

Sources of Variation in the Microbiome of Pre-Weaned Dairy Calves

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ABSTRACT

Death loss of pre-weaned dairy heifers is a major challenge confronting U.S. dairy producers, creating economic losses of at least \$100 million each year. Neonatal calf diarrhea, the leading cause of neonatal calf mortality, is controlled through colostrum feeding to ensure the passive transfer of immunity. However, these methods are not always effective in preventing calf morbidity and mortality. Therefore, other factors must play an integral role in calf health, morbidity, and mortality; the gut microbiome of the calf is likely significant. The dam's vaginal, fecal, and oral microbiomes are believed to be the primary sources of the newborn calf's gut microbiome, but the long-term health effects of the colonization by these microbes are yet to be determined. The objective of this study was to characterize the dam's fecal, vaginal, and oral microbiomes and the calf fecal microbiome. Our hypothesis stated that the dam's vaginal, fecal, and oral microbiomes will influence the early microbiome of the calf.

Multiparous Holstein cows ($n = 6$) were sampled and observed during the final weeks of pregnancy. Vaginal swab samples were collected from cows ~24 h prior to parturition. Oral, rectal, and vaginal swab samples were collected from cows immediately following parturition. Oral and rectal swabs were collected from calves ($n = 6$) at birth (h 0), h 24, d 7, d 42, and during the post-weaning period (d 60). Metagenomic 16S rDNA data was converted to inferred amplicon sequence variants (ASVs) and clustered into operational taxonomic units (OTUs) to assess microbial diversity and relative abundance

within fecal, vaginal, and oral microbiome samples. One-way ANOVA followed by Tukey's procedure for pair-wise comparisons were used to determine sample type differences. Significance was declared at $P \leq 0.05$. Using phylum count data, a negative binomial regression model was used to determine the effects of the dam's vaginal, fecal, and oral microbiomes on the calf fecal microbiome.

Metagenomic analysis revealed a diverse microbiome within the meconium of newborn calves, suggesting that microbial colonization of the neonatal calf GIT begins *in utero*. We identified 10 phyla associated with the calf meconium and fecal microbiome: Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Cyanobacteria, Verrucomicrobia, Spirochaetes, Tenericutes, Fibrobacteres, and Lentisphaerae. Principle-coordinate analysis revealed a strong relationship between the vaginal microbiome of the dam and the meconium microbiome of the calf. The dam vaginal microbiome and the calf meconium microbiome were highly similar, displaying significant differences ($P = 0.05$) in only two phyla, Bacteroidetes and Fusobacteria. The abundances of three phyla, Bacteroidetes, Proteobacteria, and Verrucomicrobia, differed significantly ($P = 0.05$) between the dam fecal microbiome and the meconium microbiome. The dam fecal microbiome was highly similar to the calf fecal microbiome post-weaning (d 60), making the fecal microbiome of the dam a strong indicator of the microbial composition of post-weaning calf feces. Overall, the calf meconium and fecal microbiome is influenced by a combination of the maternal vagina, oral, and fecal microbiomes.

Further studies will be needed to identify the transference mechanisms of maternal microbes to offspring and the associated host-microbial interactions.

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GENERAL AUDIENCE ABSTRACT

This study investigated the microbial colonization and maternal influences on the neonatal calf gut microbiome. Microbiome samples were collected from dams (n = 6) and calves (n = 6) using sterile flocked swabs. The vaginal, oral, and fecal bacterial communities were examined from the dam and the fecal community of calves was examined from birth to 60 d of age. Microbial communities varied by anatomical location and age of the calf. Metagenomic analysis 16s ribosomal DNA revealed ten phyla associated with microbiomes of the dam and the same ten phyla associated with calf feces at various time points: Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Cyanobacteria, Verrucomicrobia, Spirochaetes, Tenericutes, Fibrobacteres, and Lentisphaerae. Overall, the calf meconium and fecal microbiome is influenced by a combination of the maternal vagina, oral, and fecal microbiomes. Further studies will be needed to identify the transference mechanisms of maternal microbes to offspring and the associated host-microbial interactions.

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CHAPTER 1

REVIEW OF LITERATURE

1. INTRODUCTION

Pre-weaned dairy heifer death loss is a major challenge confronting U.S. dairy producers, with neonatal calf diarrhea accounting for more than 50% of deaths and creating substantial economic losses. Current disease prevention protocols consist of colostrum feeding practices to ensure the passive transfer of immunity. However, this method is not always effective in alleviating calf morbidity and mortality (Meganck et al., 2015). The persisting issue of pre-weaned heifer death loss and the public's growing concern of antibiotic usage in production livestock has presented a major need for an alternate method of disease prevention and treatment. Optimal microbial composition of the gastrointestinal tract (GIT) is of vital importance to the long-term health and performance of livestock. However, the gut microbiota of pre-weaned dairy calves is not well understood. This presents the need for an enhanced understanding of the calf gut microbiota for developing new approaches to improve calf gut and overall health (Malmuthuge, 2017). The GIT microbiome is responsible for a number of physiological and functional processes, including nutrient digestion and absorption, host metabolism, mucus layer development, barrier function, and mucosal immune responses (Kogut and Arsenault, 2016). Inoculation of the neonatal calf's GIT is initiated at birth and is facilitated by the dam's vaginal, fecal, oral, and colostrum microbiota. The structure and function of maternal microbial communities and the impact of these communities on maternal and neonatal health has been studied extensively in human and murine models, but these investigations are still in early phases for dairy cattle. Existing findings from

human and murine investigations have highlighted the importance of GIT microbial establishment and colonization to host development and health status (Malmuthuge et al., 2015b, Charbonneau et al., 2016). Identification of the ideal colonization processes and microbial communities of the GIT could allow for targeted microbial therapies, vital to addressing the economic and ethical concerns associated with calf mortality (Malmuthuge et al., 2015b).

2. THE MATERNAL MICROBIOME

The community of microorganisms that live in and on the human body, consisting of upwards of 100 trillion cells, is known as the human microbiome. The cells of the human microbiome outnumber host cells by a factor of ten and contains 27 times more genes than in the human genome. Different sites on and within the human body such as the skin, mouth, gut, and reproductive tract are home to discrete populations of microbes. Advancements in sequencing technologies have allowed for findings that present clear associations between microbiome composition and host health (Dunlop et al., 2015). Although most research has focused on the microbiome of the gastrointestinal tract, there is increasing interest in the female reproductive microbiome and the associated health of the offspring. The vaginal microbiome is key in successful fertilization and a healthy pregnancy. The microbiome also plays an essential role in producing healthy and reproductively fit offspring (Mueller et al., 2015). Microbial communities have been identified in niches that were traditionally considered sterile, such as the uterus, placenta, and fallopian tubes. The combination of female reproductive microbiome, postnatal environment, and infant colonization patterns heavily influence health throughout a lifetime (Younes et al., 2018).

