the bacterial community within the CON cow-calf pairs (n = 5) and the blue dots represent the bacterial community within the BLG cow- calf pairs (n = 7). The dashed circle represents the 95% confidence interval of the sample group. There was no difference in the beta diversity for the dam vaginal (*P* = 0.66) bacterial communities between the BLG vs. CON. However, there was a trend both the calf nasal microbiota (*P* = 0.08) for the BLG pairs to have altered beta diversity compared to CON.

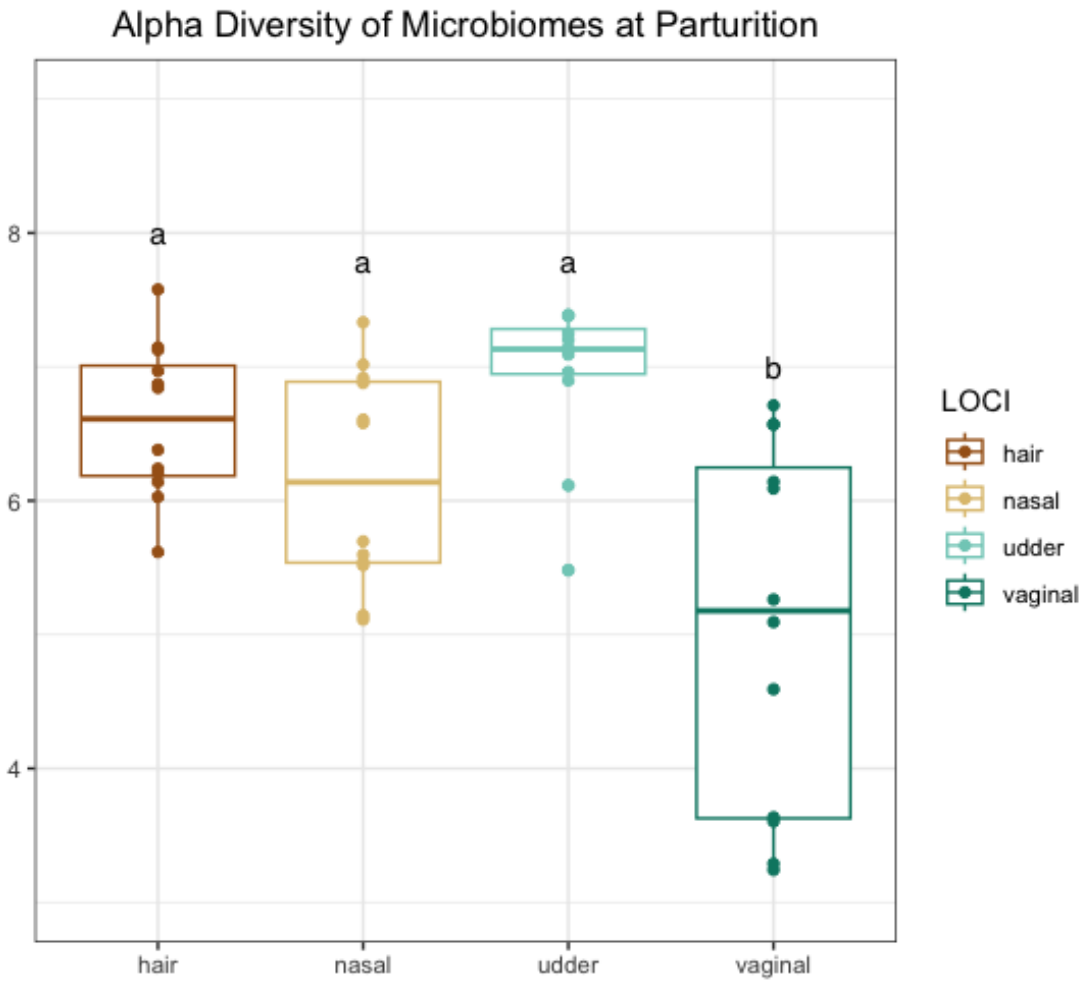

Figure 5 Alpha Diversity Between Sampling Location

Alpha diversity boxplot of the dam vaginal, calf nasal, dam udder, and dam haircoat bacterial community in Angus cow-calf pairs (n = 12) within 24 hours of parturition (d0) measured by the Shannon diversity index. The sampling locations are represented by different colored dots with dam vaginal (teal), calf nasal (yellow), dam udder (light blue), and dam haircoat (brown). The dashed circle represents the 95% confidence interval of the sample location. There was a main effect of location ($P = 0.002$) where the dam vaginal microbiota had decreased alpha diversity compared to the dam haircoat, dam udder, and calf nasal bacterial communities ($P < 0.05$).

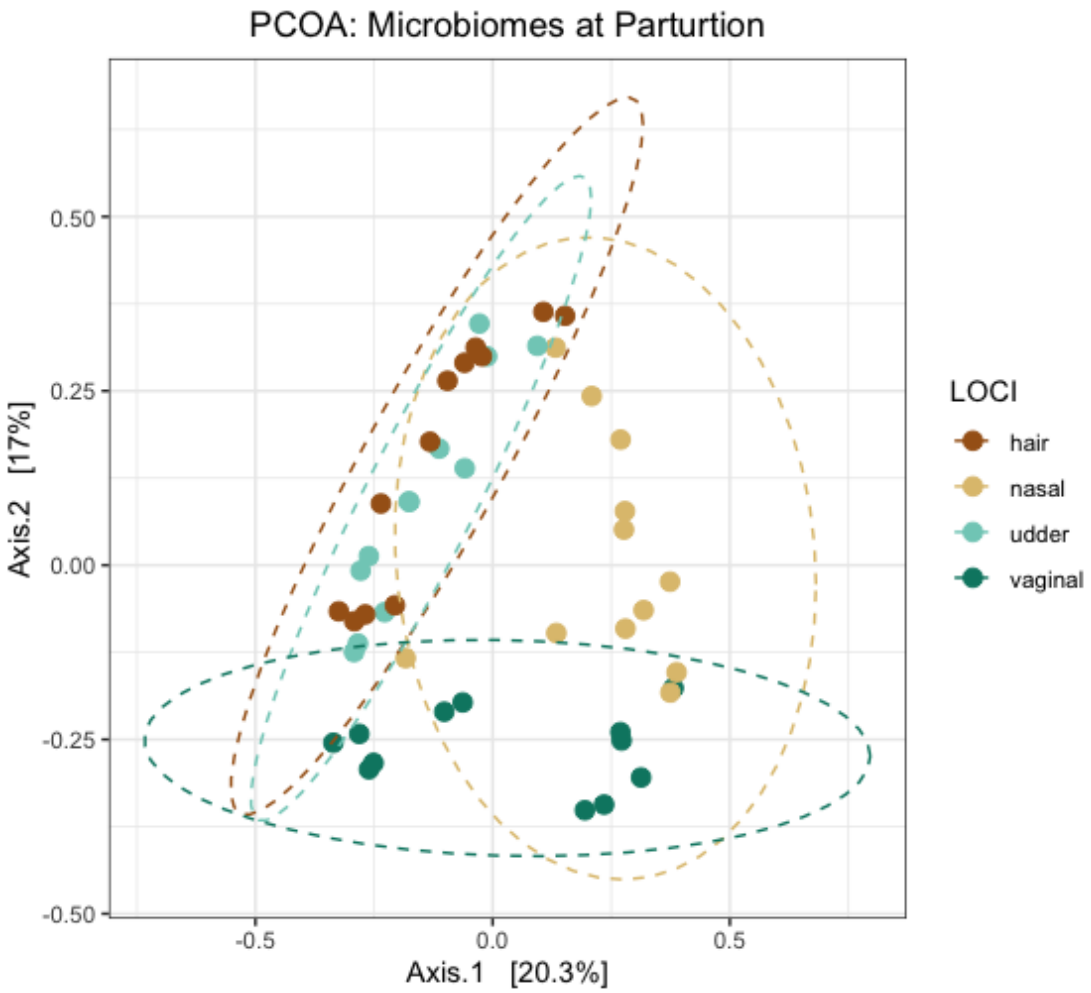

Figure 6 Beta Diversity Between Sampling Locations

Principal coordinate analysis (PCoA) depicting Bray-Curtis dissimilarities across samples of the dam vaginal, calf nasal, dam udder, and dam haircoat bacterial communities in Angus cow-calf pairs (n = 12) within 24 hours of parturition (d0). The sample locations ordered from left to right is dam haircoat (brown boxplot), calf nasal (yellow boxplot), dam udder (light blue boxplot), and dam vaginal (teal boxplot). Colored dots represent values for individual samples within the location. There was a main effect of location ($P = 0.001$) where the dam udder and haircoat locations had similar beta diversity. The dam vaginal microbiota was different from all other sample locations ($P < 0.05$). The calf nasal microbiota was different from all other sample locations ($P < 0.05$).

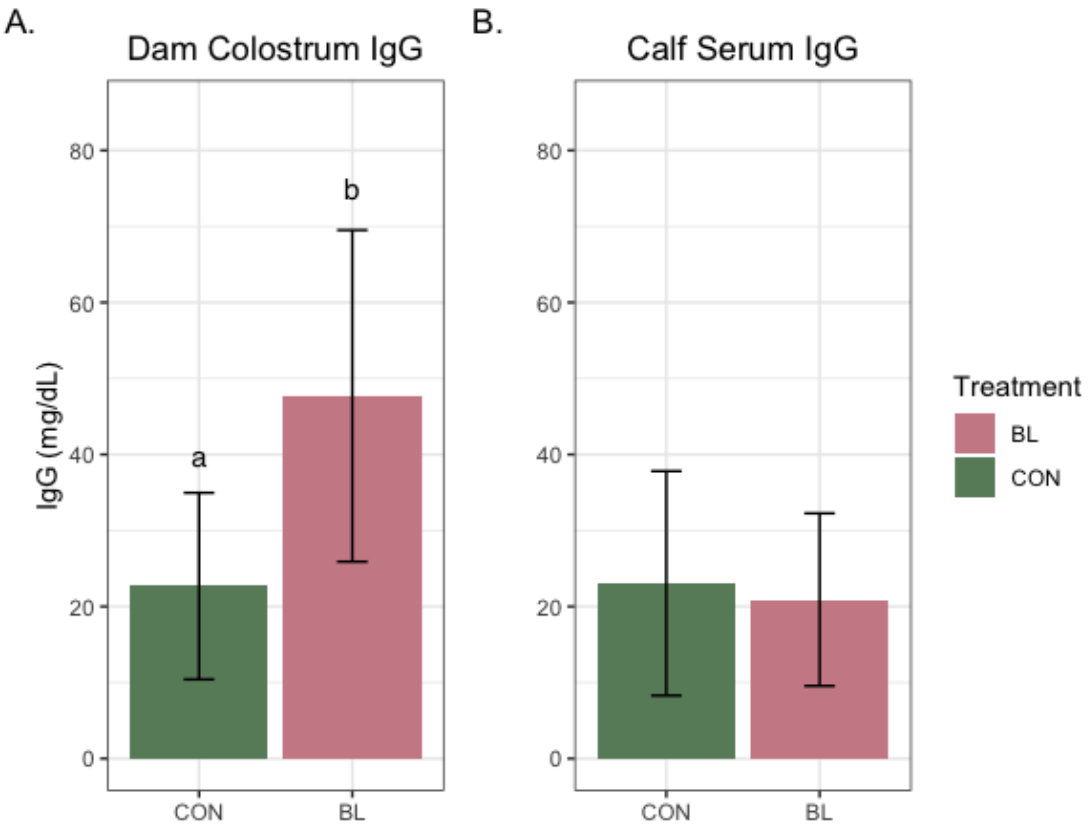

Figure 7 IgG Concentrations in Dam Colostrum and Calf Sera

Bar graph representing the IgG (mg/dL) within the dam colostrum (Panel A) and calf serum (Panel B) between BLG vs. CON cow-calf pairs (n = 22). The CON cow-calf pairs are represented by the left green bar and the BLG cow-calf pairs are represented by the right pink bar. There was a significant difference in dam colostrum IgG levels between BLG and CON dams (P = 0.04; 47.70 ± 21.8 vs. 22.66 ± 12.27). There was no significant difference in calf sera IgG between BLG and CON calves (P = 0.88; 20.9 ± 11.35 vs. 23.05 ± 14.76).

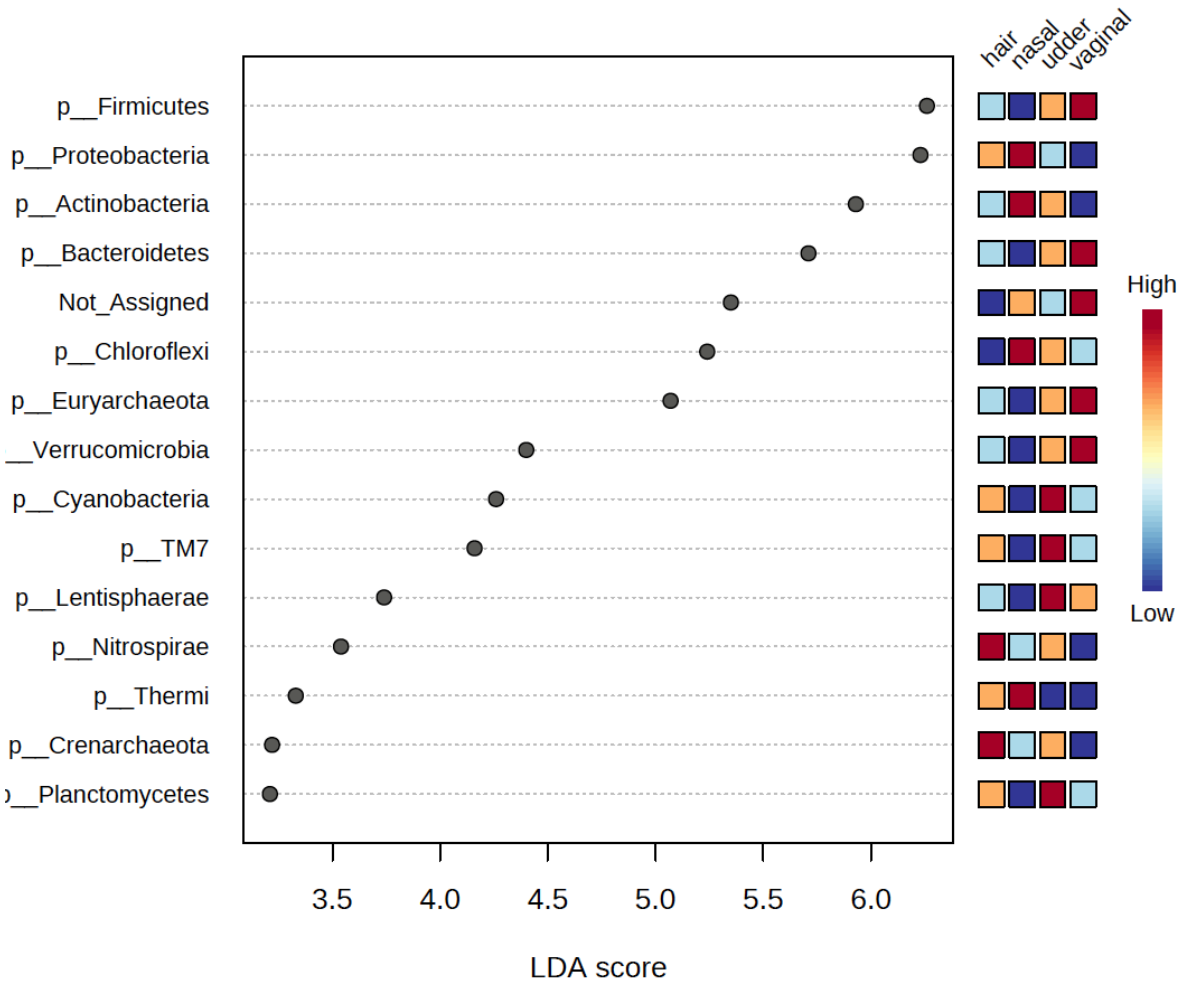

Figure 8 Phyla Differences Between Sampling Location

Dot plot depicting the LDA scores computed for differentially abundant phyla between sampling location on day 0 ($P < 0.05$).

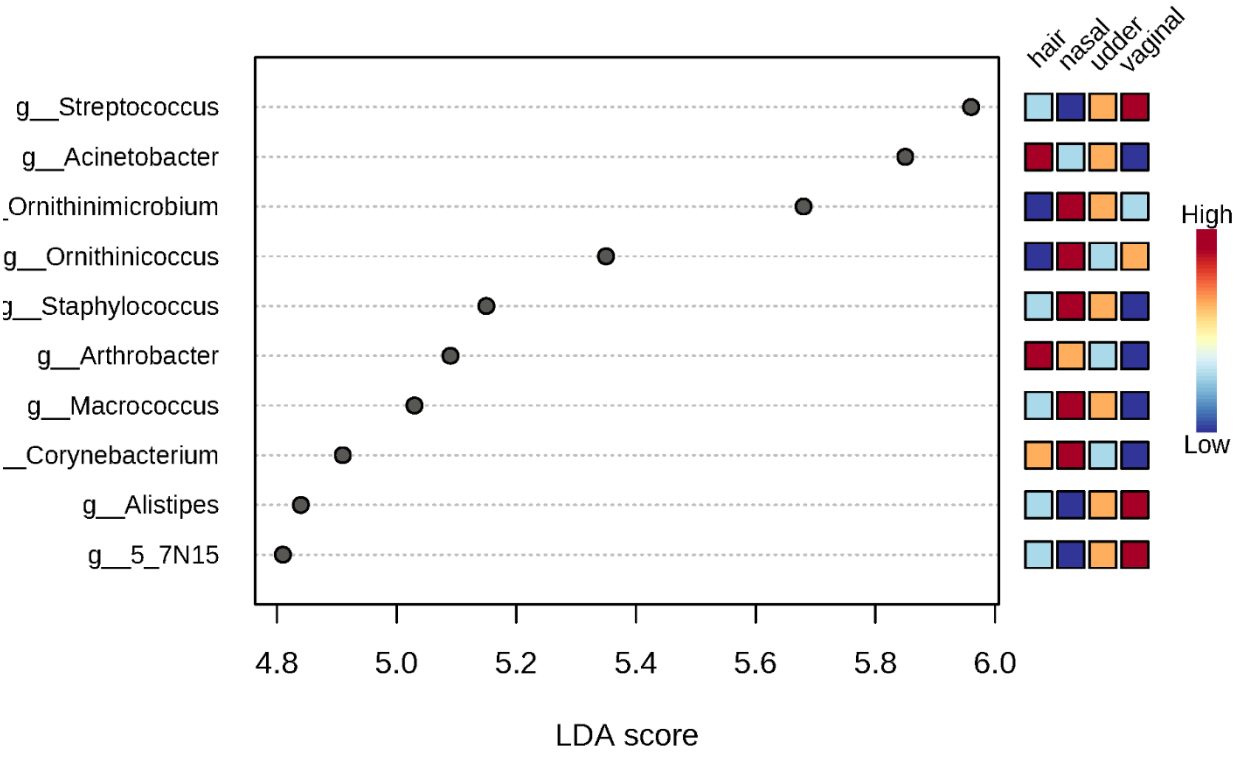

Figure 9 Genera Differences Between Location

Dot plot depicting the LDA scores computed for differentially abundant genera between sampling location on day 0 ($P < 0.05$).