Core microbiota of the human vagina

Research involving the vaginal microbiome in women has advanced in the last 15 years due to studies the development of high-throughput sequencing. Early molecular studies used general 16S ribosomal RNA (rRNA) sequencing techniques that expanded the understanding of the vaginal microbiota. These studies led to the characterization of the vaginal microbiome. The vaginal microbiome is composed of microorganisms including *Lactobacillus* sp., *Atopobium vaginae*, *Sneathia*, *Leptotrichia*, *Megasphaera*, *Dialister*, and *Eggerthella* (Martin and Marrazzo, 2016). Ravel et al (2011) characterized the “core microbiome” of the healthy, adult vagina through pyrosequencing 16S rRNA genes. The results of this study indicate that although the vaginal microbiome of healthy, reproductive-age women is dominated by *Lactobacillus iners* and *Lactobacillus crispatus*, there is no single core microbiome. Instead, there are a variety of core microbiomes that can be identified by community groups. These groups can be categorized by two criteria: (1) the constituent communities are dominated by *Lactobacillus*, and (2) the distinct species of *Lactobacillus* that are present (Ravel et al., 2011). Although there is not a core microbiome of the vagina, there are core functions, such as lactic acid production that remain among communities (Ravel et al., 2011).

Disease, sexual activity, and menstruation are events known to cause variation in the vaginal microbiome. Intervals of dysbiosis within the microbiome lead to increased susceptibility to disease (Gajer et al., 2012). Identifying factors that disrupt the stability of the vaginal microbiome could provide useful insight into predicting disease risk and women’s health.

The influence of microbes on vaginal health

Microbial communities of the vagina are composed of mutualistic bacteria that are the first line of defense against urogenital disease and infection (Kaewsrichan et al., 2006, Ravel et al., 2011). These preventable diseases include bacterial vaginosis, yeast infections, sexually transmitted infections, urinary tract infections, and human immunodeficiency virus type 1 (HIV-1) infection. Vaginal disease prevention by the microbiome is largely attributed to lactic acid-producing bacteria that commonly inhabit the vagina. Lactobacilli are found dominant in vaginal fluid of healthy women at concentrations of 10^7 - 10^8 colony-forming units (CFU) g^{-1} (Sobel and Chaim, 1996). *Lactobacillus* species are thought to provide disease protection by lowering the pH of the vagina, producing bacteriostatic and bactericidal compounds, and through competitive exclusion (Ravel et al., 2011). Some lactobacilli, such as *Lactobacillus crispatus* and *L. gasseri*, are able to control the overgrowth and infection processes of pathogens, as well as modulate systemic inflammation, cell proliferation, and apoptosis of the female (Wang et al., 2018).

A healthy vaginal environment has a pH of 4 ± 0.5 and maintenance of pH is essential to reproductive and vaginal health. The acidic environment reduces the infectivity for a variety of sexually transmitted disease pathogens, including HIV-1 (Tevi-Bénissan et al., 1997, Boskey et al., 2001). Also, elevated pH and resulting dysbiosis of the vaginal microbiome is linked to Bacterial vaginosis (BV), a prevalent condition characterized by overgrowth of certain anaerobic bacteria and reduced abundance of *Lactobacillus*. Bacterial vaginosis is associated with significant adverse health effects including abnormal pregnancy (Martin and Marrazzo, 2016), premature birth (Sagawa et

al., 1995), increased risk of HIV infection (Taha et al., 1998), and pelvic inflammatory disease (Hillier et al., 1996) (Boskey et al., 2001).

The acidic environment of the vagina is produced by lactic acid-producing bacteria. Glycogen is deposited in the vaginal epithelium during times of high estrogen, and it is metabolized into lactic acid supporting the desired pH (Boskey et al., 2001). Metabolism of glycogen was originally thought to be by the vaginal epithelium, but this theory was refuted after exploration of vaginal lactate structures. This investigation revealed that the majority of vaginal lactic acid was of the D-isoform, which cannot be produced by human metabolism (Prince et al., 2015). Vaginal lactobacilli have been found to be capable of producing acid at sufficient rates to account for the rate at which the vagina acidifies after neutralization in an *in vitro* setting (Boskey et al., 1999). Although this finding is consistent with the theory of vaginal acidity being primarily of bacterial origin, evidence is lacking to determine if this is the only mechanism responsible for acid production in the vagina (Boskey et al., 2001).

Lactobacilli are effective in restricting the growth of pathogens through the production of bacteriostatic and bactericidal compounds. Hydrogen peroxide (H_2O_2) is produced by lactobacilli present in a healthy vaginal environment. Klebanoff and colleagues (1991) found that H_2O_2 -dependent activity is bactericidal to *Gardnerella vaginalis* in an *in vitro* setting (Klebanoff et al., 1991) (Kaewsrichan et al., 2006). An overgrowth of *Gardnerella vaginalis* in the vagina is one of the primary origins of bacterial vaginosis. Lactic acid bacteria, which include lactobacilli, streptococci, and pneumococci, form and release the H_2O_2 required for the peroxidase-mediated antimicrobial system; a toxic event to a variety of microorganisms and mammalian cells.

When peroxidases are combined with H₂O₂ and a halide, the halide is oxidized by peroxidase and H₂O₂ to form a potent oxidant. This created oxidant (corresponding hypohalous acid or halogen) attacks the target cell at its oxidizable sites, resulting in cell destruction and the prevention of pathogenic infection (Klebanoff et al., 1991).

Microbial changes during pregnancy

Pregnancy causes a myriad of physiological changes to support the growth and development of the fetus. These changes include shifts in hormones, metabolism, and immunity; all are major factors inducing dramatic changes in the microbiome during pregnancy (Nuriel-Ohayon et al., 2016). Hormonal changes include the dramatic rise of progesterone and estrogen. A successful pregnancy is largely dependent on progesterone for creating a suitable endometrial environment for the growing fetus and preventing preterm labor (Kumar and Magon, 2012). Metabolic changes that occur during pregnancy include weight gain, elevated fasting blood glucose levels, insulin resistance, glucose intolerance, and changes in metabolic hormone concentration (Nuriel-Ohayon et al., 2016). Also, the female body must maintain a level of immune suppression to accept the fetus and its own developing immune system, while enabling immunity to external infectious agents. (Mor and Cardenas, 2010, Nuriel-Ohayon et al., 2016). The changes the female human body undergoes during pregnancy initiate major shifts in the microbiota at distinct sites.

The vaginal microbiome during pregnancy

The human vaginal microbiome is the primary protection against disease to offspring. A *Lactobacillus*-dominated microbiota stands as a strong biomarker for a healthy vaginal environment (Petrova et al., 2015). *Lactobacillus crispatus*, *Lactobacillus*

gasseri, *Lactobacillus iners*, and *Lactobacillus jensenii* are the most frequently isolated species. The depletion of these species is associated with several adverse consequences, including increased risk and transmission of sexually transmitted infections, infertility, preterm birth, and pelvic inflammatory disease. A *Lactobacillus*-dominated microbiota establishes anti-pathogenic mechanisms that promote health and maintain reproductive fitness of the host. *Lactobacilli* produce biochemically active compounds that directly kill or inhibit the stabilization of pathogens (Petrova et al., 2015).

The microbial community of the vagina varies in association with normal pregnancy, creating a “signature” taxonomy related to a state of pregnancy. These changes include a significant decrease in microbial diversity and an enrichment with species of *Lactobacilli* (Aagaard et al., 2012, Nuriel-Ohayon et al., 2016). Aagaard et al (2012) demonstrated significant differences between the vaginal microbiomes of pregnant and non-pregnant women (Aagaard et al., 2012) (Martin and Marrazzo, 2016). The signature vaginal taxonomy of pregnant women consists of an increased abundance of *Lactobacillus* spp, along with an overall decreased richness and diversity. The microbial community of the vagina during pregnancy varied by gestational age and proximity to the cervix (Aagaard et al., 2012). Prince et al (2015) discovered an enrichment of the orders Lactobacillus, Clostridiales, Bacteriodales, and Actinomycetales. At the species level, the vaginal microbiome during pregnancy was enriched in *L. iners*, *L. crispatus*, *L. jensenii*, and *L. johnsonii*. Enrichment in lactobacilli could be a result of the increase of estrogen induced by pregnancy (Prince et al., 2015).

Reconstruction of the vaginal microbiome during the postpartum period is induced by a sudden halt in estrogen production, which significantly reduces the

proportion of lactobacilli and enriches bacteria associated with vaginosis. Further studies are necessary for understanding the effects of sex hormones on the vaginal microbiome during and post-pregnancy (MacIntyre et al., 2015).

The gut microbiome during pregnancy

The microbial environment of the intestine undergoes considerable changes during pregnancy. The combination of stool samples, diet information, and clinical data from 91 pregnant women revealed shifts in the intestinal microbiome throughout pregnancy and that the gut microbiota is profoundly altered by pregnancy (Koren et al., 2012). The transition from the first to third trimester is characterized by a decrease in species diversity and an increase in Proteobacteria, these third trimester stool samples also exhibited markers of inflammation and energy loss (Koren et al., 2012). Microbial profiles associated with the third trimester induce physiological changes in germ-free (sterile) mice. Fecal transplants of first and third trimester stool into germ-free mice were performed to investigate if the changes in the microbiota are cause or consequence of an increased state of inflammation. After third trimester gut-microbiota were transferred to germ-free mice, the mice gained a significant amount of weight, developed insulin resistance, and showed an increased inflammatory response. The results indicate that third trimester microbiota are associated with metabolic changes in the host, suggesting a link between pregnancy-induced microbial shifts and changes in host metabolism and immunology. These microbial shifts are postulated to be necessary for proper fetal development and a healthy pregnancy (Koren et al., 2012, Nuriel-Ohayon et al., 2016).

The oral microbiome during pregnancy

The oral microbiome is also susceptible to composition changes with pregnancy. The oral cavity is home to over 600 diverse species including *Streptococci*, *Lactobacilli*, *Staphylococci*, and *Corynebacteria*, each within their own microenvironment (e.g. tongue, teeth, palates, gums, cheeks, lips). When compared to non-pregnant women, pregnant women had higher total viable microbial counts of the oral microbiome. This finding stands true in all stages of pregnancy (Fujiwara et al., 2017). Efforts have been made to elucidate the mechanisms responsible for the oral microbiome changes by pregnancy, but they remain unclear. Hormonal changes during pregnancy, such as the increase in estrogen and progesterone, may be responsible (Nuriel-Ohayon et al., 2016).

Human milk microbiome

Breast milk is considered to be the optimal source of the infant's nutritional needs. This includes proteins, lipids, and 200 different types of unique oligosaccharides. Breast milk has traditionally been considered sterile, but recent studies have shown otherwise. Breast milk is a completely adapted nutritional source for a newborn with an impressive array of immune-active molecules, acting as the leading source for establishing an infant's gut microbiome (Nuriel-Ohayon et al., 2016). Breastfeeding during the first few months of life can influence development of the immune system and modify susceptibility to disease (Vieira Borba et al., 2018).

Human milk is one of the main sources of bacteria to the breastfed infant gut. An infant consuming 800 mL of milk per day will ingest between 1×10^5 and 1×10^7 bacteria daily. *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Bifidobacterium* spp. have been isolated from human milk using culture-dependent techniques (Gomez-

Gallego et al., 2016). An in-depth analysis of breast milk using high throughput sequencing techniques revealed that breast milk has an extremely diverse microbiome that changes throughout lactation (Hunt et al., 2011). *Weisella*, *Leuconostoc*, *Staphylococcus*, *Streptococcus*, and *Lactococcus* were found to be the dominant species in colostrum. Compared to breast milk during early lactation, samples collected 1-6 months post-partum contained significantly higher amounts of bacteria that are typical inhabitants of the infant oral cavity, *Veillonella*, *Leptotrichia*, and *Prevotella* (Cabrera-Rubio et al., 2012).