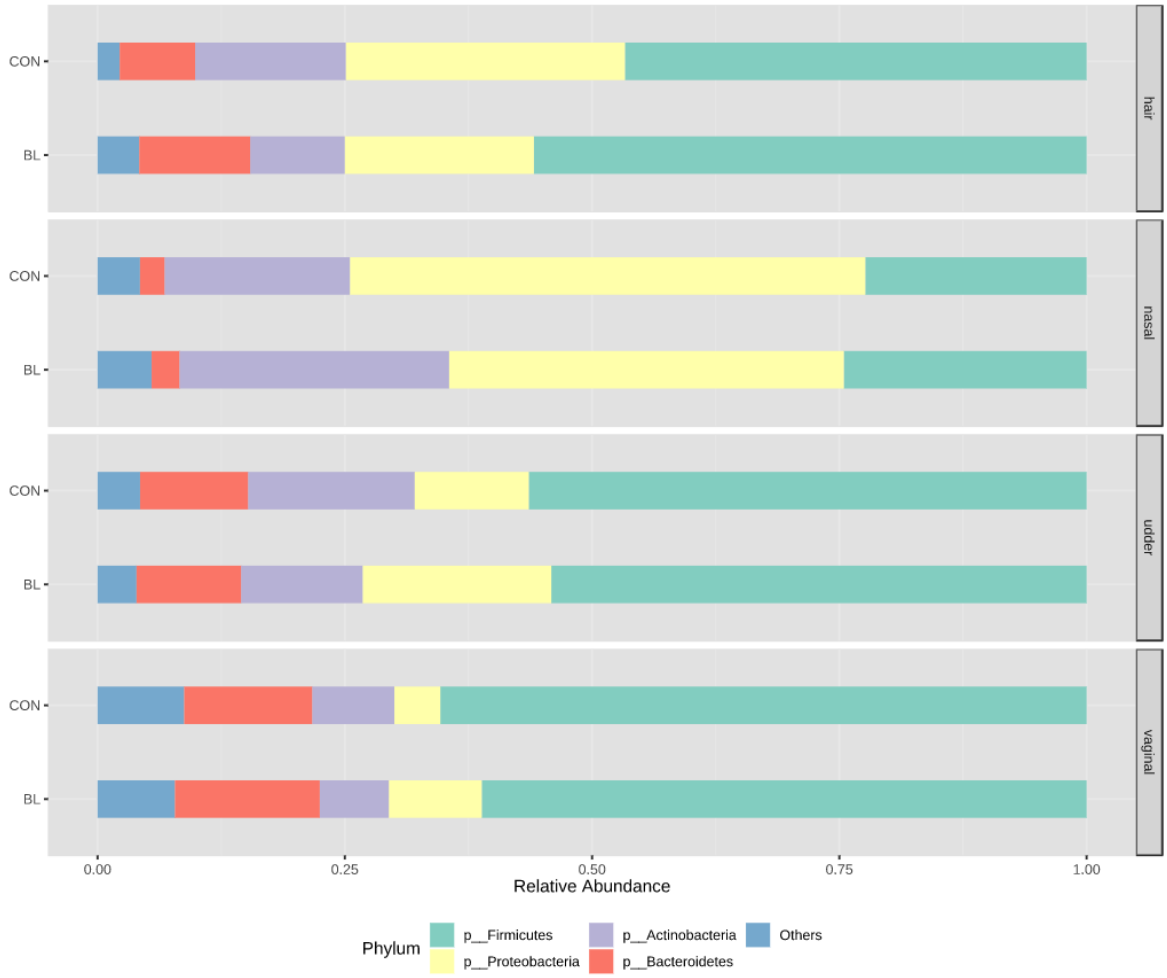

Figure 10 Most Abundant Phyla of BLG vs. CON within Sampling Location

Relative abundance of the 4 most abundant phyla level taxa of BLG (n = 7) vs. CON (n = 5) cow-calf pairs within sampling location on day 0. Each bar represents 100% of the taxa with the colors representing different phyla that are proportional to the percentage of the phyla's relative abundance.

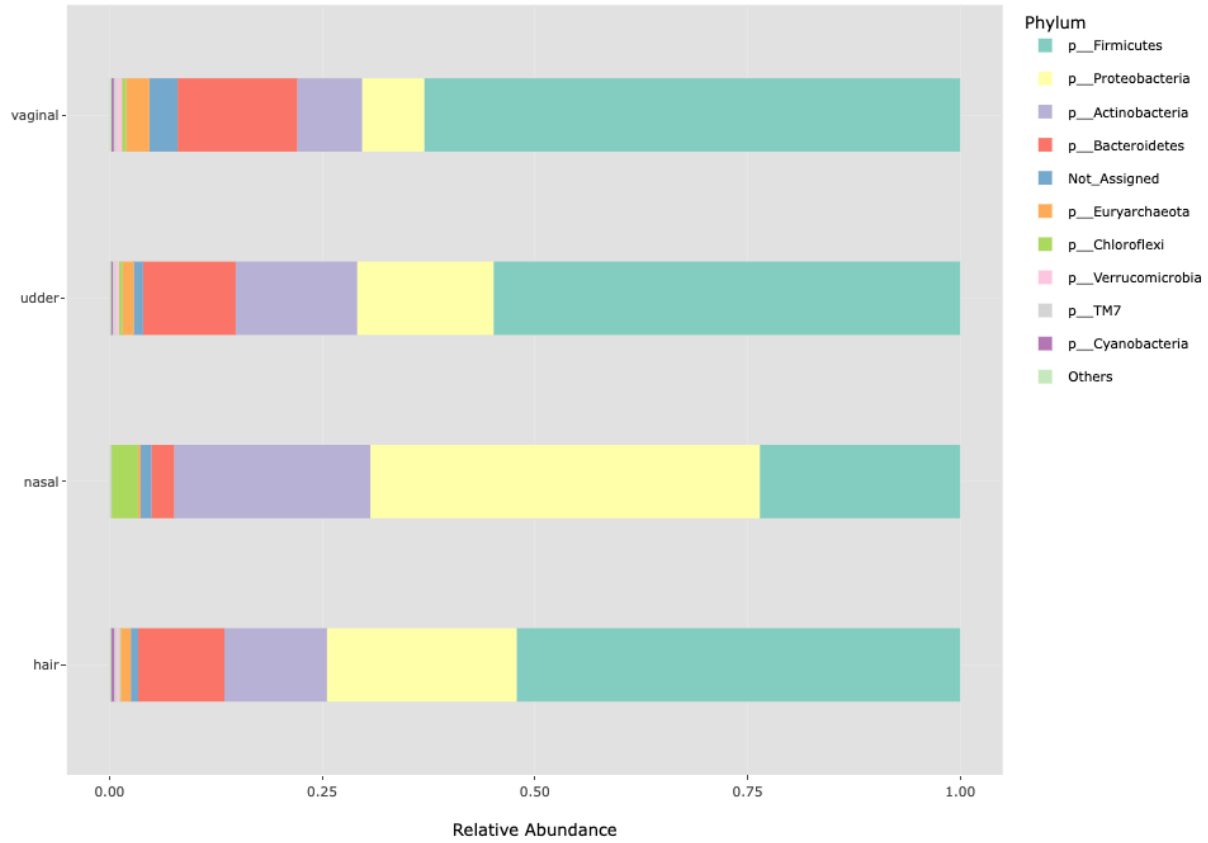

Figure 11 Top 10 Phyla within Sampling Location

Relative abundance of the 10 most abundant phyla level taxa within sampling location on day 0. Each bar represents 100% of the taxa with the colors representing different phyla that are proportional to the percentage of the phyla's relative abundance.

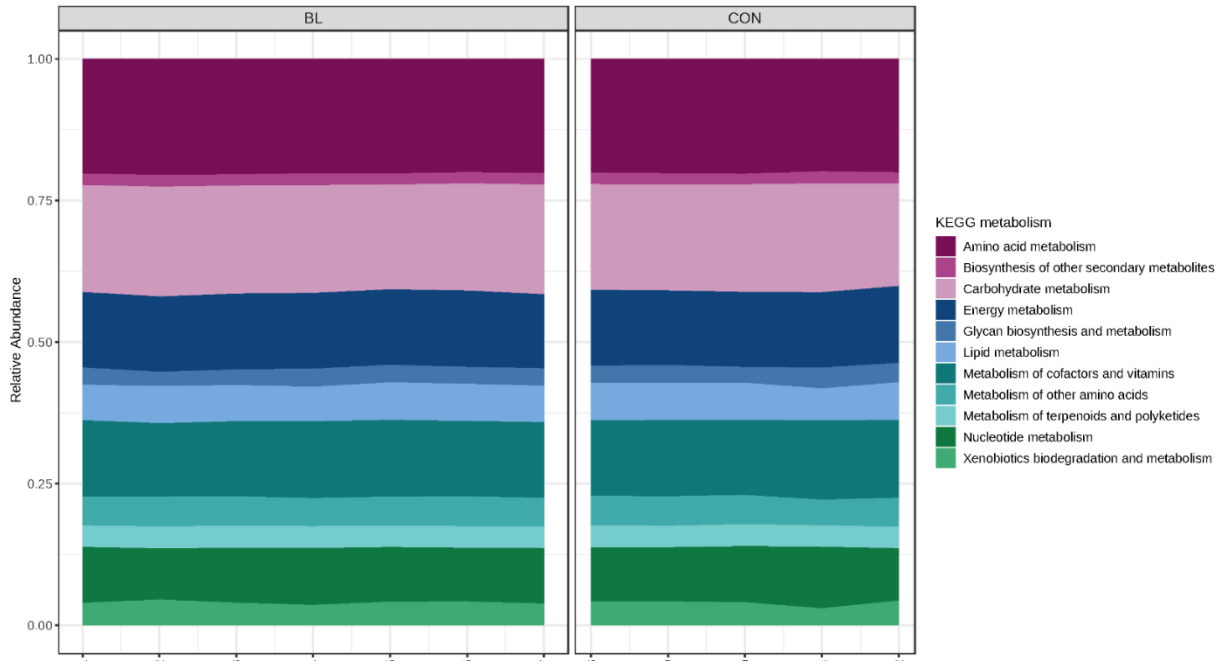

Figure 12 KEGG Functional Profiling Between BLG vs. CON Calf Nasal Microbiota

Percentage representation of the Kyoto Encyclopedia of Genes and Genomes (KEGG) functional profiling modules between the BLG calves (BL) and CON calves' nasal microbiota. There were no significant differences between BLG and CON calves ($P > 0.05$).

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CHAPTER III

COMPOSITIONAL COMPARISONS OF THE BOVINE CALF NASAL AND DAM VAGINAL MICROBIAL COMMUNITIES POST-CALVING IN RELATION TO IMMUNE STATUS

Abstract

There is limited literature characterizing the dam vaginal and calf nasal microbiota post-partum in beef cattle. In humans, the vaginal microbiota is considered the first major inoculating community within the neonate. Thus, the dam vaginal microbiota could drive commensal microbial community structure within neonates. Exposure to inoculating communities combined with calf immune responses during early life likely drives microbiome establishment. Therefore, this study aimed to characterize differences in both the dam post-partum vaginal microbiota and neonatal calf nasal microbiota after utilizing betadine lavages to alter the vaginal microbiota prior to parturition and evaluate systemic immune responses in both the dam and neonate concurrently with microbiota sampling. Beef cows (n = 12) were randomly assigned to either the control group (CON) or BL treatment group (BLG) two weeks prior to calving. Treatment BL bags were infused into the anterior vagina and cows received 1-3 treatments depending on calving date. The microbiota of the dam vaginal and calf nasal and blood samples were collected on day 0, 15, 30, and 60 post-partum and stored at -80°C. The vaginal bacterial community composition was determined through sequencing of the V3-V4 region of the 16S rRNA gene using the Illumina Miseq platform. Alpha diversity was compared via two-way ANOVA; beta

diversity was compared via PERMANOVA. Taxonomic differences were evaluated using the LEfSe platform. Immune statuses in dams and calves were analyzed via the D2Dx immunity test kit. All immune and microbiota data were analyzed using the R software package (v. 2023.03+386). Within the calf nasal microbiota, there was a main effect of time ($P = 0.001$). The calf nasal microbiota at day 0 was significantly different from the nasal microbiota at day 15, 30, and 60 ($P < 0.05$). The calf nasal microbiota at day 30 was significantly different from the calf nasal microbiota at day 15 and 60 ($P < 0.05$). The beta diversity of the calf nasal microbiota was significantly different at day 0 compared to all other timepoints ($P = 0.006$). The calf nasal beta diversity at day 15 was similar to day 30 ($P = 0.38$) but significantly different compared to day 60 ($P = 0.006$). There was no effect of time on altering the alpha ($P = 0.60$) or beta ($P = 0.06$). diversity of the dam vaginal microbiota. The alpha diversity of the dam vaginal microbiota was similar to the calf nasal microbiota at day 0 and 30 ($P = 0.06$). The beta diversity within each vaginal bacterial community (day 0, 15, 30, 60) were significantly different from the beta diversity within each nasal bacterial community (day 0, 15, 30, 60; $P < 0.05$). Calves had an elevated immune response at 60d compared to day 0 and 30 ($P < 0.001$). Together, these data indicate that there is a clear divergence of the calf nasal microbiota from the theoretical inoculating community of the dam vaginal tract on day 0. This finding is unexpected but could be linked to the maturation of the calf immune system. Further research evaluating these microbial communities and the interconnection of the immune system in beef calf neonatal inoculation is needed.

Key Words: microbiota, neonatal inoculation, immune response

Introduction

As research investigating bovine reproductive tract microbial communities increases, there is a need to understand implications of residential microbial communities throughout production lifecycle of cattle. Current research is delving into the intricacies of microbial populations within the uterine and vaginal communities related to pregnancy attainment and maintenance, especially during the embryotic phase (Poole et al., 2023; Yagisawa et al., 2023). However, there is limited literature evaluating how the physiological insult of parturition, at the end of gestation, is potentially impacting not only the dam post-partum reproductive tract microbiota but also early microbial inoculation within the neonate. Moreover, advanced sequencing practices and bioinformatics are beginning to clearly demonstrate a regulatory entanglement of the host microbiome and immune system (Zheng et al., 2020). There is a clear need to investigate general immune status within post-partum dams and calves related to microbial community composition.

In humans, infants have a diverging gut microbial community dependent on delivery method (Dominguez-Bello et al., 2010). In infants delivered vaginally, the inoculating and residential microbial communities in the infant oral cavity, nasal cavity, and gut are similar to the maternal vaginal microbiota (Zhang et al., 2021). However, in infants delivered via Cesarean section, the infant microbial communities more closely matched the maternal skin (Goedert, 2016). Compared to vaginally delivered infants, Cesarean section delivered infants had significantly decreased evenness, richness, and phylogenetic diversity within the first month of life, which sometimes persisted up to the age of 2 (Bokulich et al., 2016; Jakobsson et al., 2013). Moreover, children delivered via Cesarean section have been shown to have increased risk for food allergy (Papathoma et al., 2016), asthma (Stokholm et al., 2020), Type I diabetes (Bonifacio

et al., 2011), Type II diabetes (Chavarro et al., 2020), and obesity (Isolauri et al., 2017; Martinez et al., 2017). In cattle, Cesarean sections are less prevalent; thus, the majority of calves are inoculated with the dam vaginal microbiota during parturition. The environment in which parturition occurs in cattle is vastly different than humans, and the vaginal microbiota in cattle is compositionally and physiologically different than humans (Swartz et al., 2014). Therefore, in cattle, neonates born to cows with no vaginal microbiota or a vaginal microbiota in dysbiosis prior to parturition should be evaluated to discover if there is any link to immune function or neonatal microbiome development.

After parturition, the reproductive tract of the dam must recover from a massive physiological insult. From a microbial inoculation perspective, the vaginal microbiota naturally ascends into the uterine body during parturition (Dominguez-Bello et al., 2010), and after parturition any bacteria introduced via fecal contamination, environment, or human assistance during parturition can also migrate into the uterine body leading to infection (Sheldon et al., 2008). The majority of current bovine post-partum reproductive tract microbiota literature evaluates the effects of uterine disease (endometritis, metritis) on fertility within dairy cattle (Sheldon et al., 2020). However, in beef cattle, the compositional changes within vaginal microbiota after parturition have not been fully elucidated.

Therefore, the objective of this study was to characterize differences in both the dam post-partum vaginal microbiota and neonatal calf post-partum nasal microbiota after utilizing betadine lavages to alter the vaginal microbiota prior to parturition. We also aimed to evaluate systemic immune responses in both the dam and neonate concurrently with microbiota sampling. We hypothesized that dams receiving betadine lavages prior to parturition and their calves would have an altered vaginal microbiome and nasal microbiome, respectively, compared to controls.

Moreover, we hypothesized that as microbial communities fluctuate there will be mimicked trends within the immune status in both dams and calves.

Materials & Methods

Animal Management and Treatments

Animal care and use were approved by the Mississippi State University Institutional Animal Care and Use Committee (#21-076). Multiparous beef cows (n = 12) bred to separate sires (n = 3) were housed at the H.H. Leveck Animal Research Center (Mississippi State, MS) in a 2-acre pasture during calving and were moved to a 25-acre pasture after calving. Cows were provided ad libitum round bale hay and water throughout the project. Prior to the calving, all cows had a body condition score (BCS) of 6 ± 0.5. Diets were adjusted, 4 weeks prior to rebreeding, to include a concentrate (2.27 kg/hd/d) to address decreasing BCS of cows post-calving. All cows calved within 12 days of the expected calving date.

Three weeks prior to calving, cows were divided into two treatment groups, betadine lavage (BLG; n = 7) or control (CON; n = 5). Vaginal swabs and vaginal betadine lavages were performed once weekly until calving, cows either received one (n = 2) or two (n = 5) lavages prior to calving. The lavage bags were composed of 200mL Betadine (5% povidone-iodine) diluted in 800mL of Lactated Ringer's solution for a final dilution of 0.5% povidone-iodine per lavage Swabs were collected from the dam vaginal and calf nasal tract at (0d ± 1), (15d ± 1), (30d ± 1), and (60d ± 1) post-parturition. Blood samples were also collected at this time for immune analysis. Angus pair (n = 12) swab samples underwent 16S bacterial community analysis and functional prediction analysis.

Swab Collection

A double guarded equine uterine culture swab (Minitube Ref. 17214/2950) was utilized to sample the anterior vaginal tract of each cow and the nasal tract of each calf within 24 hours after calving. After a sample was collected, the swab unit was broken down by removing the external layer to expose the swab in the sterile tubing. The sterile tube containing the swab was snapped at a pre-determined length, then capped with sterile caps to prevent airborne contamination. All swabs were stored at -80°C until further analysis.