The presence of the microbiota in human milk was originally believed to be a result of contamination with bacteria from the mother's skin or infant's oral cavity. While suckling does create retrograde flow back into the mammary ducts, breast milk is a source of bacteria to the infant's mouth. Ecological niches in the human microbiome are not isolated environments, but rather a network of interrelated communities. Therefore, it is likely that the milk microbiome is influenced by other maternal microbial populations (Fernandez et al., 2013). Although the pathway and mechanisms have not yet been elucidated, the maternal gut is thought to be the origin of the live bacteria found in breast milk. The mechanism through which bacteria travel from the gut to the mammary glands may be modulated by maternal dendritic cells (DCs), macrophages, and hormonal changes during pregnancy. Dendritic cells penetrate the gut epithelium to take up non-pathogenic bacteria directly from the gut lumen and open tight junctions to send dendrites outside the epithelium. Progesterone is thought to increase gut permeability, which could aid DCs in penetrating the gut epithelium. Dendritic cells also retain small number of live commensal bacteria for several days in the mesenteric lymph nodes, allowing the

commensal bacteria to spread to other locations (Fernandez et al., 2013, Nuriel-Ohayon et al., 2016).

Microbiome colonization in the infant

Infant colonization of the maternal microbiome

The microbial communities within of segments of the reproductive tract have significant influence on reproductive success (Younes et al., 2018). The placenta functions not just as a method of nutrient exchange between mother and fetus; it also harbors a metabolically rich microbiome (Aagaard et al., 2014). Microbes in the uterus, placenta, and amniotic fluid make contact with a developing fetus, suggesting that the development of an organism's microbiome begins *in utero*.

The microbial colonization of the fetus appears to begin before birth (Jiménez et al., 2008). This was demonstrated using orally inoculated pregnant mice with a genetically labeled *Enterococcus faecium* strain. This strain was isolated from the meconium taken from healthy murine neonates delivered by C-section. Bacteria from the maternal microbiome are able to enter the GI tract of the fetus, clearly suggesting that the pre-birth environment of the neonate is not sterile (Jiménez et al., 2008, Nuriel-Ohayon et al., 2016).

Another study in 2012 also alludes to the ability of maternal bacteria to enter the GI tract of the fetus. In this human study, pregnant mothers received probiotics and were compared to placebo controls. In the treatment group, bacterial composition changes were detected in the infant meconium and placenta. These results clearly indicate the presence of early microbial colonization of the fetus and interactions between developing host and microbiota (Rautava et al., 2012, Nuriel-Ohayon et al., 2016).

Analogous to the fetus and placenta, meconium was also previously believed to be sterile. However, studies using a variety of methods show the presence of *Enterococcus*, *Escherichia*, *Leuconostoc*, *Lactococcus*, and *Streptococcus* (Nuriel-Ohayon et al., 2016). In comparison to adult fecal samples infant meconium shows lower species diversity, higher between-subject variation, and an enrichment of *Proteobacteria* and reduced numbers of *Bacteroidetes* (Hu et al., 2013, Nuriel-Ohayon et al., 2016).

Mode of delivery

The intestinal (Koren et al., 2012) and vaginal (Romero et al., 2014) microbiomes see the greatest shifts during pregnancy. These are particularly important because these sites are responsible for vertical microbial transmission from mother to infant (Mueller et al., 2015). While there is exposure to microbes *in utero*, the majority of colonization of an infant occurs at birth. An infant born vaginally is exposed to microbiota of the vagina, feces, and skin (Palmer et al., 2007). These microbes play an essential role in the postnatal development of the immune and metabolic system of the infant (Torrazza and Neu, 2011), so if this colonization is disrupted, the infant can suffer from lifelong consequences.

The greatest disruption to this vital assembly of microbes to the infant is the Caesarian-section (C-section) delivery. Epidemiological evidence shows that diseases such as asthma (Negele et al., 2004), type 1 diabetes (Bonifacio et al., 2011), obesity (Mueller et al., 2014), and food allergies (Eggesbø et al., 2003) are more common in infants delivered via cesarean section compared to those born vaginally (Torrazza and Neu, 2011). The contact between that infant and the maternal vaginal and intestinal flora is a major source of colonization that can only occur during vaginal delivery. During a

cesarean delivery, the absence of this maternal contact allows environmental bacteria to influence infants' intestinal colonization (Neu and Rushing, 2011).

Mode of delivery plays a key role in the development of an infant's intestinal microbiota, striking microbiological differences have been observed between C-section and vaginally delivered infants. When comparing the gut microbiome of infants born via C-section or vaginally, culture-independent techniques reveal the C-section delivered infants are deprived of strict and facultative anaerobes such as *Clostridium*, and have a decreased abundance of *Bifidobacterium* and *B. fragilis* (Penders et al., 2006, Prince et al., 2015). Unlike infants born vaginally, the gut microbiota of infants delivered via C-section is slow to gain diversity. The consequences of C-section delivery last well beyond infancy, with observations of microbial differences between C-section and vaginally delivered children seen after one month, two years, and seven years of life (Salminen et al., 2004, Mueller et al., 2015).

Breast feeding

Human milk contains a wide range of beneficial constituents, including carbohydrates, human milk oligosaccharides (HMOs), nucleotides, fatty acids, immunoglobulins, cytokines, lysozymes, lactoferrin, and other immunomodulatory factors, all of which influence the infant gut microbiome. Human milk is a unique source of nutrients and energy essential to an infant's immune maturation, metabolic and cognitive development, gut maturation, and optimal gut colonization. The composition and quality of human milk varies among individuals during lactation with maternal lifestyle, nutritional and immunological status, dietary habits, and lactation time as contributing factors (Castanys-Muñoz et al., 2016).

The interactions that occur between human milk, the developing intestinal tract, and the gut microbiota are vital to infant health. Maturation of the infant's underdeveloped intestinal epithelium is triggered by microbial colonization and linked to diet and microbiota-derived metabolites (Castanys-Muñoz et al., 2016). The rich bioactive factors in breast milk promote bacterial colonization of the infant with high amounts of Bifidobacteria and *Lactobacillus* species (Vieira Borba et al., 2018). Breast feeding is associated with a reduced risk of necrotizing enterocolitis (NEC), type 1 diabetes, type 2 diabetes, multiple sclerosis, inflammatory bowel disease, and asthma (Castanys-Muñoz et al., 2016, Vieira Borba et al., 2018).

Profound differences have been found in the gut microbiota of formula-fed vs. breast-fed infants (Mueller et al., 2015). *Bifidobacterium* are the dominant species in the gut of breast-fed infants, while the gut microbiota of formula-fed infants is dominated by *Enterococci* and *Clostridia* (Balmer and Wharton, 1989, Nuriel-Ohayon et al., 2016). An increased bacterial diversity, increased prevalence of *C. difficile*, *Bacteroides fragilis*, and *E. coli*, and decreased prevalence of Bifidobacteria is associated with formula feeding.

The differences in the gut microbiota of formula-fed and breast-fed infants is largely due to human milk oligosaccharides (HMOs), structurally diverse unconjugated glycans that are the third largest component of human milk (Bode, 2012). HMOs are bioactive compounds that are indigestible to infants due to their complex structure. HMOs travel intact through the intestinal tract to the colon, where they are liable to digestion by hydrolytic enzymes of the colonic microbiota. *Bifidobacterium*, *Lactobacillus*, and *Bacteroides* have the ability to degrade oligosaccharides into smaller sugars that the infant absorbs and uses for energy. The prebiotic effects of HMOs

selectively predominate in the large intestine of breast-fed infants. This increased proportion of Bifidobacteria in the microbiome of breastfed infants is associated with good health (LoCascio et al., 2010, Albenberg and Wu, 2014).

Reduced Bifidobacteria in the infant microbiota is associated with defects in the mucosal immune system which include, reduces production of mucosal Immunoglobulin A (IgA), reduced capacity for inflammatory responses, and decreased intestinal surface area (Kalliomäki et al., 2001). The presence of Bifidobacteria, specifically *Bifidobacteria longum*, also aids in disease prevention through competitive exclusion against infectious pathogens. Bifidobacterial strains isolated from infant feces have proven inhibitory against the colonization of *E. coli* O157:H7. *B. infantis* and *B. longum* excrete broad spectrum antimicrobial peptides associated with the bacterium's antagonistic properties that prohibit pathogenic infection (Gagnon et al., 2004).

The oral microbiome of the infant is influenced by the skin-to-skin contact involved with breast feeding. The oral cavity of breast-fed infants houses lactobacilli with antimicrobial properties that were not found in infants that were formula fed (Holgerson et al., 2013). After an infant has transitioned to solid food, the gut microbiota closely resembles the microbiota of an adult, becoming enriched with species including *Bacteroidetes*, *Firmicutes*, *Clostridium*, and *Faecalibacterium*. These changes in the gut microbiota are dependent on diet and feeding mode and enhance the infant's ability to utilize carbohydrates and synthesize vitamins (Bäckhed et al., 2015, Nuriel-Ohayon et al., 2016).

Environmental influences on host microbiota

Diet, antibiotic treatment, and maternal environment are the primary exogenous (environmental) factors influencing an infant's intestinal microbiota (Spor et al., 2011). Changes in bacterial taxa over the first two and a half years are associated with life events such as illness, dietary changes, and antibiotic treatment (Albenberg and Wu, 2014). Improved understanding of the exogenous effects on the maternal microbiome and the consequences faced by the offspring are critical for the improvement to overall health of the offspring (Nuriel-Ohayon et al., 2016).

Dietary influence of the gut microbiota

Nutrient composition affects the structure of the microbiome and provides substrates for microbial metabolism. Microbial differences observed among different societal and age groups are at least, in part, a result of different dietary practices. For example, differences are observed in adults who consume plant-based diets vs diets high in meat and fat (Albenberg and Wu, 2014). Similarly, diet is the primary factor shaping microbiome diversity during pregnancy (Nuriel-Ohayon et al., 2016). Pregnant mice fed a high-fat diet were found to have high amounts of microbes involved in pathways contributing to fatty acid, ketone, vitamin, and bile synthesis and significant differences exist in the abundance of genes favoring lipid metabolism, glycolysis, and gluconeogenic metabolic pathways (Gohir et al., 2015, Nuriel-Ohayon et al., 2016).

Of all the life events that influence an infant's intestinal microbiota, weaning and the introduction of solid foods induces the greatest change. This begins a microbial shift toward an adult-like microbiota, with the intestinal microbiota resembling that of an adult by three years of age (Yatsunenکو et al., 2012, Albenberg and Wu, 2014).

Influence of antibiotic exposure on gut microbiota

Antibiotic treatment of mothers during pregnancy influences intestinal colonization and microbiome composition of their infants; this has been well established using murine models. The administration of antibiotics during pregnancy significantly reduces bacterial diversity and encourages weight gain (Khan et al., 2016).

Administration of category B antibiotics (antibiotics that do not cause pregnancy complications or birth defects), during pregnancy increases the relative abundance of Proteobacteria and *Enterobacter*, while reducing amounts of Firmicutes and *Lactobacillus* in feces (Gonzalez-Perez et al., 2016). Maternal antibiotic treatment during pregnancy and lactation also reduced adaptive antiviral immune responses in the infants, suggesting a broad immune effect on the offspring (Gonzalez-Perez et al., 2016, Nuriel-Ohayon et al., 2016)

Antibiotics are the most commonly prescribed drug for children, but use of these drugs can disrupt the delicate ecosystem of the neonatal microbiome (Tamburini et al., 2016). Epidemiologic evidence suggests that frequent courses of antibiotics during infancy and childhood alter the intestinal microbiota and could serve as a significant risk factor in the development of allergic and autoimmune diseases (Vangay et al., 2015, Gonzalez-Perez et al., 2016). The effects of antibiotic exposure on the microbiome depends on body site, dose, and type of antibiotic used, but these are not well understood, leaving a gap in our knowledge during a critical developmental period (Tamburini et al., 2016).

The response of the gut microbiome to a course of antibiotics transitions through four stages: pre-treatment, mid-treatment, recovery, and long-term stasis. Antibiotic

treatment results in dysbiosis, provoking loss of keystone taxa and short-term metabolic shifts. The gut microbiome begins a process of recovery immediately after the course of antibiotics, but permanent metabolic consequences can persist after the gut microbiome has reached a new stasis, including a loss of biodiversity, bloom of pathobionts, and increased risk of infectious disease (Vangay et al., 2015). Mouse models have demonstrated significant long term disruptions of the gut microbiome, including a reduction in *Lactobacillus*, *Allobaculum*, and segmented filamentous bacteria, creating a T helper 17 cell response in the colon (Ivanov et al., 2009, Cox et al., 2014).

3. THE MATERNAL MICROBIOME OF THE DAIRY COW

Gut microbiota in ruminants

The gut microbiome, mainly of the rumen, provides 70% of a ruminants' daily energy requirements through the fermentation of indigestible dietary substrates. The microbiome of the rumen is composed of bacteria, archaea, protozoa, and fungi that are all involved in the fermentation process. The microbial composition of the rumen is affected by the species of ruminant, diet, age, season, and geographic region (Malmuthuge et al., 2015b).