Vaginal Swab Collection

Cows were restrained in a hydraulic chute and the vulva was cleaned by wiping with a paper towel to prevent swab contamination. The double guarded unit containing the swab was removed from sterile packaging and immediately inserted through the vulva into the vaginal tract. The swab was angled upward, over the pelvic shelf, and towards the anterior vagina. Once the swab would not move forward with pressure, the cotton swab was exposed from the sterile guarding to make direct contact with the anterior vagina. The swab was rotated for approximately 30 seconds then retracted back into the sterile guarding. The entire double guarded swab unit was removed from the cow's vaginal tract. The swab was closely examined in the sterile guarding for any urine (yellow staining) or feces. If contamination looked possible, the cow was re-swabbed.

Calf Nasal Swab Collection

Calves were restrained manually by two trained personnel. The double guarded unit containing the swab was removed from sterile packaging and placed within the cranial portion of the nasal canal. The cotton swab was exposed from the sterile guarding and advanced into the nasal canal (approximately 8-10 cm) to make direct contact with the nasopharynx. The sampling

continued for approximately 30 seconds, then the swab was retracted back into the sterile guarding. This process was then repeated, using the same swab unit in the opposite nasal canal. Thus, each nasal swab sampled both the right and left nasal tracts of the calf.

Blood Collection & D2Dx Immunity Test Kit

Whole blood was collected via jugular venipuncture from dams and calves within 24 hours post-partum. Blood was allowed to clot at room temperature and placed on ice until transported to the laboratory for processing. Approximately one hour after collection, blood tubes were centrifuged at 2000xg at 4°C for 10 minutes. Serum was immediately collected and transferred into sterile 2 ml tubes and then stored at -80°C until further analysis.

Immune status was evaluated using the D2Dx immunity test kit (NanoDiscovery Inc., Orlando, FL). Briefly, the D2Dx kit detects antibody-mediated immune responses within a blood sample by using a gold nanoparticle as a pseudo pathogen; the proteins from the humoral immune system (IgG, IgM) and complement proteins interact with the gold and the interaction is quantified by monitoring average nanoparticle size change via dynamic light scattering giving a final test score (Zheng et al., 2020). In this study, samples were thawed for 3 days at -20°C and allowed to equilibrate at room temperature for 3 hours prior to the assay in accordance with the company instructions. The gold nanoparticle was added to the serum sample, vortexed for 10s, then placed in the D2Dx CT-100 reader. A measurement is taken immediately after the sample is placed in the reader and 30s later; the difference between the two measurements is the test score for that sample.

Bacterial Community Analysis 16S

Samples from Angus dam-calf pairs (n = 12) were selected to undergo 16S bacterial community analysis. The 16S bacterial community analysis was performed by Zymo Research Corporation located in Irvine, CA. Genomic DNA was extracted using the ZymoBIOMICS -96 MagBead DNA Kit (Zymo Research; Irvine, CA) following the manufacturer's protocol. ZymoBIOMICS microbial community standard (Zymo Research; Irvine, CA) was used as a positive control for each DNA extraction. The ZymoBIOMICS microbial community DNA standard (Zymo Research; Irvine, CA) was used as a positive control for each targeted library preparation. Negative controls (blank extraction control, blank library preparation control) were included to assess contamination during the wet-lab process. The DNA samples were prepared for targeted sequencing with the *Quick*-16S Primer Set V3-V4 Plus NGS Library Prep Kit (Zymo Research; Irvine, CA). The sequencing library was prepared using real-time PCR to control cycles and limit PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned up with the Select-a-Size DNA Clean and Concentrator (Zymo Research; Irvine, CA), then quantified with TapeStation (Agilent Technologies, Santa Clara, CA) and Qubit (Thermo Fisher Scientific, Waltham, WA). Sequencing was done with an Illumina MiSeq (Illumina, San Diego, CA, USA) with a v3 reagent kit (600 cycles). The sequencing was performed with 10% PhiX spike-in.

16S sequence data were processed and analyzed using the plugin-based microbiome bioinformatics framework QIIME 2 (Bolyen et al., 2019)(V 2022.8). Cutadapt (v.4.1) (Martin, 2011) was used to remove the primer sequences, from both forward, and reverse reads. DADA2 (Callahan et al., 2016) was used (via the q2-dada2 QIIME 2 plugin) to quality filter the sequence

data, removing chimeric, and erroneous reads. Sequences were further trimmed to remove reads where the average quality score dropped below 25 and clustered to ASVs (Amplicon sequence variants) after denoising with DADA2 in QIIME2. Taxonomy was assigned to each sequence variant using q2-feature-classifier plugin (Bokulich et al., 2018) in QIIME 2 with a pre-trained classifier from the SILVA database (Janssen et al., 2018) . The final output table of amplicon sequence variants (ASVs) was used to analyse bacterial community diversity, structure, and composition.

Statistical Analysis

The vaginal bacterial community comparisons of interest were the dam vaginal bacterial communities over time, calf nasal communities over time, and the effect of treatment within each timepoint in the dam vaginal and calf nasal microbiota. The R software program (R Core Team, 2013) was used to conduct the statistical analyses, specifically using the Phyloseq package pipeline (McMurdie & Holmes, 2013). Alpha diversity was calculated using the Shannon index and significance was tested using ANOVA. Beta diversity was computed using the Bray-Curtis dissimilarity and visualized using the principal coordinate analysis (PCoA) plot. Differences in community structure were assessed using the permutational multivariate analysis of variance (PERMANOVA) with the microbial community as the main fixed factor and using 9,999 permutations for significance testing in R (Adonis function from the Vegan package). Microbiome Analyst was applied to evaluate taxonomic differences via LEfSe analysis, differences in relative abundance, and functional predictions (Dhariwal et al., 2017). The predetermined p-value cut off was set to ($P < 0.05$) for all Microbiome Analyst statistical analysis. The PROC mixed procedure in SAS was used to analyze the D2Dx immune responses

with repeated measures. Significance was set to ($P < 0.05$); tendencies were set to ($0.05 < P < 0.01$).

Results

Bacterial Community Structure

A total of 96 swabs were analyzed for 16S sequencing. Vaginal, nasal swabs from BLG cow-calf pairs (n = 7) or CON pairs (n = 5) were analyzed. Both negative and positive controls were utilized throughout laboratory preparation for contamination checks, and contamination was accounted for within the analytic pipeline. A total of 12,589,488 quality filtered reads were obtained with an average of 95,374 quality filtered reads per sample that were assigned to 16,932 ASVs, after quality control analyses and ASV filtering. The four most abundant phyla within the calf nasal microbiota samples were Proteobacteria (46.5%), Actinobacteria (28.6%), Firmicutes (17.8%), and Chloroflexi (2.6%). The four most abundant phyla within the dam vaginal microbiota samples were Firmicutes (61.3%), Bacteroidetes (12.2%), Proteobacteria (9.8%), and Actinobacteria (7.5%; Fig 20).

Treatment Effects on Calf Nasal Microbiota

On day 0, the alpha diversity of the calf nasal microbiota was significantly different ($P = 0.03$; Fig13A) and the beta diversity was not different ($P = 0.08$) between BLG vs. CON calves. However, this difference did not persist over time. There were no differences in alpha diversity between the BLG calf nasal microbiota on day 15 ($P = 0.79$), day 30 ($P = 0.76$), or day 60 ($P = 0.80$; Fig 13A) compared to CON calves. There were no differences in beta diversity between the BLG calf nasal microbiota on day 15 ($P = 0.67$), day 30 ($P = 0.42$), or day 60 ($P = 0.14$) compared to CON calves.

There was a main effect of time ($P = 0.001$; Fig13C). The calf nasal microbiota at day 0 was significantly different from the nasal microbiota at day 15, 30, and 60 ($P < 0.05$; Fig 13C). The calf nasal microbiota at day 60 was significantly different from the calf nasal microbiota at day 30 and 60 ($P < 0.05$; Fig 13C). The alpha diversity of the calf nasal microbiota was different at day 0 compared to all other timepoints ($P < 0.001$). The alpha diversity at day 15 was similar to day 60, but significantly different compared to day 30 ($P < 0.001$). The beta diversity of the calf nasal microbiota was significantly different at day 0 compared to all other timepoints ($P = 0.006$; Fig 14A). The calf nasal beta diversity at day 15 was similar to day 30 ($P = 0.38$) but significantly different compared to day 60 ($P = 0.006$; Fig 14A).

A linear discriminant analysis (LDA) was conducted to evaluate differences at the phyla and genera level between BLG and CON within the calf nasal microbiota. There were no significantly different phyla or genera with a p-value cut-off of ($P = 0.05$) between the BLG and CON calves' nasal microbiota at day 0, 15, 30, or 60.

Treatment Effects on Dam Vaginal Microbiota

There were no differences in alpha diversity between the BLG dam vaginal microbiota on day 0 ($P = 0.42$), day 15 ($P = 0.79$), day 30 ($P = 0.76$), or day 60 ($P = 0.80$) compared to CON dams (Fig 13B). Moreover, there were no differences in beta diversity between the BLG dam vaginal microbiota on day 0 ($P = 0.66$), day 15 ($P = 0.67$), day 30 ($P = 0.42$), or day 60 ($P = 0.14$) compared to CON calves. There was no effect of time on altering the alpha ($P = 0.60$; Fig 13D) or beta ($P = 0.06$; Fig 14B) diversity of the dam vaginal microbiota.

A linear discriminant analysis (LDA) was conducted to evaluate differences at the phyla and genera level between BLG and CON within the dam vaginal microbiota. There were no

significantly different phyla or genera with a p-value cut-off of ($P = 0.05$) between the BLG and CON dams' vaginal microbiota.

Similarities Between Dam Vaginal Microbiota and Calf Nasal Microbiota Over Time

The alpha diversity of the dam vaginal microbiota was similar over time. The species richness and evenness (alpha diversity) was similar in the calf nasal microbiota at day 0 and 30 to the dam vaginal microbiota at day 0, 15, 30, and 60 ($P < 0.05$; Fig 15), and the dam vaginal microbial communities at day 0, 15, and 30 were similar to the calf nasal microbiota at day 15 (Fig 15). However, there were no compositional similarities between the calf nasal microbiota or dam vaginal microbiota post-partum at any timepoint. This was represented by beta diversity within each vaginal bacterial community (day 0, 15, 30, 60) being significantly different from the beta diversity within each nasal bacterial community (day 0, 15, 30, 60; $P < 0.05$; Fig 16).

A linear discriminant analysis (LDA) was conducted to evaluate differences at the phyla and genera level between the dam vaginal microbiota and calf nasal microbiota. There were 16 significantly different phyla (Fig 21) and 82 genera (Fig 22) with a p-value cut-off of ($P = 0.05$) between the dam vaginal and calf nasal microbiota communities post-partum.

Functional Predictions

Functional predictions were utilized to further understand compositional differences utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) via the MicrobiomeAnalyst platform (Kanehisa et al., 2021; Dhariwal et al., 2017). There were no significant functional feature differences (KEGG pathways) in the dam vaginal microbiota over time (day 0, 15, 30, 60) when evaluating using a single factor analysis (Kruskal-Wallis test) or LDA with a p-value cut-off of ($P = 0.05$).

Moreover, functional differences were evaluated in the calf microbiota over time (day 0, 15, 30, 60) There were 4519 differentially expressed KEGGs over time in the calf nasal microbiota (P = 0.05).

Dam and Calf Immune Status Post-Partum

In dams, there was a main effect of day with immune responses decreasing at 60d postpartum compared to 0d ($P < 0.001$), 15d ($P < 0.001$), and 30d ($P < 0.001$; Fig 17). In calves, there was a main effect of day where immune responses were increased on 15d compared to 30d ($P = 0.046$) and decreased compared to 60d ($P = 0.005$; Fig 6). Moreover, there was a decreased immune response in calves at 0d ($P < 0.001$) and 30d ($P < 0.001$) compared to 60d (Fig 18). In calves, there was a tendency for BLG calves to have a decreased immune response compared to CON ($P = 0.08$; Fig 19).

Discussion

Literature evaluating the nasopharyngeal microbiota in healthy neonatal beef calves is extremely limited (McDaneld et al., 2019). Lima et al. (2019) evaluated the calf upper respiratory tract, calf fecal, dam vaginal, and dam fecal microbiota in Holstein cow-calf pairs (n = 100) at 3, 14, and 35 days of life; calves were separated from their dams at day 1 of life and house separately within calf hutches. In dairy calves, the dam vaginal microbiota and calf nasal microbiota shared 253 OTU and authors indicated the dam vaginal microbiota as a potential driver of calf nasal colonization until at least 35 days of life (Lima et al., 2019). This finding was surprising due to the separation of dam and calf at day 1 of life, which drove the current study to evaluate the dam vaginal and calf nasal microbiota in beef cattle post-partum. Due to the clear implications of the microbiota in neonatal health (Zheng et al., 2020) compounded with the

inoculating potential of the dam vaginal microbiota (Lima et al., 2019), drove the objective to alter the dam vaginal microbiota, via betadine lavage, prior to calving to evaluate effects on microbial composition and immune response.

Povidone iodine (Betadine) is a water-soluble iodine-releasing agent with broad spectrum antimicrobial activity against both Gram-negative, Gram-positive bacteria, and mature biofilms like those found in the vaginal tract (Yasuda et al., 1997; Hoekstra et al., 2017). Within this study, betadine lavages only altered alpha diversity of the calf nasal microbiota at day 0 and tended to alter the beta diversity. However, this difference in treatment groups did not persist over time. This difference could be attributed to BLG calves tending to a decreased immune response compared to CON calves.

To evaluate immune responses, the D2Dx immunity test was utilized. This relatively simple and quick blood test can be utilized chute side or on frozen samples (Zhang et al., 2021). Tsai et al. (2021) evaluated systemic immunes responses in post-partum lactating dairy cattle with the D2Dx test, verifying its implications in predicting or identifying adverse health conditions. Due to the broad effects of the microbiota on the immune system, the D2Dx immune test was a logical choice to understand immune fluctuations at given timepoints that can be further explored with more targeted analysis.

The overall immune status of calves at day 0, 15, and 30 was negligible (0.01-0.02 dx value) compared to healthy adult cattle (0.03-0.06 dx value; Tsai et al., 2021). This finding can be attributed to varying colostrum quality and quantity consumed within each neonate at day 0 and their subsequent immune system development. Although healthy calves are born with all immune system components, their adaptive immune system is not function until 2-4 weeks of age and maternal antigens have a half-life of 16 to 28 days (Fulton et al., 2004; Reber et al.,

2006). The D2Dx immune status was increased at day 60 compared to all other timepoints; this is likely due to the maturation of the calves' adaptive immune system.

Following a similar trend, the beta diversity of the calf nasal microbiota was similar at day 15 and 30 but diverged at day 60. Specifically, the nasal microbiota had increased variation between animals (beta diversity) at day 15 paired with decreased species richness (alpha diversity) compared to day 0 and 30. At day 30, there was a stark increase in species richness with similar variation between animals. However, by day 60, there was a decrease in the richness and evenness of the nasal microbiota compared to day 0 and 30 with a tighter clustering at day 60. This timeline is interesting as the majority of immune influence on the calf microbiota from 0-30 days would be dependent on passive transfer from the dam (Baintner, 2007). However, at day 60, the adaptive immune system of the calf has likely developed antibodies against environmental antigens, including virulent bacteria. Thus, the decrease in alpha diversity accompanied with a tighter clustering of individuals at day 60 could be driven by increased immune status within the calves.

Dam and calf pairs were moved from the 2-acre calving pasture to a 25-acre pasture within a week after calving. It is plausible that microbiome alterations from day 0 to 15 were a result of environmental change, but Lima et al. (2019) also observed a decreased in the calf nasal microbiota from day 0 to 14 of life. This consistent finding suggests a recolonization period between day 0 and 15 of life in calves; this period has potential for strategic therapeutic delivery to drive specific microbial colonization within the calf nasal tract. However, more research is needed to determine the effectiveness of altering recolonization in the neonate during this timepoint.

The dam vaginal microbiota was not altered by betadine lavage prior to calving at day 0, 15, 30, and 60. Moreover, there were no differences in alpha diversity or beta diversity of the dam vaginal microbiota between day 0, 15, 30, or 60. This is consistent with previous research in beef cattle describing the vaginal microbiota as consistent and difficult to alter (Messman et al., 2021). In dairy cows, reproductive microbiome research is focused on determining the bacterial communities that drive endometritis leading to decreased fertility (Pascottini et al., 2020). Dysbiosis within the reproductive tract is described as a decrease diversity and loss of heterogeneity within the biome, typically caused by pathogen overgrowth (Galvao et al., 2019). Within our study, all cows were within the same environment and showed no clinical signs of endometritis or reproductive tract infection post-partum. Thus, the lack of clear differences post-partum suggests that, in beef cattle, the reproductive tract microbiota can recover not only from a physiological insult prior to calving (betadine lavage) but also parturition itself. Authors hypothesize a plasticity within the vaginal microbiome after insults resulting in the lack of diversity indices differences in healthy post-partum beef cattle. The D2Dx values for dam immune responses post-partum were similar to values observed in post-partum dairy cows (Tsai et al., 2021) with an increased immune response observed until day 30 during the post-partum interval until a stark decrease at day 60 likely after the succession of uterine involution.

Finally, there were similarities in alpha diversity between calf and dam microbial communities post-partum. This finding suggests that calf nasal and dam vaginal microbial communities have similar high amounts of species diversity that ceases at day 60 post-partum; this result is likely due to the maturation of the calf's adaptive immune response decreasing species diversity within the calf. However, the beta diversity of the calf nasal microbiota was significantly different from the dam vaginal microbiota at all timepoints. This result was

inconsistent with previous literature in dairy calves (Lima et al., 2019). Husbandry differences between beef and dairy calves could alter the inoculating and commensal bacteria within the calf nasal tract leading to these observed differences.

Summary

There were no similar microbial communities in the calf nasal and dam vaginal microbiota at 0-, 15-, 30-, or 60-days post-partum. The dam vaginal microbiota composition did not change from parturition to 60 days post-partum. However, the calf nasal microbiota and immune responses fluctuated over time which can be attributed to the adaptive immune development in the calf. Thus, research focusing on the microbial composition combined with measuring specific cytokine responses in post-partum beef calves is needed to further understand this relationship. Moreover, it appears the plasticity and upregulation of immune responses post-partum within the dam could be advantageous for reproductive tract health. The lack of overlap in post-partum dam vaginal microbiota and calf nasal microbiota is contradicting of the current literature. However, more research investigating neonatal calf microbial inoculation and development is needed.