The bacterial composition of the gastrointestinal tract of preweaned calves influences early development as well as performance and health after weaning. The GIT of preweaned calves is dominated by with Firmicutes, Bacteroidetes, and Proteobacteria. The rumen and large intestinal regions are primarily composed of Bacteroidetes and Firmicutes, while the small intestine contains a bacterial composition of more than 95% Firmicutes. The small intestine mucosa is home to primarily Bacteroidetes, Firmicutes,

Proteobacteria, and 17 other genera of bacteria that are specific to this region of the GIT (Malmuthuge et al., 2014).

Distinct mucosa-attached bacterial phylotypes are established by three weeks of life. The mucosa-associated bacterial communities have a higher concentration of bacteria than the content-associated community. Analysis of these mucosa-specific bacteria may provide a better understanding of early gut function and development (Malmuthuge et al., 2014, Malmuthuge et al., 2015b). There is more variation in microbial composition in young ruminants in comparison to adults (Jami et al., 2013). This suggests that the gut microbiome is more susceptible to change during early life and could explain why prebiotics and probiotics have a much greater effect in young than in mature animals. Improved understanding of early gut colonization and microbiome establishment could provide strategies to improve the health and performance of ruminants (Malmuthuge et al., 2015b).

The vaginal microbiota of dairy cows

Data characterizing the microbial community in the bovine vaginal tract is limited (Nesengani et al., 2017). Bacterial composition and diversity of the vaginal microbiome are breed-dependent. In attempt to evaluate and compare microbial diversity among breeds, Nesengani et al. (2017) characterized the vaginal microbiota of Holstein and Fleckvieh cows. Holstein cattle have less abundant and diverse bacteria than the Fleckvieh cattle, while *Fusobacteria* were more abundant in Holsteins (Nesengani et al., 2017). Characterization of the vaginal microbiome in Criollo Limonero cows revealed a microbiome composed of Gram-positive aerobic and anaerobic bacteria. *Staphylococcus aureus*, *Staphylococcus epidermis*, *Arcanobacterium pyogenes*, *Erysipelothrix*

rhusiopathiae and *Escherichia coli* were the primary aerobic species isolated from vaginal swab samples. Isolated anaerobic species included *S. aureus*, *Staphylococcus intermedius*, *A. pyogenes*, *Peptostreptococcus* spp., and *Bacteroides* spp (Zambrano-Nava et al., 2011). Laguardia-Nascimento et al. (2015) found that the vaginal microbiota of Nellore cattle is dominated by Firmicutes (40-50%), followed by Bacteroidetes (15-25%) and Proteobacteria (5-25%) (Laguardia-Nascimento et al., 2015b). Swartz et al. (2014) reported that the vaginal microbiota of crossbred beef cows is predominated by Bacteroidetes, followed by Fusobacteria and Proteobacteria (Swartz et al., 2014). A low abundance of Fusobacteria is associated with healthy cattle, while a high abundance is associated with uterine diseases and infections, such as postpartum fever and metritis (Bicalho et al., 2017). Characterization of the vaginal microbiome could predict susceptibility and reduce the occurrence of reproductive-associated infections (Nesengani et al., 2017).

Microbial colonization and maternal influences of the newborn calf

The fetal calf develops in the absence of maternal antigens leaving both the systemic and mucosal immune systems premature at birth. There is, however, a progressive development of various immune defense mechanisms *in utero*. The thymus, spleen, and lymph nodes begin to develop during the first nine weeks of gestation, while the mucosa-associated lymphoid tissue (MALT) develops during late gestation with the mesenteric lymph nodes, diffuse lymphoid tissues, and Peyer's patches fully formed at birth. As the fetal calf develops, the abundance and functional capacity of leukocytes increases (Taschuk and Griebel, 2012). Fetal neutrophils and macrophages developed *in utero* and are released into the bloodstream after 130 days of gestation, however these

phagocytes have a reduced functionality and are unable to defend the host against pathogens at birth (Sherwin and Down, 2018). The adaptive immune system also develops throughout gestation, with naïve T-cell and B-cell populations present at birth. Newborn calves are born with an immune repertoire that is quickly educated by the microbial bombardment that begins at birth (Taschuk and Griebel, 2012).

Colonization of the newborn calf's GI tract is mediated by successional and host-mediated factors. During birth, the calf is first exposed to *Firmicutes* and *Bacteroidetes*, the dominant phyla in the bovine birth canal (Zambrano-Nava et al., 2011). As discussed earlier, human and murine studies have identified the importance of the neonate's first exposure to maternal flora. Vaginally-delivered infants acquire a gut microbiota closely resembling populations of the maternal birth canal, while infants delivered by Caesarian-section obtain a GIT microbiota that closely resembles the maternal epidermal flora. This is accompanied by lifelong health consequences. Vaginal birth provides pioneering bacteria necessary for the development of a superior-functioning mucosal immune system (Dominguez-Bello, Blaser, Ley, & Knight, 2011; Taschuk & Griebel, 2012).

4. EARLY IMMUNE DEVELOPMENT AND HOST HEALTH

Microbiota-induced maturation of epithelial barrier function

Colonization of the ruminant gastrointestinal tract begins at birth with the birth canal, along with feces, colostrum, and saliva from the dam providing the inaugural microbes. This early period of colonization, within the immediate hours after birth, is vital to the development of the neonate's immune system by providing fundamental microbes and favorable substrates for bacterial growth (Jost et al., 2012).

The intestinal mucus barrier allows the calf to tolerate mutualistic microorganisms that reside in the intestinal lumen while concurrently restricting the growth of pathogens (Sommer and Backhed, 2013). Thickness of the intestinal mucus barrier layer is stimulated and highly influenced by intestinal microbes, as germ-free animals that retain a thinner mucus layer than conventional animals (Deplancke and Gaskins, 2001). The use of gnotobiotic and conventionally raised mice has revealed that microorganisms act as a major influence on mucus composition and thickness. In comparison to conventionally raised mice, gnotobiotic animals have fewer goblet cells, a higher percentage of neutral mucins, and a thinner mucus layer (Sharma et al., 1995). Petersson et al (2011) found that when microbe-derived lipopolysaccharides and peptidoglycans were administered to germ-free mice, production of the colonic mucosal surface was stimulated and restored thickness to that of conventional mice, demonstrating that the gut microbiota is essential for the secretion of intestinal mucus (Petersson et al., 2011).

The gut microbiota plays an important role in shaping the development of lymphoid structures including Peyer's patches, mesenteric lymph nodes, and isolated lymphoid follicles (Sommer and Backhed, 2013). These secondary lymphoid structures allow lymphocytes to effectively encounter antigen-presenting cells that bring antigens from tissue (van de Pavert and Mebius, 2010). The development of intestinal immune cells is subservient to host-specific microbiota. Chung et al (2012) found low amounts of CD4+ and CD8+ T cells, proliferating T cells, and dendritic cells when gnotobiotic mice were inoculated with human microbiota in comparison to mice inoculated with mouse microbiota. Interestingly, no changes were observed in the microbiota of germfree mice colonized with the human microbiota (Chung et al., 2012). These observations suggest

that microbial interactions in the developing gut must be studied within relevant host species (Malmuthuge et al., 2015b).

Effect of antimicrobials on gut microbiota composition

Antimicrobial use in the dairy industry has traditionally focused on promoting growth while lowering the risk of enteric infections; however, there is limited understanding of the effects of antimicrobials on the neonatal gut microbiota in pre-weaned heifers (Malmuthuge, 2017). Oultram et al (2015) observed microbial alterations in feces of calves treated with antibiotics (oxytetracycline, tulathromycin, or florfenicol). All antibiotic treatments resulted in a decreased abundance of *Lactobacillus*, with oxytetracycline creating the greatest reduction in abundance (Oultram et al., 2015, Malmuthuge, 2017). Also, feeding calves with waste milk containing residual antibiotics decreases the relative abundance of *Clostridium* and *Streptococcus* in the feces (Van Vleck Pereira et al., 2016). Waste milk containing traces of β -lactam antibiotics increased the presence of β -lactamase resistance genes in the *E. coli* population of preweaned calves (Maynou et al., 2016). Early exposure to antibiotics influences microbial composition, which could lead to an increase of enteric infection in preweaned calves (Malmuthuge, 2017).

Colostrum feeding and the bacterial colonization process

The timely feeding of three to four liters of clean, high-quality colostrum (IgG > 50 mg/mL of colostrum) is a principal practice for limiting instances of diarrhea in neonatal calves (Weaver et al., 2000). Maternal colostrum is a vital source of Immunoglobulin G (IgG), carbohydrates, fat, and protein, essential to passive transfer of immunity and metabolic fuel to the newborn calf, preventing enteric disease (Morrill et

al., 2012). While the importance of colostrum management for the passive transfer of immunity is well known, the effects of colostrum on establishing a microbial community in the gut is not clearly understood (Malmuthuge et al., 2015b).

Colostrum acts as a natural probiotic by promoting the establishment of commensal bacteria in the gut, leading to the suppression of enteropathogens and reduced prevalence of enteric infection (Barrington and Parish, 2001, Malmuthuge, 2017).

Malmuthuge et al (2012) found that feeding colostrum within 1 hour after birth established bacterial colonization of the small intestine within 12 hours postpartum. Calves fed high quality colostrum within one hour of birth retained bacterial counts similar to older calves, while calves deprived of colostrum acquired significantly fewer bacteria (Malmuthuge et al., 2012). A single feeding of heat-treated colostrum soon after birth promotes the colonization of Bifidobacteria and reduces the colonization of *E. coli* in the small intestine, while calves deprived of colostrum showed a significantly lower abundance of *Bifidobacterium* and a higher abundance of *E. coli* in intestinal samples (Malmuthuge et al., 2015a).

Enteric infection can be prevented by the presence of Bifidobacteria in the intestinal microbiota through pathogen inhibition. The ability of Bifidobacteria to protect its host is largely related to how easily these bacteria adhere to the intestinal epithelium. Strains including *B. bifidum* RBL 71, *B. bifidum* RBL 460, and *B. pseudolongum* ATCC 25526 are efficient in reducing the adhesion of *E. coli* O157:H7 on Caco-2 (colonic) cells by over 50% in a dose-dependent manner (Gagnon et al., 2004). Multiple studies have highlighted the effectiveness of certain strains of Bifidobacteria in reducing the adhesion of pathogens including, *L. monocytogenes*, *E. coli*, *C. difficile*, *Ent. sakazakii*, and *S.*

enterica serovar Typhimurium. Although Bifidobacteria have been shown effective in pathogen displacement, the exact mechanisms of this phenomena are not known. Another possible mechanism through which *Bifidobacteria longum* protects a host from an infectious pathogen, *E. coli* O157:H7, is by the production of acetate. Acetate, a byproduct of *B. longum* metabolism, promotes the defense functions of host epithelial cells by inducing the presence of anti-inflammatory genes in Caco-2 cells. The presence of acetate prohibits the Stx2 toxin, produced by *E. coli* O157, from reaching the intestinal lumen. The presence of *B. longum* preserved the life of murine hosts by preventing the lethal dose of Stx2 from entering the bloodstream, suggesting that short-fatty acids produced by Bifidobacteria are effective in preventing pathogen-induced death to the host (Fukuda et al., 2011).

Supplementation of direct-fed microbials (probiotics)

Growing concern of antimicrobial-resistance by the public as contributed to pressure for limiting antibiotic uses in livestock (Sneeringer et al., 2017). Therefore, interest in the effects of feeding probiotics as an alternate method for disease prevention and health in livestock has increased in recent years. The feeding of freeze dried microbes has been widely studied as a strategy to improve health and production in livestock. The term probiotic has been defined as “*a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance*” (Fuller, 1989, Krehbiel et al., 2003). Probiotics are also referred to as direct-fed microbials (DFMs) and the terms are used interchangeably to describe viable microbial cultures, culture extracts, enzyme preparations, and various combinations of the above (Quigley, 2011). Live microbial cultures have been shown to reduce mortality and

morbidity through preventing pathogen colonization (Krehbiel et al., 2003). DFMs reduce the colonization of enteric pathogens through the production of antimicrobials and competing with pathogens for nutrients and space in the gut (Malmuthuge et al., 2015b).

Direct-fed microbials can increase daily gain and feed efficiency in beef cattle, enhance milk production in dairy cows, and decrease incidence of scours in neonatal calves (Krehbiel et al., 2003). Direct fed *Lactobacillus acidophilus* significantly lowered scour index scores in calves (Abu-Tarboush et al., 1996). Supplementation of *L. acidophilus* BT1386 in the diets of pre- and post-weaned calves, impacted bacterial community structure of the ileum in the calves. *L. acidophilus* supplementation significantly reduced potentially pathogenic bacteria genera *Streptococcus* and *Tyzzerella_4*, while increasing *Fibrobacter*, a beneficial bacterium (Fomenky et al., 2018). There are no reported calf performance benefits attributed to the supplementation of *Lactobacillus*, but in many cases improved health and reduction in severity and incidence of neonatal diarrhea is a sufficient response to microbial supplementation in pre-ruminants (Krehbiel et al., 2003).