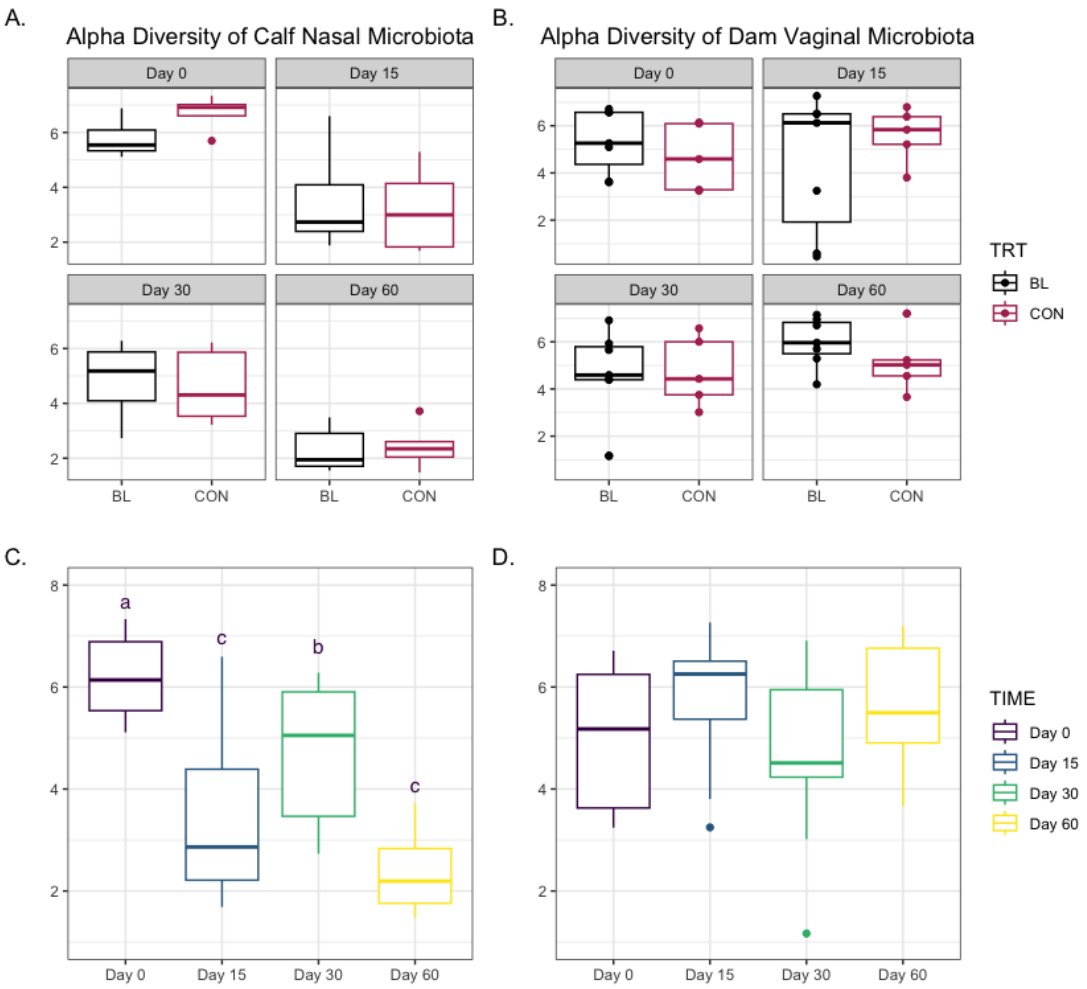

Figure 13 Alpha Diversity Boxplot of Treatment within Time and Effect of Time

Alpha diversity boxplot of the effect of treatment (BLG vs. CON) within each sampling timepoint (Day 0, 15, 30, 60) of the calf nasal microbiota (Panel A) and dam vaginal (Panel B) bacterial communities measured by the Shannon diversity index. The left black box represents the bacterial community within the BLG cow-calf pairs (n = 7), and the right maroon box represents the bacterial community within the CON cow- calf pairs (n = 5). The alpha diversity of the calf nasal microbiota was not different between BLG vs. CON within any sampling timepoint. Moreover, there were no differences in alpha diversity between BLG vs. CON within any sampling timepoint in the dam vaginal microbiota. Alpha diversity boxplot of the effect of location within the calf nasal microbiota (Panel C) and the dam vaginal microbiota (Panel D). There was a main effect of time in the calf nasal microbiota with day 0 being different from day 15, 30, and 60. The 30-day calf nasal microbiota was different from all other sampling timepoints. However, the 15 and 60-day calf nasal microbiota were similar. There was no effect of time within the dam vaginal microbiota. Significance was set at $P < 0.05$.

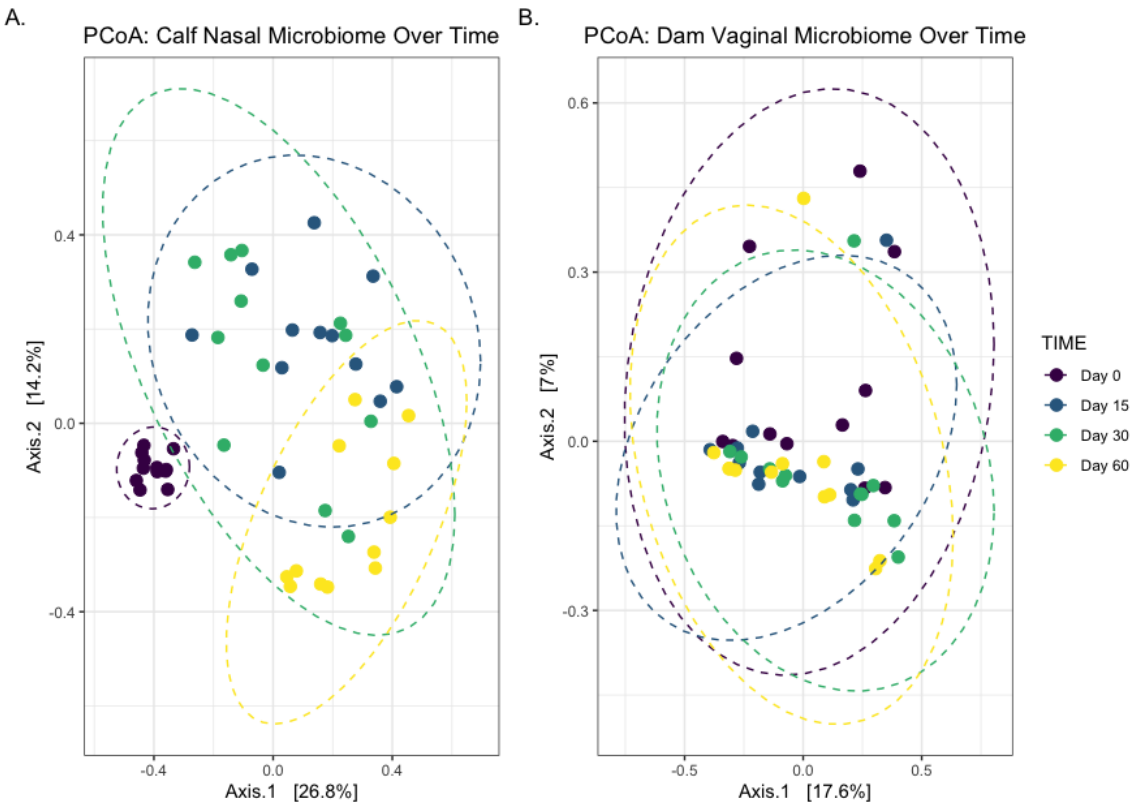

Figure 14 Beta Diversity of Dam Vaginal & Calf Nasal Microbiota Over Time

Principal coordinate analysis (PCoA) depicting Bray-Curtis dissimilarities across samples of the calf nasal (Panel A) and dam vaginal (Panel B) bacterial communities in Angus cow-calf pairs at 0-, 15-, 30-, and 60-days post-partum. The purple dots represent the bacterial community on day 0, the blue dots represent the bacterial communities on day 15, the green dots represent the bacterial communities on day 30, and the yellow dots represent the bacterial communities on day 60. The dashed circle represents the 95% confidence interval of that timepoint. There was no difference in the beta diversity for the dam vaginal bacterial communities between sampling timepoints. However, the beta diversity of the nasal microbiota on day 0 was different from all other timepoints. The calf nasal microbiota was similar on day 15 and 30 but was different from all other sampling timepoints at day 60. Significance was set at $P < 0.05$.

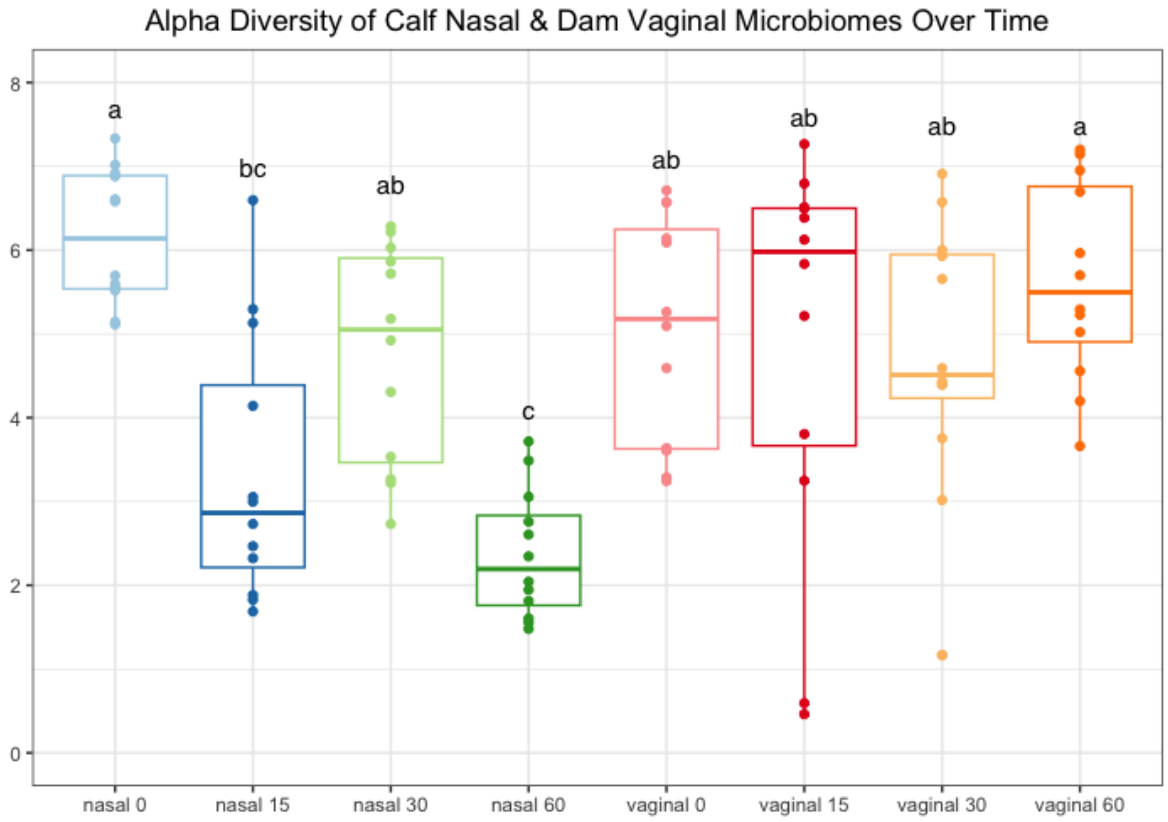

Figure 15 Alpha Diversity of Calf Nasal and Dam Vaginal Microbiota Over Time

Alpha diversity boxplot of the calf nasal and dam vaginal microbial communities over time. The alpha diversity of the dam vaginal microbiota was similar over time and similar to the calf nasal microbiota at day 0 and 30. The dam vaginal microbial communities at day 0, 15, and 30 were similar to the calf nasal microbiota at day 15. The calf nasal microbiota at day 15 and 60 were similar. Significance was set at $P < 0.05$.

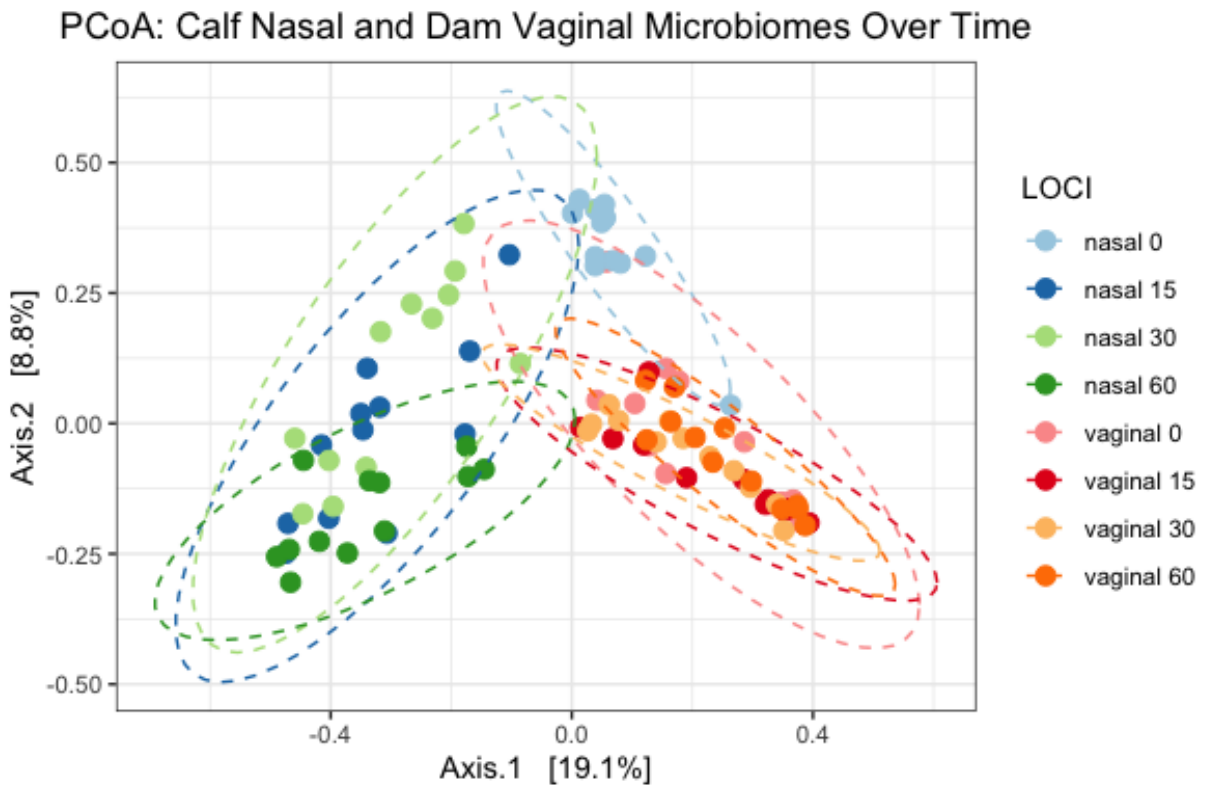

Figure 16 Beta Diversity of Calf Nasal and Dam Vaginal Microbiota Over Time

Principal coordinate analysis (PCoA) depicting Bray-Curtis dissimilarities across samples of the calf nasal dam vaginal bacterial communities in Angus cow-calf pairs at 0-, 15-, 30-, and 60-days post-partum. The light blue dots represent the calf nasal bacterial community on day 0, the dark blue dots represent the calf nasal bacterial communities on day 15, the light green dots represent the calf nasal bacterial communities on day 30, and the dark green dots represent the calf nasal bacterial communities on day 60. The light pink dots represent the dam vaginal bacterial community on day 0, the red dots represent the dam vaginal bacterial communities on day 15, the light orange dots represent the dam vaginal bacterial communities on day 30, and the dark orange dots represent the dam vaginal bacterial communities on day 60. The dashed circle represents the 95% confidence interval of that timepoint. All vaginal bacterial communities (day 0, 15, 30, 60) were significantly different from all nasal bacterial communities (day 0, 15, 30, 60). Moreover, the calf nasal microbiota at day 0 was significantly different from the nasal microbiota at day 15, 30, and 60. Moreover, the calf nasal microbiota at day 60 was significantly different from the calf nasal microbiota at day 30 and 60. Significance was set at $P < 0.05$.

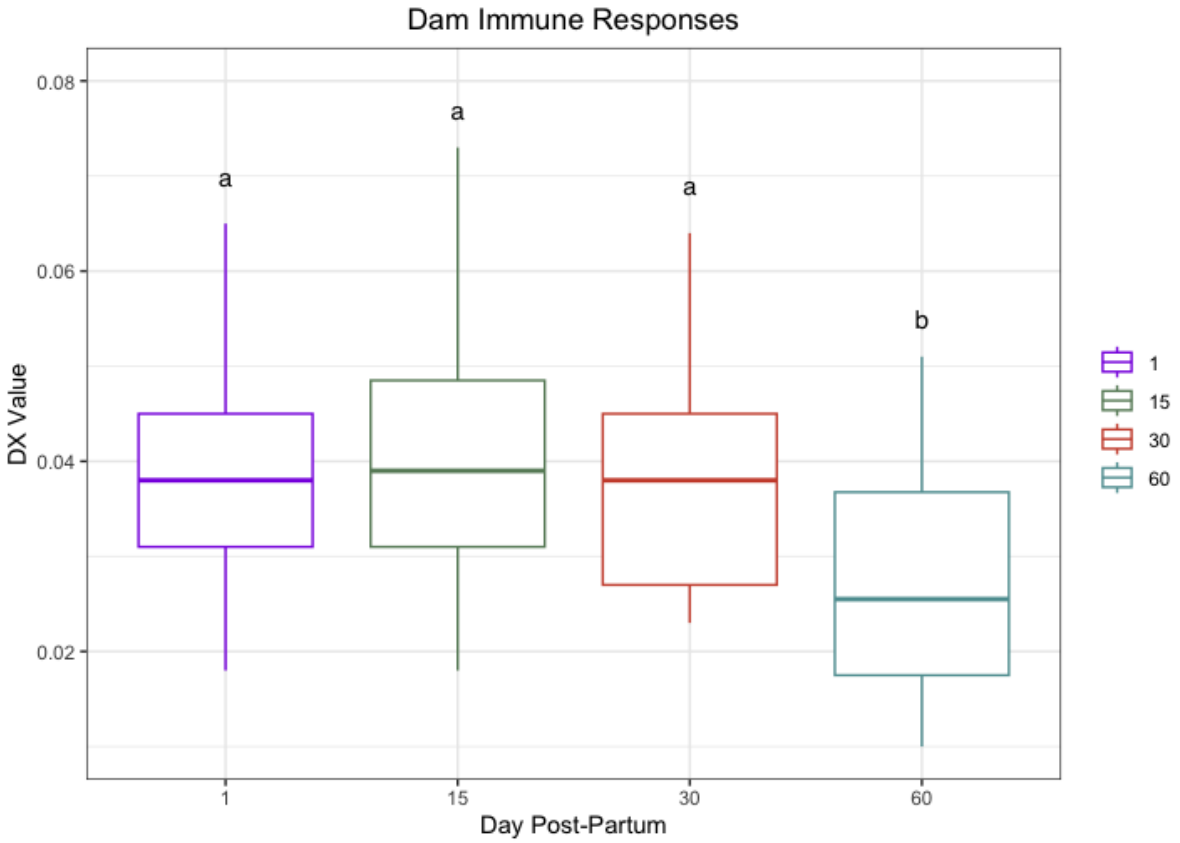

Figure 17 Immune Status of Dams Post-Partum

Boxplot depicting the D2Dx immune test kit values within dam serum at day 0, 15, 30, and 60 post-partum. There was a main effect of time with the dx immune response values on day 0, 15, and 30 being increased compared to day 60. Significance was set at $P < 0.05$.

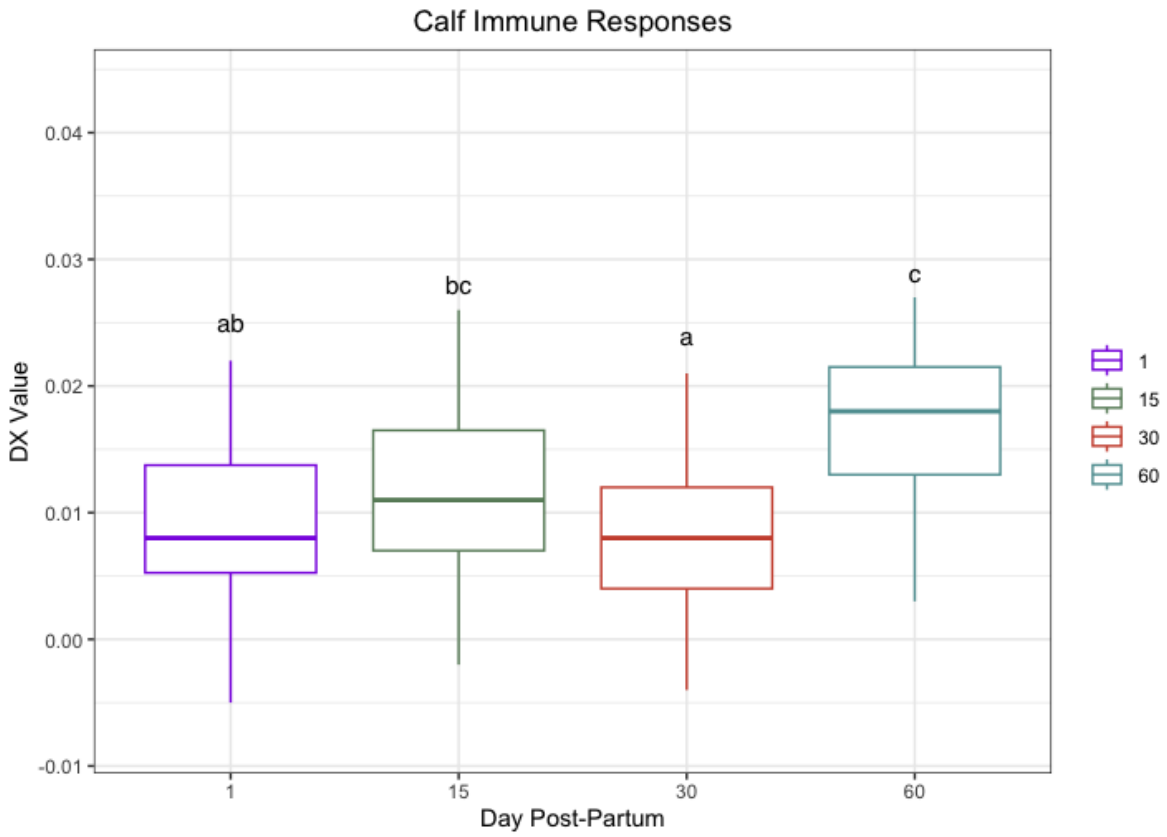

Figure 18 Immune Status of Calves Post-Partum

Boxplot depicting the D2Dx immune test kit values within calf serum at day 0, 15, 30, and 60 post-partum. There was a main effect of time with the dx immune response on day 0 was similar to day 15 and 30. The immune response on day 15 was similar to day 0 and day 60. Significance was set at $P < 0.05$.

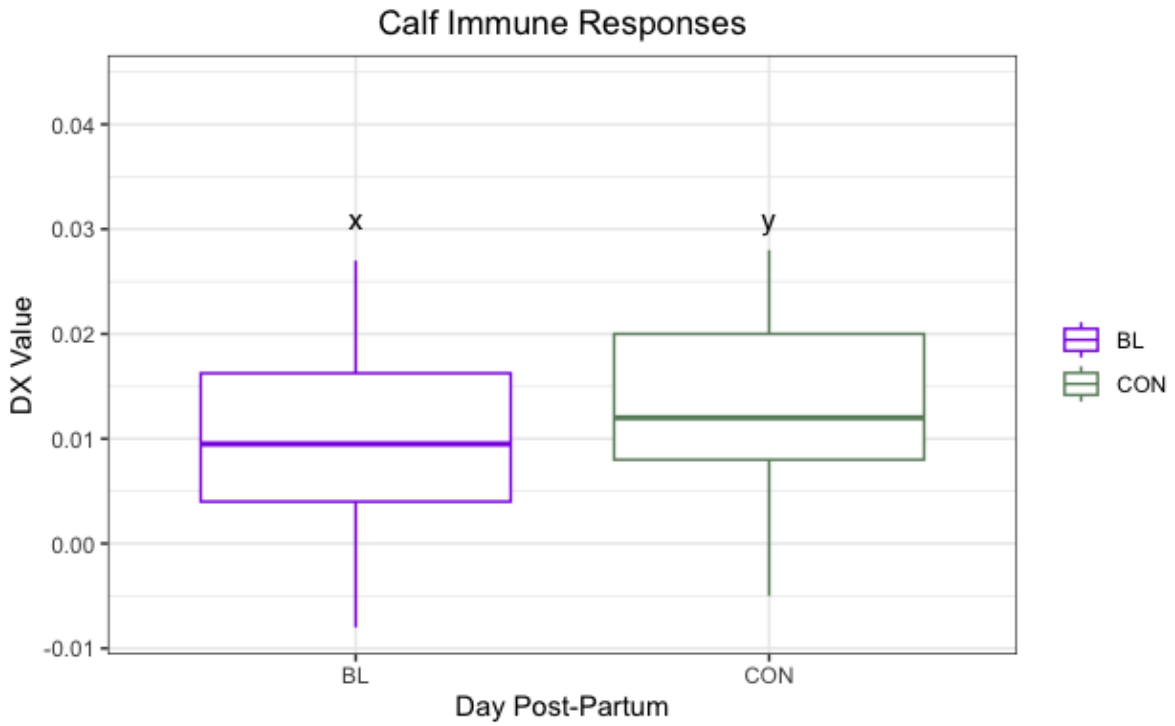

Figure 19 Immune Status Between BLG vs. CON Calves

Boxplot depicting the D2Dx immune test kit values between BLG (BL) calves compared to CON calves. There was a tendency ($P = 0.08$) for BLG calves to have a decreased immune status compared to CON calves.

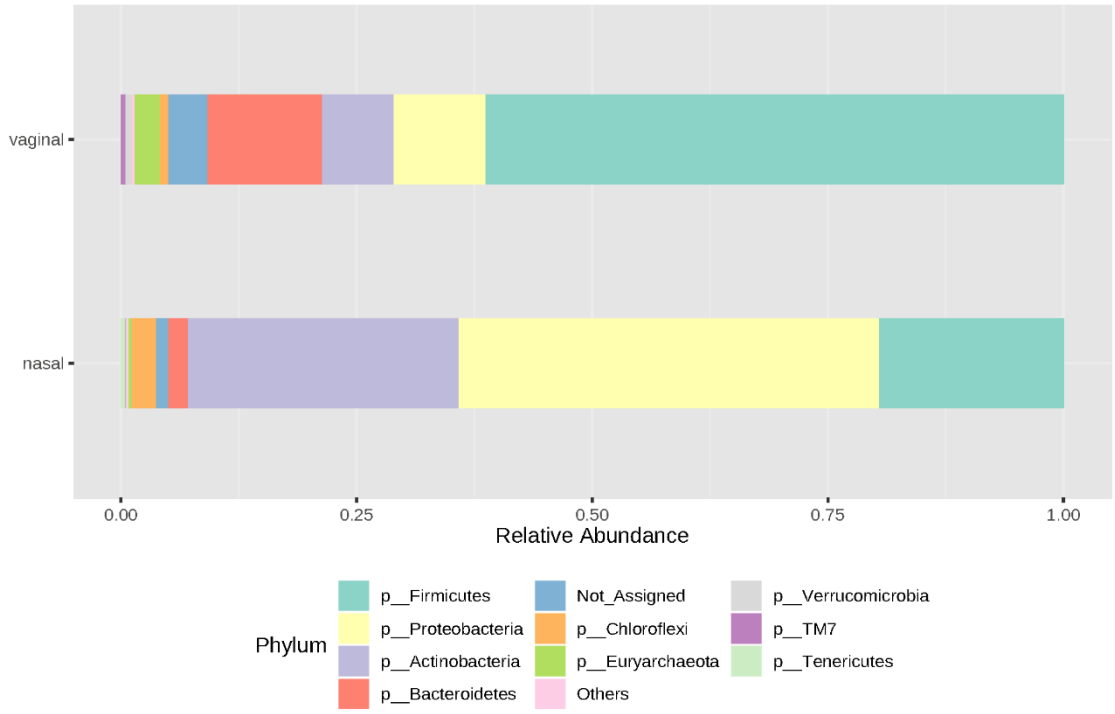

Figure 20 Phyla Relative Abundance in Dam Vaginal & Calf Nasal Microbiota

Relative abundance of the most abundant phyla level taxa within the dam vaginal and calf nasal microbiota. Each bar represents 100% of the taxa with the colors representing different phyla that are proportional to the percentage of the phyla's relative abundance.

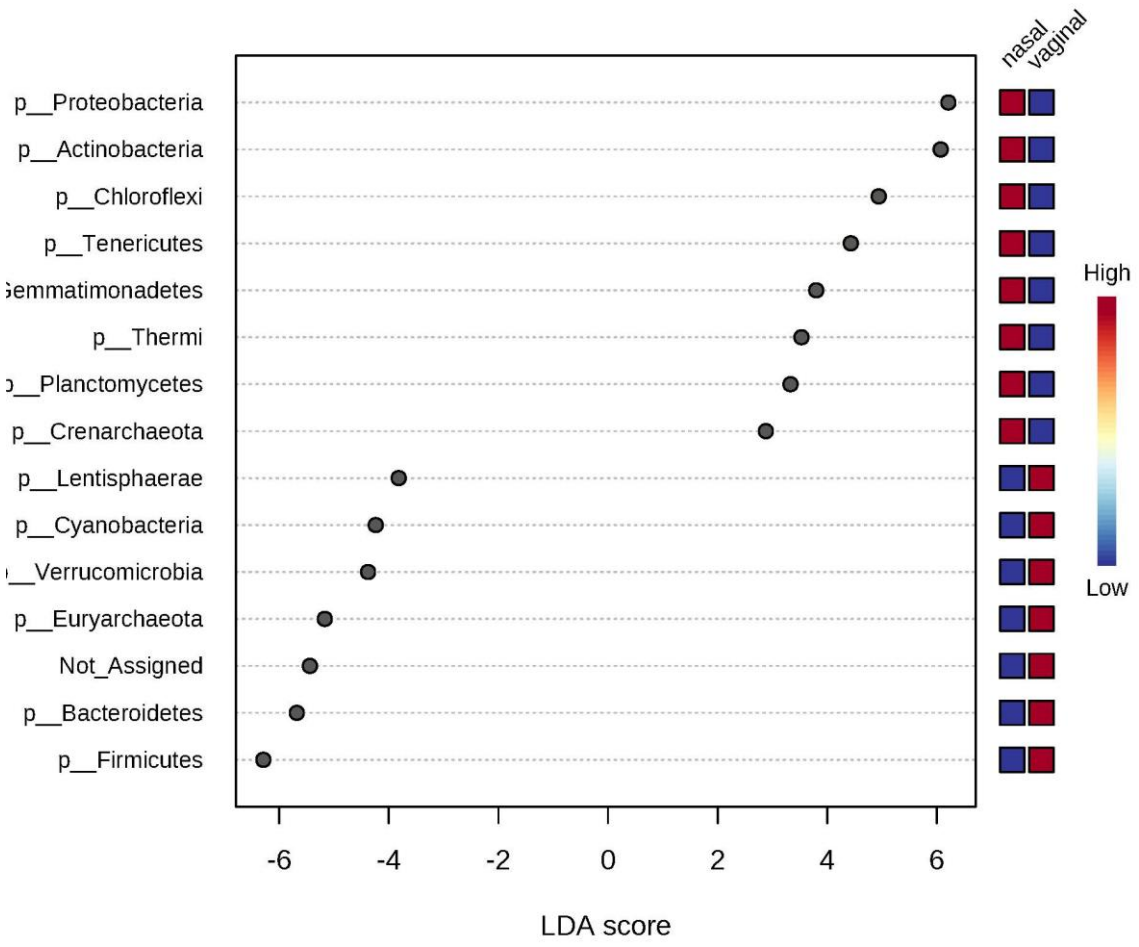

Figure 21 Phyla Differences Between Dam Vaginal & Calf Nasal Microbiota

Dot plot depicting the LDA scores computed for differentially abundant genera between the dam vaginal and calf nasal microbiota post-partum ($P < 0.05$).

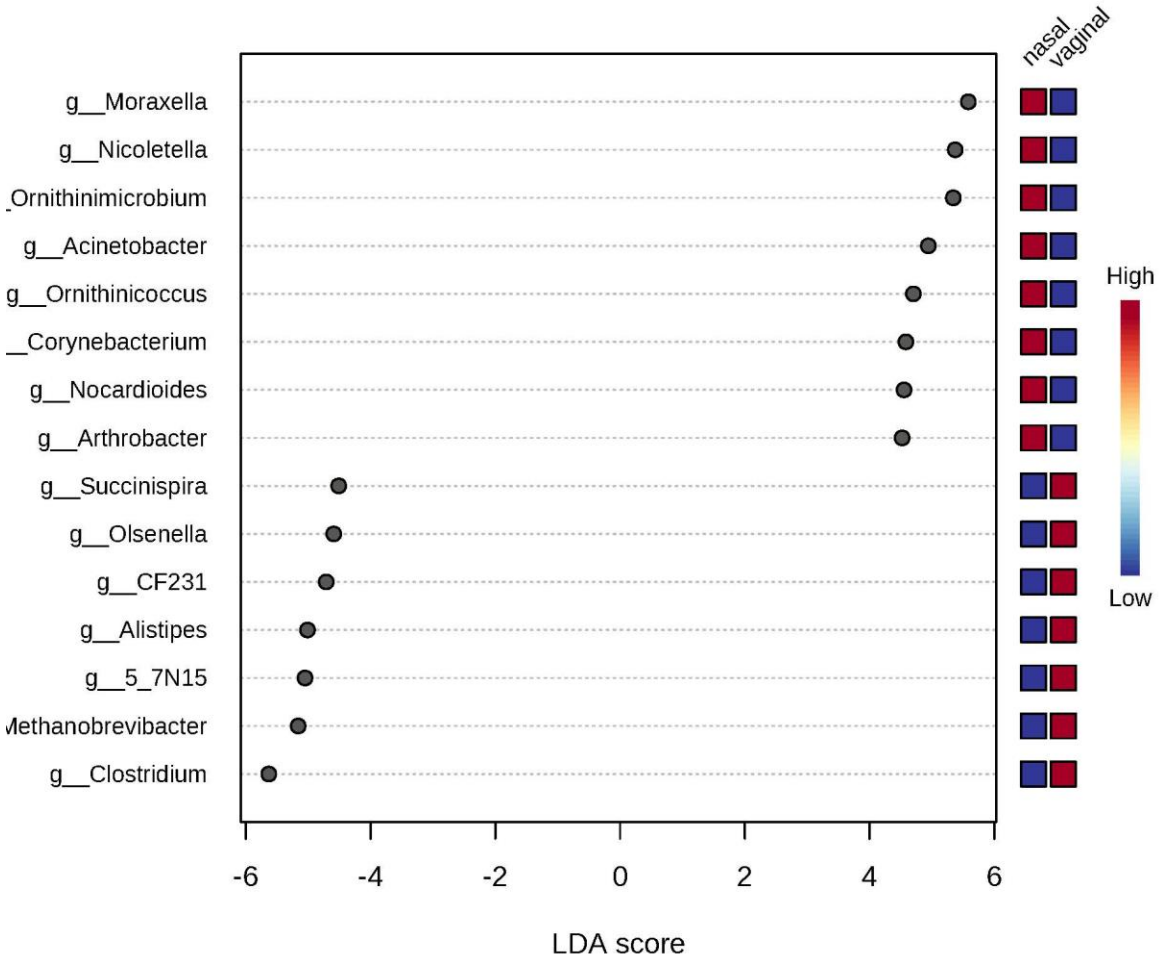

Figure 22 Genera Differences Between Dam Vaginal & Calf Nasal Microbiota

Dot plot depicting the LDA scores computed for differentially abundant genera between the dam vaginal and calf nasal microbiota post-partum ($P < 0.05$).

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CHAPTER IV

GENERAL DISCUSSION

The data and literature presented within this dissertation addresses the current paucity within beef cattle microbiota research, specifically characterization of the dam vaginal and calf nasal microbiota at calving and through the post-partum period. The amount of literature within cattle reproductive microbiota research has increased substantially over the last decade. The first chapter of this dissertation aimed to summarize all proposed microbial communities that could inoculate the conceptus from conception to colostrum consumption. Throughout this review, authors noticed clear inconsistencies in sample collection, data analysis, and discrepancies within the literature. There is clearly a need for more research to verify the presence of a commensal uterine or placental microbial community utilizing newer sequencing techniques, such as shotgun analysis. Moreover, authors proposed the inoculation vs. maturation hypothesis; this hypothesis is centered around the fact that commensal microbial communities are present in both the maternal and paternal reproductive tract prior to conception. The likelihood of bacteria being transferred within the uterus during copulation is high, and subsequently, those bacteria either mature or die within the uterine environment with the conceptus. Research in this area used 16S sequencing technology that cannot verify the metabolic status (alive vs. dead) of bacteria within the uterine environment. Therefore, although bacteria have been identified within the placenta, fetus, and uterus during gestation the viability of these bacteria and their contributing roles are still widely unknown.

Within the first study, there were no similarities between the dam microbial communities and the calf nasal microbiota. However, betadine lavages in the dam vaginal tract prior to calving altered the calf nasal microbiota at day 0. Betadine lavages also increased colostrum IgG. These results were unexpected. Authors expected to observe a clear overlap of the dam vaginal and calf nasal microbiota with additional microbial variation being explained by the dam udder/haircoat samples. However, it appears that physiological insults (betadine lavage) to the dam prior to calving likely caused an immune response that altered colostrum. Thus, it is likely that the specific IgG profiles were different between BLG vs. CON calves leading to separate inoculating communities. These findings led authors to conclude that immune status of the calf may dictate microbial colonization more than microbial exposure within 24 hours post-calving.

The second study aimed to understand fluctuations within the dam vaginal and calf nasal microbial communities at day 15, 30, and 60 post-partum in conjunction with immune status. The treatment of betadine lavage did not have any effect on the microbiota post-partum. Similar results were observed with the dam vaginal and calf nasal microbial communities not being similar at any given timepoint. The dam vaginal and calf microbial communities had similar species richness and evenness (alpha diversity) until day 60 when calves had a stark decrease. This decrease in microbial presence could be attributed to the maturation of the calf adaptive immune responses prior to day 60, resulting in the downregulation of bacteria that could colonize within the nasal tract. Moreover, there was an increased immune response at day 15 in calves paired with a difference in beta diversity from day 0; together, these results are suggestive of a recolonization timepoint that occurs between day 0 and 15 of life in calves.

Finally, the dominant phyla, within both studies, agree with previous literature explored in the first chapter. Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria dominated both

the calf nasal and dam vaginal tract but at different ratios dependent on sampling timepoint and treatment group. Interestingly, Actinobacteria was consistently increased in calf nasal samples compared to dam vaginal. The ratio and presence of this phyla within the literature is inconsistent, leading authors to hypothesize Actinobacteria has implications in the development of commensal microbial communities in calves. Further research to understand the role of this phylum within the calf nasal and dam vaginal microbiota is needed.

To conclude, the data within this dissertation adds valuable characterization research to the beef cattle literature. Repetition of this study with increased animal numbers and sample collection timepoints is needed. Understanding the immune system's role in neonatal inoculation has major implications in the field of livestock microbiota research. Thus, future research should focus on identifying specific immune modulations in the dam peri-partum and the subsequent effects on colostrum IgG composition, calf microbial communities over time, and calf immune status post-partum. These investigations could further elucidate how microbial colonization occurs and the intricate relationship between the microbiome and immune system in livestock.

APPENDIX A

2022 BI-ANNUAL EVALUATION REPORT: MISSISSIPPI STATE CATTLE ARTIFICIAL INSEMINATION SCHOOL

EXECUTIVE SUMMARY

Background and Purpose

The focus of this evaluation is the Mississippi State Artificial Insemination School (MS-AI) hosted by the extension agents within the Department of Animal and Dairy Sciences at Mississippi State University. The purpose of the Mississippi State Artificial Insemination School is to provide producer education in cattle reproductive management, semen handling, and insemination techniques. The purpose of this evaluation is to understand the knowledge and skills gained by cattle producers as they prepare to implement an artificial insemination program within their production scheme and their satisfaction with the program. The evaluation results will be used by the Mississippi State extension agents to improve program implementation and strategize for program evaluation in the future.

The big E questions addressed by the evaluation include:

1. What knowledge did participants increase by attending the program?
2. What skills did participants gain by attending the program?
3. Were participants satisfied with the program?

Exploring these evaluation questions will help identify if participants are meeting the following learning objectives:

1. After the Thursday/Friday sessions, participants will demonstrate a better understanding of cattle reproductive management, specifically estrous synchronization, reproductive anatomy, nutrition, genetics, and health.
2. After the Friday/Saturday sessions, participants will be able to identify different equipment and protocols used for artificial insemination.

Methods

Data were collected directly from participants before and after the program. All data was collected via pen and paper copies and surveys were collected immediately after completion. Data collected prior to the program included a pre-test and demographic survey. Data collected at the end of the program included a post-test, self-evaluation on skill knowledge, and satisfaction survey. Quantitative data was analyzed using R software program (R Core Team, 2020). The pre-posttest differences in means were evaluated with a student's t-test. Qualitative measures were utilized to describe the self-evaluation and satisfaction survey results.

Key Findings and Conclusions

Big E Question 1: Knowledge Gained

There was a total of 31 participants that completed both the pre- and post-test. This test was comprised of 10 questions that did not change between the pre- and post-test. The average score on the pre-test was a 6.45/10 (65%). The average score on the post-test was an 8.16/10 (82%). These scores were confirmed to be significantly different ($P < 0.05$) indicating that the program was successful in increasing producer knowledge after the program. However, 6/10 questions had 'all of the above' as a multiple-choice answer. Thus, these results could partially be due to testing bias rather than actual knowledge gained.

Big E Question 2: Skill Gained

Producers (n = 28) were asked in a self-evaluation survey at the conclusion of the program 'how many times during the program had they passed an artificial insemination gun' and 'what estrous synchronization program would you implement at your operation'. These questions were aiming to look at how many participants executed the skill and if they could

correctly select an estrous synchronization program. All participants passed an artificial insemination rod at least one time, with an average of four passes per participant. Furthermore, 17/28 (61%) producers selected the correct estrus synchronization program, 4/28 (14%) selected an incorrect estrus synchronization protocol, and 8/28 (29%) did not answer. These results indicate that producers are successful in executing the artificial insemination skill throughout the program, but that producers (~40% of participants) are not able to select an estrus synchronization protocol at the conclusion of the program.

Big E Question 3: Satisfaction with Program

A satisfaction survey filled out by participants (n = 31) at the end of the program allowed evaluators to gauge topic, speaker, and overall satisfaction. Participants were asked to rate each topic and speaker on a scale from 1-5 with 1 being very dissatisfied and 5 being very satisfied. The average rating for topics was 4.8, and the average rating for speakers was 4.6, indicating that the topics and speakers for the program were more than satisfactory. Finally, the participants (n =30) were asked if they would recommend this program to other producers. All respondents answered either very likely (n = 27) or likely (n = 3), indicating enough overall satisfaction with the program to recommend to others.

Recommendations

Four recommendations were made based on the evaluation results:

1. ***Provide AI equipment displays and demonstrations throughout the entire program.*** Evaluations indicated that participants felt most unsatisfied with the AI equipment talk (4.5/5) and open responses indicated that many participants wanted more time handling equipment for AI. Evaluators strongly recommend having a table set up throughout the program with all equipment needed for AI, semen handling, and estrus synchronization.

2. ***Review and update estrus synchronization content.*** Participants did not meet the short-term goal for estrus synchronization selection, indicating an adjustment is needed in content delivery and dose.
3. ***The pre-posttest needs to exclude 'all of the above' from all questions.*** This recommendation is due to the results of the pre-posttest potentially being skewed due to testing bias. It is apparent that even without general knowledge of cattle artificial insemination 6/10 questions could be answered fairly easily. Thus, evaluators encourage that the pre-posttest be altered to remove testing bias.
4. ***The amount of evaluation paperwork needs to be condensed.*** There are varying numbers of participants that turned in each set of paperwork due to an overwhelming number of evaluation documents (n = 5) that each participant was expected to fill out. Evaluators recommend condensing the demographic survey, satisfaction survey, and self-evaluation into one document (front and back) with more focused questions. Additionally, these documents had several repeated questions that could be prevented if combined.

Introduction

Overview

The focus of this evaluation is the Mississippi State Artificial Insemination School extension program hosted by the Department of Animal and Dairy Sciences at Mississippi State University. The purpose of the Mississippi State Artificial Insemination School is to provide producer education in cattle reproductive management, semen handling, and insemination techniques. The evaluation described within this report is intended to assess if the program

improves participants knowledge in cattle reproduction, artificial insemination skills during its duration; moreover, this evaluation also considered participant satisfaction during the program.

Organizations Involved

The primary organization involved is Mississippi State University Department of Animal and Dairy Sciences, which comprises the management team and staff that design, plan, and implement the program. Secondary organizations include the Mississippi State College of Veterinary Medicine and the College of Agriculture and Life Sciences. These secondary organizations are not directly involved with program planning or design; these organizations primarily provide many of the speakers and artificial insemination technicians that assist during program implementation.

Stakeholders

Stakeholders for the program include multiple entities at Mississippi State University including the Department of Animal and Dairy Sciences, the College of Agriculture and Life Science, and the College of Veterinary Medicine who help in the sponsorship and implementation of the program by providing speakers and staff. The participants have financial ($450/person) stake and personal stake in the program. Other stakeholders include Southeast United States beef cattle extension agents, United States' cattle producers, semen companies (American Breeders Service and Select Sires), and all staff who participate in the program.

Intended Audience

The intended audience of the evaluation report is the Mississippi State extension agents, specifically Dr. Brandi Karisch, who is directly in charge of the theory, process, implementation, and evaluation of the Mississippi State Artificial Insemination School.

Intended Use

The purpose of this evaluation is to ascertain if the short-term learning objectives were achieved during the execution of the Mississippi State Artificial Insemination School. Specifically, did participants increase knowledge and skills relating to artificial insemination after participation in the program, and were the participants satisfied with the topics covered, speakers, and overall program. This information will be utilized by Mississippi State University Extension agents to refine and improve the program for the future.

Structure

The following document includes an in-depth review of the Mississippi State Artificial Insemination School program, including the theory, process, implementation, and activities within the program. Immediately following is a detailed overview of the evaluation process, including the key evaluation questions, evaluation criteria, performance criteria and standards, methods, and materials for conducting the evaluation. This report concludes with a description of the evaluation results and recommendations based on these findings.

Mississippi State Artificial Insemination Program Description

Need for Program

Overall Problem:

Artificial insemination (AI) is an easily accessible advanced reproductive technology that producers can utilize to improve herd genetics, reproductive efficiency, and economic gains (Foote, 2001). The technique was widely adapted by the dairy industry; today, over 90% of dairy producers utilize AI within their herds. However, less than 10% of beef producers utilize AI despite its positive effects. One must consider the vast differences in production schemes and

goals between dairy and beef cattle, but AI remains a viable option for improvement for either producer. ***Thus, there is an apparent problem in the adaptation and implementation of AI within beef cattle producers in the United States***. More than likely, the delayed adaptation of AI in beef producers is due to lack of extension programs communicating the benefits of AI and teaching the technique to producers.

Benefit 1: Decreased Bull Cost

The use of AI within dairy production eliminates the need for a bull. Dairy bulls are characteristically larger and more aggressive than beef bulls; dairy bulls are more likely to cause producer death during handling as well (Haskell et al., 2014). In contrast, beef bulls typically have a more docile temperament; it is not uncommon for beef producers to have several bulls on their property year-round. This translates to having to provide feed and maintain for multiple bulls year-round that are only being used during the breeding season. Bulls can very easily translate into economic loss for producers.

A well-bred young bull can cost between $2000-5000 to purchase. Feed costs for a bull are estimated at ~$500 per year and an annual breeding soundness exam to ensure fertility is ~$150. Thus, the annual expense for a new bull is between $2650-5650. Excluding the original purchase cost, keeping a bull for a single breeding season will cost $650. A bull can cover 25 cows with a 92% pregnancy rate (Berger, 2017). Thus, depending on the number of cows a producer has within a breeding season determines the number of bulls needed and annual costs (250 cows = 10 bulls = $6,500 per year). However, if a producer uses AI for all 250 cows, then turns out 5 clean up bulls, he could lower his annual bull expense to $3250 and decrease the risk of bull related injuries at his operation.

Benefit 2: Decreased Spread of Venereal Diseases

Venereal diseases, or sexually transmitted infections, can be massively destructive for a producer if introduced within the herd. In cattle, venereal diseases are those that can be spread through natural service between a bull and a cow. Typically, venereal diseases lead to reproductive failure within the female classified by failure to conceive or abortion during mid- to late gestation. Major venereal diseases in cattle include *Camplyobacter fetus* (Vibrio) and *Trichomonas fetus* (Trich) (Overbay, 1999). Moreover, bacteria from the environment, such as *Mycoplasma, Ureaplasma*, and *Hemophilus,* can have negative effects on pregnancy within the female reproductive tract. When using natural service, all bulls must undergo testing for venereal diseases to prevent a herd-wide outbreak, but this does not prevent a bull from contracting a venereal disease during the breeding season from an infected cow and subsequently infecting all other cows within the pasture. Thus, when using natural service there is an increased risk for venereal disease spread within the herd compared to AI.

Benefit 3: More Uniform Calf Crop

A huge advantage to AI is the ability to breed all cows within the herd early in the breeding season at the same time due to estrus synchronization protocols (Senger, 2012). All cows conceiving at the same time will translate to a shorter and earlier calving season with all calves typically being born within 1-2 weeks of the expected due date. Calves that are born earlier in the calving season have more time between birth and weaning to gain muscle and adipose tissue. The price per head at weaning is based off of weight; calves that are on the ground longer have more time to gain weight. Therefore, AI calves are born around the same time early in the calving season giving these calves the most time to grow prior to weaning translating to a uniform and heavier calf crop.

Benefit 4: Improved Calf Genetics

Semen used for AI in cattle is collected from bulls that have the best genetics within a breed that is owned by large scale semen companies. The bulls utilized undergo extensive genetic testing and data is collected on their calf crop to create an Expected Progeny Difference (EPD) that producers can utilize to select a bull that fits their production goals. For example, producers who aim to sell their calves for finishing can select for a bull that has high genetic potential for quality carcass characteristics (Detweiler et al., 2019). However, if the same producer also needs to produce replacement heifers that year, a bull that produces maternal cows with high fertility can be selected. Finally, EPDs can also be used to select for calving ease, birth weight, and weaning weight to prevent difficult births (and potential mortality) within the herd while also selecting for calves that will grow quickly after birth. The unique ability for producers to be able to select genetics to integrate within their herd with such precision from bull EPDs is specific to AI and cannot be done with a herd bull.

Benefit 5: Increased Post-Partum Anestrus Interval

As previously mentioned, AI allows for cows to become pregnant early in the breeding season translating to her calving earlier. The advantage to early calving is two-fold: increased time for the calf to grow prior to weaning and increased post-partum interval for uterine involution to occur. Cows undergo a period of anestrus after calving that can last between 40-80 days. Therefore, it is important that cows are giving a minimum of 40 days before rebreeding. However, this can become an issue if cows are becoming pregnant at the end of the breeding season and calving late. Typically, cows that have a short post-partum interval before rebreeding do not conceive to AI, get pregnant late in the breeding season, calve late the following year and the cycle repeats itself until the cow unable to stay within the herd due to her inability to conceive during the correct timeframe. However, AI decreases the number of cows with a short

post-partum interval because it increases the number of cows becoming pregnant at the beginning of the breeding season.

Benefit 6: Economic Gains

Finally, all the aforementioned benefits translate to an economic gain for the producer. Typically. the producer can expect to see a $100-200 increase per AI calf weaned, depending on genetics. Therefore, the benefits and improved economic gains clearly demonstrates the effectiveness of AI in beef cattle operations and the lack of implementation of AI demonstrates a need within cattle producers.

Needs Assessment

There was no formal needs assessment conducted. Based on AI rates in the United States being less than 10% within beef cattle producers, there is an apparent lack of resources to assist producers with developing their confidence and skill in estrus synchronization and AI implementation on their operation. After the refinement of AI in the late 1940s, AI schools began to emerge across the nation (Foote, 2001). These schools were hosted by land-grant universities, major companies within the AI industry, and extension agents. Therefore, the Mississippi State AI School is a result of efforts within land-grant universities and is still needed today within the beef industry specifically.

Program Purpose

The purpose of the Mississippi State Artificial Insemination School is to provide producer education in cattle reproductive management, semen handling, and insemination techniques.

Goal and Objectives

Program Goal:

Artificial insemination is an economically important advanced reproductive technology that producers can implement to achieve maximal reproductive efficiency within their cattle production scheme.

Program Objectives:

Ultimate Objective: Within 10 years, the number of cattle producers utilizing artificial insemination and estrous synchronization in the United States will increase by 5%.

Behavioral Objectives:

- Within one year, 50% of the participants will implement an estrous synchronization protocol and artificial insemination program within their herd.
- Within two years, 40% of the participants will start selecting for genetic improvement via artificial insemination resulting in increased overall value of their annual calf crop.

Learning Objectives:

- After the learning sessions, 80% of the participants will demonstrate a better understanding of cattle reproductive management, specifically estrous synchronization, reproductive anatomy, nutrition, genetics, and health.
- After the hands-on sessions, 80% of the participants will be able to correctly identify the proper estrus synchronization protocol for their production scheme.
- At the conclusion of the program, 80% of the participants will successfully complete two artificial insemination passes.

Program History & Description

The Mississippi State Artificial Insemination School began in 1977 as part of a collaborative effort of Southeastern extension agents to bring knowledge of artificial insemination to cattle producers in the Southeast. Throughout the program, participants attend multiple classroom sessions (totaling 7 hours) covering various aspects of reproduction and artificial insemination techniques. Then, participants spend 8 total hours gaining hands-on experience practicing artificial insemination at the farms under the supervision trained artificial insemination technicians. The program offers producers hands-on practice in both beef and dairy cattle using chutes and headlocks. Typically, it is offered bi-annually and fills to capacity (30 producers). A total of 1,052 participants from over 14 states have attended this program since its founding (Marks et al., 2019).

Target Audience

The target audience includes all cattle producers within the United States interested in learning the technique of artificial insemination. There is little marketing done for the program; word-of-mouth is how the participants typically hear about the program and register. Many of the participants are from the Southeast United States, but there have been participants from across the United States. Participants most commonly travel from Mississippi, Alabama, Arkansas, Tennessee, and Louisiana for the program.

Program Theory

Process Theory

The program consists of 7 hours of lecture presentations and 8 hours of hands-on practice broken into 2.5 days of learning.

Day 1: Introduction to Bovine Reproduction (4 hours)

This session begins at 6pm on a Thursday night and focuses on the introduction of bovine reproductive anatomy, physiology, artificial insemination, and estrus synchronization. Additionally, participants are introduced to program staff through hands-on stations.

Sign in, Surveys, & Pre-Test (30 min)

- All participants sign in and receive a binder containing relevant information, including PowerPoint slides, handouts, and technique instructions. Participants are asked to fill out a demographic survey and the pre-test which are both collected prior to program onset.

Lecture Content (2 hours)

- Four lectures are given introducing participants to general knowledge needed for the hands-on activities. These lectures include 'economics of artificial insemination', 'reproductive anatomy', 'estrous cycle, estrus synchronization and sexed semen', and 'equipment for artificial insemination'. Lectures are given by an agricultural economist from the College of Agriculture and Life Sciences and reproductive physiology graduate students from the Department of Animal and Dairy Sciences.

Hands-on Activities (1.5 hours)

- Participants are split into three groups and rotated between three stations with 30 minutes per station. The stations include:
    - Semen handling and loading an AI gun
        - Led by employees of semen companies that sell the semen for artificial insemination
    - Passing an AI gun through reproductive tracts

    - Led by graduate students and AI technicians that will be assisting at the farm
- Overview of an online estrus synchronization calculator
    - Led by Dr. Karisch and economists

Day 2: Industry Related Lectures & Hands-on AI skill practice

Lecture Presentations (3 hours)

- Four lectures are given introducing participants to selection parameters and the benefits of incorporating artificial insemination within their herd. These lectures include 'economics of artificial insemination', 'reproductive anatomy', 'estrous cycle, estrus 'heat detection and heat detection aids', 'nutritional programs for AI success', 'sire selection', and 'reproductive herd health and biosecurity'. Lectures are given by professors from the Department of Animal and Dairy Sciences and veterinarians from the College of Veterinary Medicine.

Lunch (1 hour)

- Lunch is provided for the participants before heading to the farms for the hands-on portion of the day. This time is used for content clarification and general discussion amongst the group and staff.

Hands-on practice (4 hours)

- Hands-on practice with artificial insemination at either the Mississippi State beef unit or dairy unit. During this time, participants are led through palpation, AI rod insertion, semen loading, and passing the rod through the cervix. During this time, AI technicians are on stand-by to assist, answer questions, and walk participants through the technique.

Day 3: Hands-on AI skill practice & close

Hands-on practice (4 hours)

- Hands-on practice with artificial insemination at either the Mississippi State beef unit or dairy unit. During this time, participants are led through palpation, AI rod insertion, semen loading, and passing the rod through the cervix. During this time, AI technicians are on stand-by to assist, answer questions, and walk participants through the technique.

Post-Test, Satisfaction survey, & Self-evaluation (15 minutes)

- To complete the program and receive their certificate, all participants must complete the post-test, satisfaction survey, and self-evaluation prior to leaving. Once these documents are collected, the participants are free to leave.

Impact Theory

Program: Mississippi State Artificial Insemination School Logic Model

Situation: Artificial insemination is an emerging technique within the cattle industry, but to successfully implement the technique, training is required. Cattle producers could advance their herd genetics, improve profit margins, decrease bull associated risks, and advance their operation if trained on advanced reproductive techniques.

Inputs	Outputs		Outcomes -- Impact		
	Activities	*Participation*	*Short*	*Medium*	*Long*
Mississippi State University Staff Graduate Students from Animal and Dairy Sciences Time Money Semen Handling and Artificial Insemination Equipment Extension Research Dairy and Beef Cattle Mississippi State cattle working facilities Catered lunches	2.5-day workshop Teach reproductive anatomy of cattle Train producers on how to select an estrous synch protocol Build relationships with producers and semen company representatives, MS state staff, and local veterinarians Teach producers proper semen handling, palpation, and artificial insemination techniques Assess producers' ability to perform the artificial insemination technique Train producers on implementing artificial insemination within their own operation	Cattle producers within the United States Beef cattle producers Dairy cattle producers Show industry producers Mississippi Cattlemen's Association	Producers increase awareness relating to artificial insemination Producers improve their attitudes regarding advanced reproductive techniques Producers are motivated to design an artificial insemination program that matches their operation goals Producers increase their knowledge about cattle reproduction Producers increase their confidence performing the artificial insemination technique	Producers implement an estrous synchronization protocol on their operation Producers implement an artificial insemination protocol at their operation Producers utilize expected progeny differences to advance their herd genetics and increase profit margins Producers collaborate with their local semen company representatives, MS state staff, and local veterinarians to improve cattle reproductive performance	Use of the artificial insemination technique within the cattle industry increases Cattle genetics are improved via artificial insemination Higher quality meat products are produced from each animal Cattle producers increase their profit margins

Assumptions	External Factors
Cattle producers are interested in implementing the artificial insemination technique within their herd. Producers are physically capable of performing artificial insemination in cattle.	Covid-19 pandemic Weather Travel restrictions

Evaluation Background

Purpose and Intended Use

The purpose of this evaluation is to understand the knowledge and skills gained by cattle producers as they prepare to implement an artificial insemination program within their production scheme and their satisfaction with the program. This information will be used by extension agents at Mississippi State University, specifically Dr. Brandi Karisch, to improve and refine the bi-annual artificial insemination program. Additionally, this evaluation report can be used by extension agents throughout the Southeast to create a similar AI school and evaluation process within their program.

Stakeholders

The stakeholders from the Department of Animal and Dairy Sciences are directly involved in the creation, implementation, and collection of the evaluations.

Evaluation Questions

The big E questions that are addressed by the evaluation are:

1. What knowledge did participants increase by attending the program?
2. What skills did participants gain by attending the program?
3. Were participants satisfied with the program?

These questions were developed by discussing current program goals and objectives with the stakeholders. Exploring these big E questions will allow the evaluators to determine if the program meets the following learning objectives:

- After the learning sessions, 80% of the participants will demonstrate a better understanding of cattle reproductive management, specifically estrous synchronization, reproductive anatomy, nutrition, genetics, and health.
- After the hands-on sessions, 80% of the participants will be able to correctly identify the proper estrus synchronization protocol for their production scheme.
- At the conclusion of the program, 80% of the participants will successfully complete two artificial insemination passes.

Additionally, the third Big E question allows for evaluators to gauge participant satisfaction with the program.

Scope

The evaluation will focus on the Spring 2022 replicate of the Mississippi State Artificial Insemination School extension program. The evaluation paperwork including pre-posttest, demographic surveys, self-assessment, and satisfaction surveys completed by participants during the program is the data that will be focused on in this evaluation.

Response to Culture and Context

All evaluation documents were reviewed and approved by the Mississippi State Extension Service. This approval process includes screening documents for cultural bias, misappropriation, and exclusivity.

Evaluation Methodology

Evaluation Design

The evaluation design is pre-experimental with no randomly chosen or assigned groups and a one group pre/post study using a pre-posttest to evaluated knowledge gain.

Participants

Participants in the spring 2022 Mississippi State Artificial Insemination School extension program were United States cattle producers (n = 31) from Mississippi (n = 11), Alabama (n = 15), Tennessee (n = 2), and Michigan (n = 2). There were both males (n = 17) and females (n = 14) that participated in the program. The age of participants ranged from 17-58 years old. The majority of participants (87%) were Caucasian with only four participants (13%) indicating a Native American or Hispanic ethnicity.

Data Collection

Data were collected from participants prior to the program and immediately following the program in a pre/post design manner. Gain of knowledge was measured using quantitative data by administering a pre-posttest. Qualitative demographic data was collected prior to the program start. Self-evaluation data measuring the producer's ability to select an estrus synchronization protocol (qualitative), number of successful AI rod passes during the program (quantitative), and producer confidence in AI implementation was collected at the end of the program (qualitative). Qualitative data regarding program satisfaction was also collected at the culmination of the program. All data was collected by participants filling out physical paperwork. Documents were reviewed or graded by evaluators to collect relevant data that was transferred into computer records.

Instruments

A total of five separate evaluation handouts were given to participants during the program. Two evaluations were given at the beginning of the program and three at the end. All

evaluation hand-outs were printed and color coded. These hand-outs included a pre-posttest, demographic survey, satisfaction survey, and self-evaluation (see Appendix).

Performance, Criteria, & Standards

A summary of the performance evaluation tools, criteria, and standards can be found in Table 1.

1. *What knowledge did participants increase by attending the program?*
    a. Evaluation Tools
        i. All items on pre and post test
    b. Criteria
        i. Ability to perform better on written exam after attending the program
    c. Standards
        i. A significant increase ($P < 0.05$) between the pre- and post- test assessment
2. *What skills did participants gain by attending the program?*
    a. Evaluation Tools
        i. Self-Evaluation Survey (post-only)
    b. Criteria
        i. Ability to select and estrous synchronization protocol that is best for their operation
        ii. Ability to pass artificial insemination rod into the uterine body
    c. Standards
        i. 80% of the participants can select an estrous synchronization protocol that is best for their operation

ii. The average time of skill execution during the program will be 2 times per person

3. *Were the participants satisfied with the program?*
    a. Evaluation Tools
        i. Satisfaction survey (post-only)
    b. Criteria
        i. Satisfaction with speakers
        ii. Satisfaction with topics
        iii. Overall satisfaction with the program
    c. Standards
        i. The average satisfaction score for speakers and topics is > 4.5
        ii. 80% of the program participants would recommend the program to other producers

Data Collection Procedures

All data was collected during program implementation. The demographic survey and pre-test were collected within the first 30 minutes of the program on March 10, 2022, prior to any content presentation. The post-test, satisfaction survey, and self-evaluation were collected during the last 30 minutes of the last day on March 12, 2022. Participants were given ample time to complete all documents. These documents had to be submitted before participants received their certificate of completion.

Data Analysis

Quantitative data analysis from the pre-posttest was analyzed using the R software program (R Core Team, 2020). A student's t-test was utilized to analyze differences between means. Qualitative survey data were summarized via Microsoft Excel; numbers were assigned to survey responses to indicate a satisfaction score from a 1-5 with 1 being extremely dissatisfied and 5 being extremely satisfied. This scoring system was used for the satisfaction survey. AI rod passes were recorded an averaged. Qualitative data including participants comments, demographics, and ability to identify estrus synchronization protocols were transcribed into Microsoft Excel.

Evaluation Standards, Guidelines, and Ethics

Systematic Inquiry

The principle of systematic inquiry mandates that evaluators should, "adhere to the highest appropriate standards in conducting their work to increase its accuracy and credibility" (Rossi et al., 2004). This evaluation report is transparent, including all evaluation documents used and evaluation methods are clearly described. The evaluation was created by evaluating the short-term learning objectives of the program and creating big E questions directly related to measuring if the short-term objectives were achieved.

Competence

The principle of competence refers to the ability of the evaluators to conduct a professional evaluation (Rossi et al., 2004). This evaluation was conducted as part of a graduate level college course to increase the skills of the evaluator while still under the direction of the course instructor.

Integrity and Honesty

The principle of integrity and honesty focuses on ensuring that honesty is implemented throughout the entire evaluation process including stakeholder discussions, changes, concerns, conflicts of interest, and data representation (Rossi et al., 2004). All information within this evaluation document is true to the evaluator's knowledge. All evaluation methods are clearly outlined within the document; relevant data has been included within the document but excluded data does not change the overall findings of the evaluation.

Respect for People

The principle of respect for people refers to, "evaluators respecting the security, dignity, and self-worth of program participants, clients, and stakeholders with whom the interact" (Rossi et al., 2004). Anonymity was provided to participants when completing the self-evaluation and satisfaction survey. Participant names will not be shared within this evaluation report and all recommendations will be communicated in a respectful and professional manner.

Responsibilities for General and Public Welfare

The principle of responsibilities for general and public welfare states that when evaluating programs, the diversity of public interests, perspectives, and broad implications should be considered (Rossi et al., 2004). This evaluation has the potential to impact the implementation of AI schools at land-grant universities. Therefore, the conclusions made within this evaluation are tailored to the general and public audience that could implement this program in the future.

Evaluation Results

Big E Question One

What knowledge did participants increase by attending the program?

Findings

All program participants (n = 31) completed both the pre- and posttest (Appendix). Both tests were identical and comprised of 10 questions relating to content covered throughout the program. There was a significant increase in knowledge from the pre-test to the post-test ($P < 0.05$). The average score for the pretest was a 64% (6.4/10) compared to the posttest average of 81% (8.1/10; Fig 24).

Conclusions

Results from the spring 2022 participants pre-posttests indicate a significant increase in knowledge after completion of the program with a test score increase of 17%. Thus, according to this data, the program was successful in completing the short-term objective of increasing producer knowledge relating to cattle reproduction. The performance standard was met by the significant difference ($P < 0.05$) between the pretest and posttest scores when analyzed via the R software package.

Limitations

Evaluators strongly believe there was a testing bias within the pre-posttest. Of the 10 multiple-choice questions, six of these had the correct answer being ‘all of the above’. Thus, the improved scores from the pre-posttest could be a reflection in test taking skill rather than actual knowledge gained. Additionally, participants had the posttest within their binders during the entirety of the program, so there was potential for participants to complete the posttest using notes instead of closed book as intended.

Big E Question Two

What skills did participants gain by attending the program?

Specifically, we are interested in the ability of producers to successfully complete the technique by passing an AI rod through the cervix and their ability to select an estrus synchronization protocol that meets the need of their operation.

Findings

On the self-evaluation (Appendix), participants were asked the following:

- How many times during the artificial insemination practice did you successfully execute the technique?
- What estrous synchronization protocol would you implement at your current operation?

All participants executed the technique at least one time, with an average of 4 passes per person. The participants filled out information relating to their production scheme immediately before the estrus synchronization implementation questions. Their answers about the production scheme allowed evaluators to determine if participants selected an estrus synchronization protocol that best fit their production goals. Results indicated that 59% of the respondents (n = 29) were able to correctly identify an estrus synchronization protocol for their operation while 41% were either incorrect or did not respond (Fig 25).

Conclusions

Based on the current findings, the program was successful in improving participants skills in relation to the standards set for artificial insemination. The set standard was that the participants would average 2 passes per person; this mark was exceeded with the average for the spring 2022 cohort being 4 passes per person. However, there was a large range in number of passes (1-14). Thus, for future evaluations averaging the number of passes may not be the best standard to adhere to. Evaluators would recommend evaluating passes on an individual basis.

However, this data indicates that the program was successful in accomplishing the short-term objective of participants successfully completing two passes.

Secondly, the program was not successful in increase producer's ability to select an estrus synchronization protocol. The standard was set that 80% of participants could select the correct protocol for their operation but only 59% of participants properly executed this skill. Therefore, the short-term objective of improving producer's ability to select an estrus synchronization protocol was not achieved. Evaluators strongly believe that the amount of content related to estrus synchronization should be re-evaluated and more frequently discussed throughout the program.

Limitations

There were only twenty-nine of the 31 participants that completed the self-evaluation. Thus, results could be different if all responses were collected. Moreover, the standards set for these evaluation parameters need to be revisited. It may be more beneficial to evaluate technique execution on an individual basis rather than taking the average for the group. Finally, the failure to meet the short-term objective relating to estrus synchronization was potentially a reflection of course content and delivery, but also could be related to the open-ended question being more difficult to answer compared to a selection. Future evaluation hand-outs could have estrus synchronization protocols to choose from which would eliminate the high number of 'unsure' responses.

Big E Questions Three

Were participants satisfied with the program?

Findings

Participants were asked to complete a satisfaction survey where they rated each topic and speaker on a scale from 1-5 with 1 being extremely dissatisfied and 5 being extremely satisfied. Moreover, participants were asked to give any suggestions for program improvement on this survey as well. Finally, overall satisfaction of the program was based on the following questions using the Likert scale:

- How likely are you to recommend MSU AI School to other producers?
- How likely are you to implement an AI program at your operation?

Participants indicated they were very satisfied with the topics (4.8 avg.; Table 1) and speakers (4.6 avg.; Table 2) throughout the program (Table 1). Moreover, 90% of respondents (n = 30) indicated they were very likely to recommend this program to others with the remaining 10% of respondents indicated they were likely to recommend (Fig 26). Finally, the overall satisfaction translating to implementation had mixed responses 10% of participants responded with somewhat likely, 38% responded with likely, and 52% responded with very likely when asked if they were going to implement an AI program at their operation (Fig 27).

The themes of qualitative responses included incorporating more equipment displays, availability to purchase equipment, pregnancy palpation demonstration, CIDR insertion demonstration, and earlier end time on Thursday night (Table 3).

Conclusions

Based on these findings, it can be concluded that participants were satisfied with the topics, speakers, and overall program. This was determined by average satisfaction scores being over the standard (4.5) and 100% of respondents were likely to recommend the program to other

which exceed the standard of 80%. Overall satisfaction was also determined by evaluating the likelihood of producers to implement an AI program at their operation, but results were not as positive as evaluators expected. Thus, future evaluations should incorporate a follow up question asking participants why they chose a specific response. This data could be used to incorporate additional resources or topics to increase the likelihood of participants implementing AI at their operation.

Many of the qualitative response themes are easily addressed. Evaluators will be recommending that staff have equipment displays throughout the program, contacts for equipment purchase within the binders, and demonstrate all equipment usage during the hands-on portion of the program. Time adjustments within the program are unlikely due to work-hour constraints of staff.

Limitations

Not all participants responded to each satisfaction survey, resulting in a variable number of responses for each satisfaction parameter. All participants responding could alter the results presented within this report. With the scoring system, the results obtained are not further explained. Thus, it is difficult for evaluators to provide clear direction for improvement based on these scores. It would be beneficial in the future to include a space for the participant to provide information of how to improve the likelihood or satisfaction scores provided.

Recommendations

Developmental Process

Recommendations are based on evaluation findings and conclusions of the Big E questions while considering the limitations of the evaluation. Recommendations are intended to

increase the future success of the program's ability to meet the short-term objectives and improve participant satisfaction while condensing and refining the current evaluation process.

Recommendations for the Program

Based on the evaluation results, the evaluators noticed two program theory areas that could be improved to increase participants' knowledge, skill, and overall satisfaction.

1. Provide AI equipment displays and demonstrations throughout the entire program.

Evaluations indicated that participants felt most unsatisfied with the AI equipment talk (4.5/5) and open responses indicated that many participants wanted to visualize equipment needed and have demonstration. Thus, evaluators strongly recommend having a table set up throughout the program with all equipment needed for AI, semen handling, and estrus synchronization. Evaluators feel that it would be beneficial to include the opportunity to practice injections, CIDR insertion, semen handling, AI gun loading, and resources to purchase equipment to participants. This can easily be incorporated during the hands-on learning activities and resources can be added to the participant binders.

2. Review and update estrus synchronization content.

Participants did not meet the short-term goal for estrus synchronization selection indicating an adjustment is needed in content delivery. Estrus synchronization is a harder topic covered within the program, but crucial for producers to be able to implement AI within their operation. Thus, evaluators recommend that the estrus synchronization lecture content is refined for clarity and ease of learning and that estrus synchronization is discussed frequently during the program including during hands-on activities. Finally, it could be beneficial to incorporate an

activity within the lecture period where participants choose an estrus synchronization protocol for their operation and practice calendar planning with that protocol.

Recommendations for Future Evaluations

Based on the evaluation results, the evaluators noticed two evaluation parameters that could be improved to increase the efficacy of the evaluation process.

1. ***The pre-posttest needs to exclude 'all of the above' from all questions.*** This recommendation is due to the results of the pre-posttest potentially being skewed due to testing bias. It is apparent that even without general knowledge of cattle artificial insemination 6/10 questions could be answered fairly easily. Thus, evaluators encourage that the pre-posttest be altered to remove testing bias.

2. ***The amount of evaluation paperwork needs to be condensed.*** There are varying numbers of participants that turned in each set of paperwork due to an overwhelming number of evaluation documents (n = 5) that each participant was expected to fill out. Evaluators recommend condensing the demographic survey, satisfaction survey, and self-evaluation into one document (front and back) with more focused questions. Additionally, these documents had several repeated questions that could be prevented if combined.

Dissemination Plan for Evaluation Results

This section discusses alternative report formats that could be utilized to communicate evaluation findings with the identified stakeholders.

Standalone Executive Summary

A standalone executive is a two-page maximum report that summarizes the major findings and recommendations of the evaluation report. This is a condensed version of the evaluation report that allows stakeholders to easily understand the major findings and alterations that are needed to improve the program for future replicates without reading a 20+ page document.

Verbal and Video Presentations

A brief (2-5 min) video presentation could be very beneficial when reporting results to stakeholders. It allows evaluators to provide more visual representations of the data, communicate results clearly, and elaborate on recommendations beyond text.

Brochure

Results from this evaluation could be used to market the future program by creating a one-page aesthetically pleasing brochure to reinforce public relations and generate interest in the program. In addition to communicating results in an efficient manner with stakeholders, this method also provides stakeholders with a marketing tool to use for future program recruitment.

Lessons Learned

The evaluation process of this program was very in-depth. There were 31 participants with each participant filling out 5 evaluation sheets equating to 155 evaluations that needed to be collected, inputted into Excel, and analyzed. Personally, I would transition most of this data collection to an online survey that participants complete during the program. This would greatly decrease labor on the evaluator side, decreased data entry error, and allow for quick analysis.

I also found that many of the evaluation documents were repetitive and could be easily condensed. I only obtained data from 50% of the questions on each evaluation sheet. If given the opportunity, I would restructure the evaluation paperwork (I recommended within the report) to collect data that is directly relevant to the big E questions.

The next evaluation should also have a space for clarification on how to improve overall program satisfaction because it was hard to give recommendations for program improvement if results only showed dissatisfaction but no directionality.

I would also completely redo the pre-posttest. The current questions lead to testing bias and are not directly reflective of knowledge gained in my opinion. I believe a lot of the previous data showing differences is skewed due to testing bias.

To conclude, if I was repeating this evaluation, I would transition all evaluations to an online interface. I would restructure the evaluation questions to be directly related to answering the big E questions, ensure there was a response area for program satisfaction improvement/suggestions, and I would also write the evaluation report while doing the evaluation process instead of after all data was collected.

Table 1 Performance criteria and standards for each big E question.

Big E Question	***Evaluation Tools***	***Criteria***	***Standard/Indicators***
1. *What knowledge did participants increase by attending the program?*	All items on pre and post test	Ability to perform better on written exam after attending the program	A significant increase (P< 0.05) between the pre- and post- test assessment
2. *What skills did participants gain by attending the program?*	Self-Evaluation Survey (post-only)	Ability to select estrous synchronization program Ability to pass artificial insemination rod into the uterine body	80% of participants can select an estrous synchronization program that is best for their operation The average times of skill execution during the program will be 2 times per person
3. *Were participants satisfied with the program?*	Satisfaction Survey (post-only)	Satisfaction with speakers Satisfaction with topics Overall satisfaction with program	The average satisfaction score for speakers and topics is >4.5 80% of the program participants would recommend the program to other producers

Table 2 Satisfaction scores of topics covered with the average topic score of 4.8.

TOPIC	**1**	**2**	**3**	**4**	**5**	**MEAN**
ECONOMICS OF ARTIFICIAL INSEMINATION	0	0	3	6	18	4.6
REPRODUCTIVE ANATOMY	0	0	0	2	26	4.9
ESTROUS CYCLE AND ESTRUS SYNCH	0	0	1	3	23	4.8
ARTIFICIAL INSEMINATION EQUIPMENT	0	0	1	4	22	4.8
HEAD DETECTION AND HEAT DETECTION AIDS	0	0	1	5	22	4.8
NUTRITIONAL PROGRAMS FOR AI SUCCESS	0	0	0	2	25	4.9
SIRE SELECTION	0	0	0	4	23	4.9
REPRODUCTIVE HERD HEALTH AND BIOSECURITY	0	0	0	8	20	4.7
INSEMINATION PRACTICE TRAINING	0	0	0	6	21	4.8
SEMEN HANDLING	0	0	0	3	22	4.9

Table 3 Satisfaction scores of speakers with the average speaker score of 4.6.

SPEAKER	**1**	**2**	**3**	**4**	**5**	**MEAN**
ECONOMICS OF ARTIFICIAL INSEMINATION	0	0	1	6	20	4.7
REPRODUCTIVE ANATOMY	0	0	2	2	27	4.8
ESTROUS CYCLE AND ESTRUS SYNCH	0	0	5	5	23	4.5
ARTIFICIAL INSEMINATION EQUIPMENT	0	0	5	5	23	4.5
HEAD DETECTION AND HEAT DETECTION AIDS	0	0	2	2	26	4.8
NUTRITIONAL PROGRAMS FOR AI SUCCESS	0	0	5	5	24	4.6
SIRE SELECTION	0	0	3	3	25	4.7
REPRODUCTIVE HERD HEALTH AND BIOSECURITY	0	0	6	6	20	4.4
INSEMINATION PRACTICE TRAINING	0	0	4	4	25	4.6
SEMEN HANDLING	0	0	3	3	25	4.7

Table 4 Themes within participant responses to improve satisfaction.

THEMES	**QUOTES FROM PARTICIPANTS**
INCREASED EQUIPMENT USE AND EXPOSURE	"AI tools on display for viewing during breaks" "More equipment talks" "Equipment for sale" "Demonstration of CIDR insertion"
PREGNANCY PALPATION	"More on palpation techniques" "Pregnancy detection"
TIMING AND HANDOUT CONTENT	"Larger pictures provided on PowerPoint slides" "End earlier than 10pm the first day" "Provide more time for some speakers that communicate too quickly" "More anatomy notes within the PowerPoints" "Smaller class size"

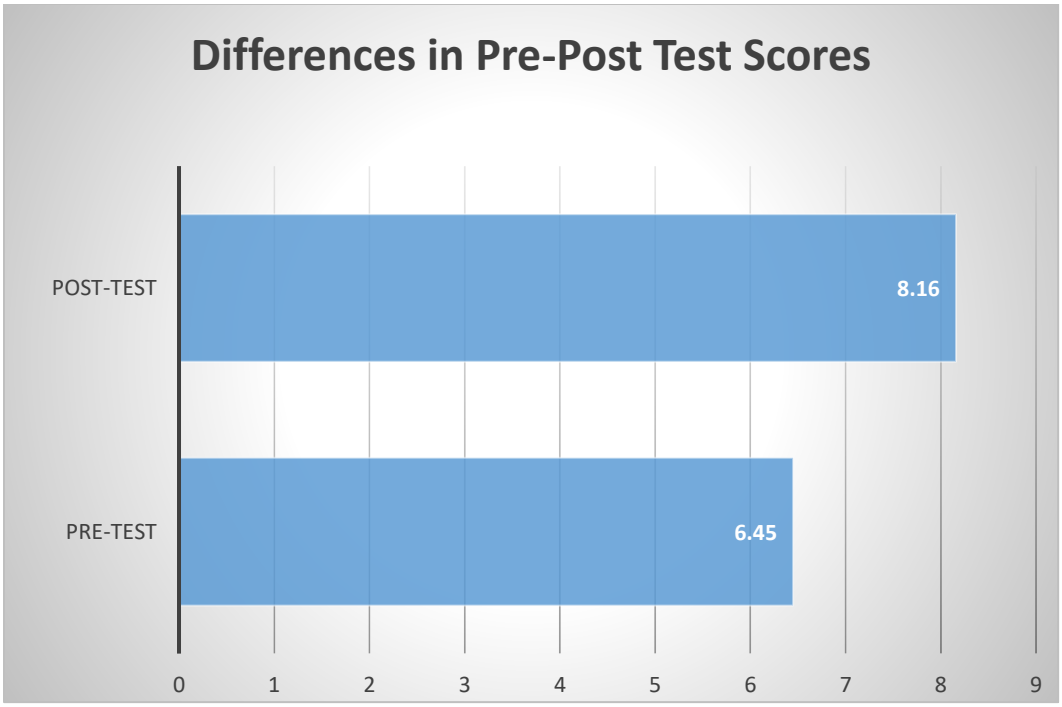

Figure 23 Artificial Insemination School Pre-Post Test Score Difference

Bar graph depicting the differences between pre-test and post-test scores from participants in Mississippi State Artificial Insemination School Spring 2021.

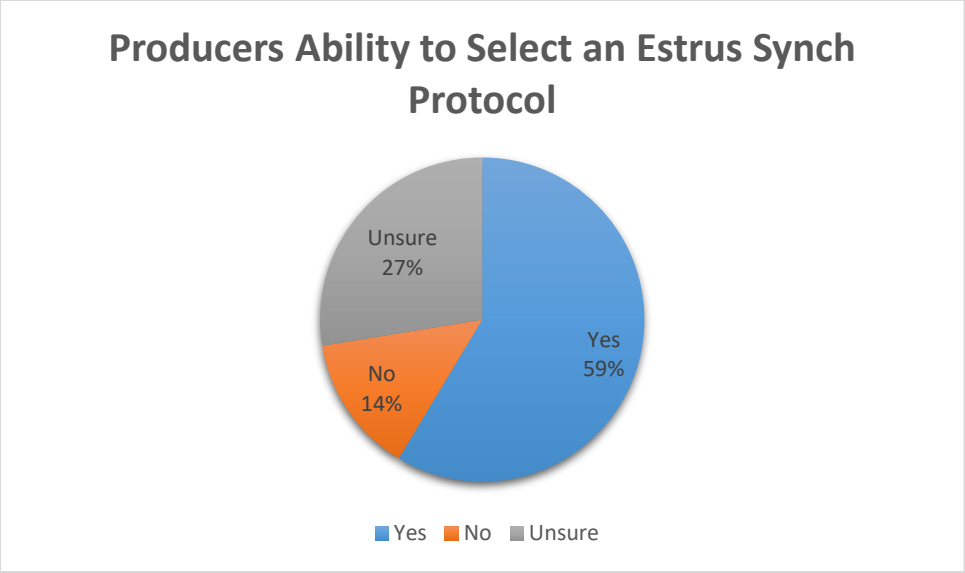

Figure 24 Producer Ability to Select an Estrus Synchronization Protocol (Post)

Pie chart of participant's ability to select the correct estrus synchronization protocol for their operation.

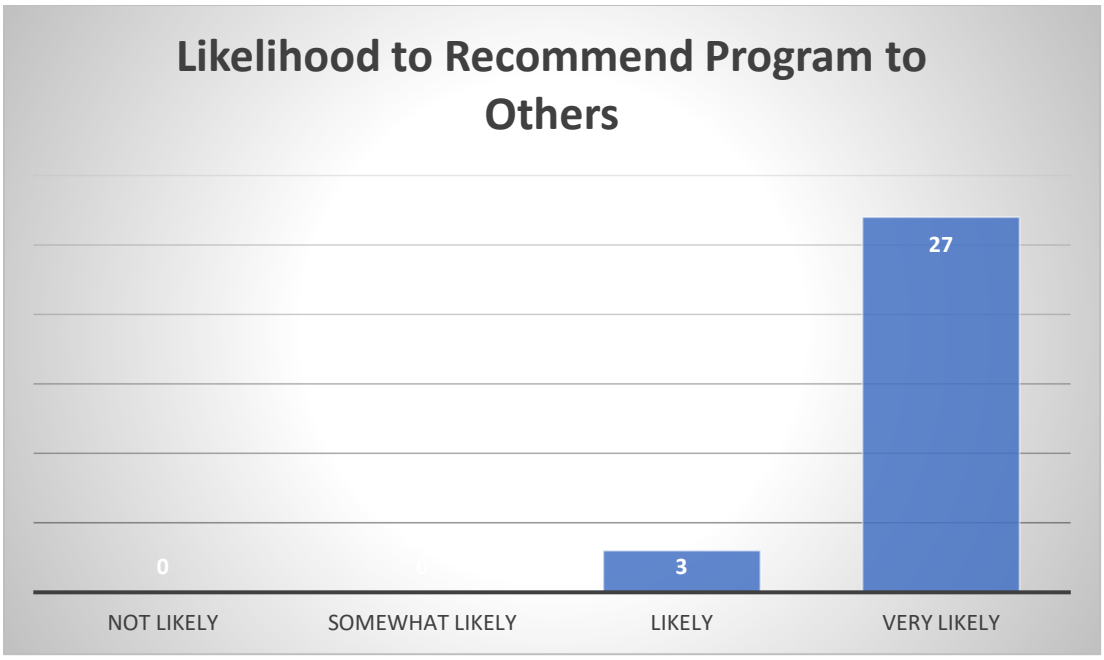

Figure 25 Recommendation Results of MS-AI Program

Bar graph depicting the likelihood of participants to recommend the Mississippi State Artificial Insemination School extension program to others.

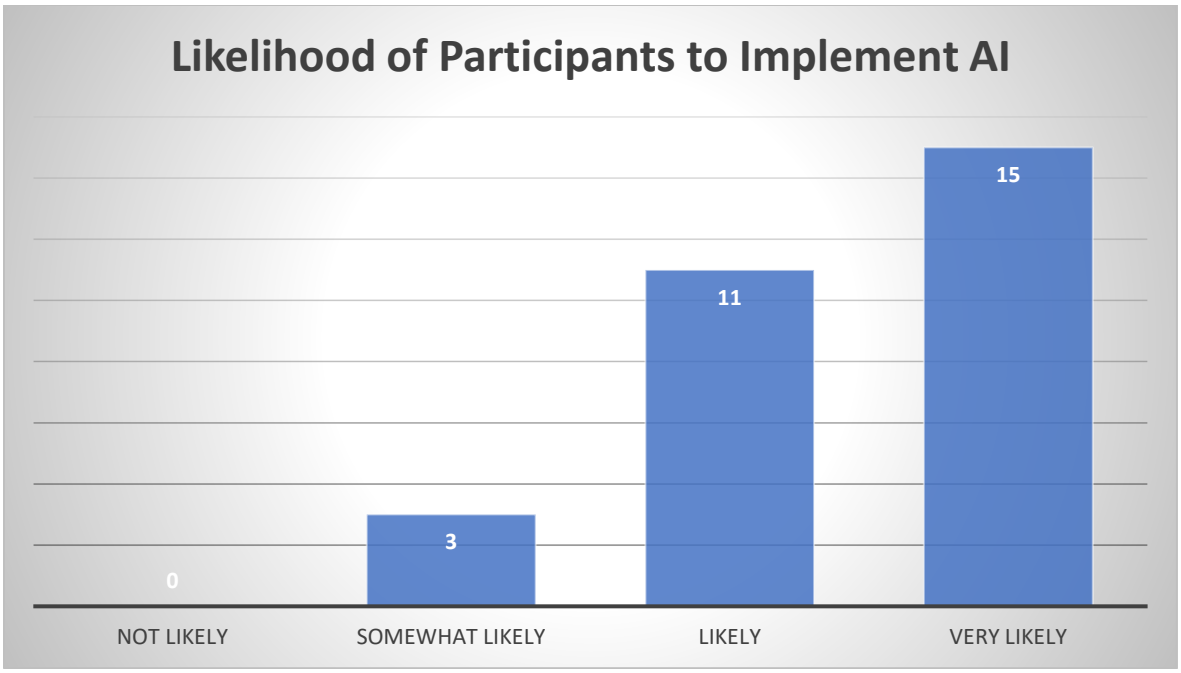

Figure 26 Implementation Results of MS-AI Program

Bar graph depicting the likelihood of participants to implement an artificial insemination program at their operation.

Data Collection Documents

Pre- & Post-test

Mississippi State Artificial Insemination School Pre- & Post-Test

1. Which of the following is an advantage to AI?
    a. Access to superior genetics
    b. Tighter calving interval
    c. Reduced bull requirements
    d. All of the Above
2. When artificially inseminating, semen should be deposited…
    a. in the uterine horn
    b. in the cervix
    c. in the uterine body
    d. anywhere in the reproductive tract
3. Estrus, sexual receptivity in the female, lasts approximately ____ to ____ hours, and occurs, on average, every ____ days.
    a. 2, 6, 28
    b. 8, 10, 21
    c. 6, 24, 21
    d. 16, 48, 21
4. A standard straw of semen is _____ mL in volume.
    a. 0.25 mL
    b. 0.75 mL
    c. 0.5 mL
    d. 1.0 mL
5. Which of the following is a secondary sign of heat in cattle?
    a. Riding other cows
    b. Roughened hair on tailhead
    c. Mucous discharge
    d. All of the above
6. Which of the following is a consequence of nutritional mismanagement?
    a. Increased age at puberty
    b. Poor re-breeding rates
    c. Lower conception rate
    d. All of the Above
7. When selecting an AI sire to use on virgin heifers, which of the following parameters would be most beneficial?
    a. Visual appraisal
    b. Actual Birth Weight of Sire
    c. Calving Ease Direct EPD
    d. Birth Weight Ratio of Sire
8. Higher weaning weights will typically be associated with which of the following?
    a. Higher Yearling Weights
    b. Larger Scrotal Circumferences
    c. Higher Birth Weights
    d. All of the Above
9. Which of the following is a cause of economic loss due to decreased reproductive herd health?
    a. Bovine Viral Diarrhea Virus
    b. Ringworm
    c. Trichomoniasis
    d. Both a. & c.

10. Benefits of estrus synchronization include
    a. inseminating your females at the time of your choosing
    b. having more calves bred at the beginning of the breeding season
    c. more uniform calf crop
    d. all of the above

Self-Evaluation Survey

Mississippi State University Animal and Dairy Sciences
Artificial Insemination School – Self Evaluation

How many times during the artificial insemination practice did you successfully execute the technique?

_____

Based on the information provided in the Artificial Insemination School program, what is the likelihood that you will implement estrous synchronization and artificial insemination protocols within your herd?

○ Not likely ○ Somewhat Likely ○ Likely ○ Very Likely

What type of operation do you currently own/operate? Circle below:

Beef / Dairy

Commercial / Seedstock

Under 100 hd / Over 100 hd

Bos taurus / *Bos indicus*

What estrous synchronization protocol would you implement at your current operation?

_____

Do you have any additional comments/concerns about implementing the artificial insemination technique at your own operation?

Satisfaction Survey

Mississippi State University Animal and Dairy Sciences
Artificial Insemination School - Participant Survey

1. Please rate the topic on its value to you.
   Please rate the speaker on presentation, content, and effectiveness in answering your questions.
   Circle one number per topic and speaker (1 = poor and 5 = excellent).

Topic		**Speaker**
1 2 3 4 5	Economics of Artificial Insemination	1 2 3 4 5
1 2 3 4 5	Reproductive Anatomy	1 2 3 4 5
1 2 3 4 5	Estrous Cycle and Estrus Synchronization	1 2 3 4 5
1 2 3 4 5	Artificial Insemination Equipment	1 2 3 4 5
1 2 3 4 5	Heat Detection and Heat Detection Aids	1 2 3 4 5
1 2 3 4 5	Nutritional Programs for A.I. Success	1 2 3 4 5
1 2 3 4 5	Sire Selection	1 2 3 4 5
1 2 3 4 5	Reproductive Herd Health and Biosecurity	1 2 3 4 5
1 2 3 4 5	Insemination Practice Training	1 2 3 4 5
1 2 3 4 5	Semen Handling Training	1 2 3 4 5

2. Please indicate the number of **beef cows** and the number of **acres** in pasture or hay that you manage.

Beef cows	◯ 1-50	◯ 51-100	◯ 101-200	◯ 201-500	◯ Over 500	◯ N/A
Acres of pasture and hay	◯ 1-100	◯ 101-200	◯ 201-500	◯ 501-1,000	◯ Over 1,000	◯ N/A

3. Will something you learned from this program have an economic benefit on your operation?
   *Please estimate the anticipated economic **benefit per head over an entire year.***

◯ $0 (no impact) ◯ $1-$5 ◯ $6-$15 ◯ $16-$25 ◯ More than $25 ◯ Not applicable

4. The anticipated economic benefit can be attributed to which of the following? (Select all that apply)

   ○ Increased production ○ Improved marketing ○ Improved efficiency
   ○ Improved sustainability ○ Other _____

5. Based on the information provided in the Artificial Insemination School program, what is the likelihood that you would recommend the Mississippi State University Extension Service to your family and friends as a contact on beef cattle?

   ○ Not likely ○ Somewhat Likely ○ Likely ○ Very Likely

6. What is your primary state and county of residence? _____

7. How did you learn about the Artificial Insemination School program?

   ○ Radio ○ Newspaper ○ Extension Newsletter ○ Word of Mouth
   ○ Website ○ TV ○ Flyer ○ Other _____

8. What would you suggest to improve this or future programs?

9. What was the most important or beneficial thing you learned from this program?

10. Please provide any additional comments below.

Demographic Survey

The demographic survey asked producers the following:

- Name
- Sex
- Ethnicity
- Permanent Address

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