Instances of neonatal diarrhea are followed by dysbiosis, microbial imbalances associated with intestinal disorders (Carding et al., 2015) (Oikonomou et al., 2013). Inflammatory bowel disease in humans is accompanied by a reduced prevalence of *Bifidobacterium* and *Faecalibacterium*, suggesting that these bacterial groups play key roles in the prevention of infection (Malmuthuge et al., 2015b). *Faecalibacterium prausnitzii*, a bacterium negatively associated with neonatal diarrhea (Oikonomou et al., 2013, Foditsch et al., 2016, Malmuthuge, 2017), administered during the first week of life, effectively reduced the incidence of diarrhea and death during the first seven weeks

of life in calves. Supplementation of *F. prausnitzii* may be effective in decreasing the susceptibility of preweaned calves to enteric infection (Foditsch et al., 2016, Malmuthuge, 2017). *F. prausnitzii* secretes anti-inflammatory metabolites that could explain the negative association between *F. prausnitzii* supplementation and diarrhea (Oikonomou et al., 2013). Although the responses to direct-fed microbials have been positive, the underlying mechanisms are not clearly understood, presenting the need for further studies to better define the mechanisms related to these effects (Fomenky et al., 2018). Clearly understanding the variation in the abundance of bacterial species of the gut microbiota between healthy and diseased individuals presents the opportunity to use targeted microbials as a method of treatment. Expanding our understanding on the mode of action of feeding of live microbials would allow for individual supplementation protocols, relating to a specific animal or pathogen (Krehbiel et al., 2003).

5. THE ROLE OF METAGENOMICS IN ADVANCING MICROBIOME UNDERSTANDING

Metagenomics: methods and applications

Metagenomics is a fast growing and diverse field that is applied to obtain knowledge on genomes of microbial communities. Metagenomics is the study of genetic material found within environmental samples, which provides insight into the metabolic potential of uncultivated microbes and has led to the discovery of novel genes and metabolic pathways. Two methodologies of metagenomics are recognized: function-based and sequence-based. Function-based metagenomics relies on cloning environmental DNA into expression vectors and propagating them in appropriate hosts. Once the clone is determined, the gene of interest is further analyzed for its

biotechnological potential. Sequence-based metagenomics is applied using prior knowledge on proteins, allowing for a screening of genes that are predicted to encode proteins indicative of their functionality (Chistoserdova, 2010). As a result of the availability of ever-expanding gene databases, the experimental component of searching for specific proteins has been eliminated. Instead, searches for genes, proteins, or metabolic pathways can be conducted electronically. In recent years, the area of metagenomics has been revolutionized by the application of whole genome shotgun (WGS) sequencing. Advances in next-generation sequencing led to a significant price drop of DNA sequencing, allowing for sequencing to be performed on a significantly larger scale than with traditional technologies. These developments have introduced the ability to address new, previously unattainable questions and have a potential to significantly accelerate genome based discoveries (Chistoserdova, 2010).

16S-Based Metagenomics

Amplicon analysis of the 16S ribosomal DNA (rDNA) gene has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria (Woo et al., 2008). This is the most common sequencing method used to analyze the microbiome, which was used to compile most of the data collected by the Human Microbiome Project (HMP) (Group, 2012). The 16S rDNA, which codes for the subunit of ribosomal RNA, is present in all prokaryotic cells. The 16S subunit is the most widely used informational macromolecule for bacterial systematic studies at the family, genus, species, and subspecies levels. The 16S subunit contains sequences that are used to infer relationships between distantly related species and variable regions that can be used to separate closely related ones. 16S rDNA sequencing is particularly useful when

identifying bacteria that are rare, slow-growing, uncultivable, from culture-negative infections, and display unusual phenotypic traits (Woo et al., 2008). Sequencing 16S rDNA makes it possible to isolate and sequence the complete or selected regions of DNA from unknown isolates, compare them with the 16s rDNA sequences in a database, and identify most similar species (Cocconcelli et al., 1997, Chen et al., 2000). This provides a functional identification technique for bacteria that are unculturable or difficult to culture (Amann et al., 1995) (Chen et al., 2000).

Whole-genome Shotgun sequencing

Whole-genome Shotgun sequencing is known as the most powerful method to identify genomic diversity among closely related strains or isolates (Fraser et al., 2002). A major advantage of WGS sequencing is the ability to sequence broad regions of the genome, while 16S-based methods only sequence a single region of the bacterial genome (Ranjan et al., 2016). Sequencing large regions of the bacterial genome allows for a broad and in-depth coverage of the genome, even in bacteria of rare abundance (Ranjan et al., 2016). When performing WGS sequencing, an entire bacterial genome is isolated and broken into small fragments. Portions of these small fragments are then assembled into larger pieces by identifying overlaps between fragments. The complete genome is established by filling in gaps between the larger pieces (Eisen, 2007).

Classification of bacterial sequences

Precise classification of bacterial sequences remains to be a challenge for both 16S and shotgun libraries. Classification limitations observed by either clustering phylotypes or grouping bacteria into operational taxonomic units (OTUs), can be overcome by using a hybrid method that combines both approaches (Jovel et al., 2016).

Comprehensive reference databases have been compiled for the classification of bacterial metagenomes. The Greengenes database, the Ribosomal Database Project (RDP), and SILVA are used for the annotation of 16S sequenced genes. These databases are publicly available and provide extensive catalogs of 16S rDNA and rRNA sequences with bioinformatics tools for analysis (Jovel et al., 2016).

6. SUMMARY

There is growing evidence that abnormal bacterial communities in early life can alter immune development and lead to disease (Tamburini et al., 2016). The establishment of a desired gut microbiome in early life plays a vital role in the long term health of the host by stimulating mucosal epithelium development, maintaining the integrity of the intestinal barrier, and promoting overall immune efficacy (Malmuthuge, 2017). Specific species of commensal bacteria can contribute to proper immune system development, pathogen evasion, and other health promoting functions (Ruiz et al., 2016). However, our understanding of the microbiome and the macromolecular mechanisms that influence immune development in dairy cattle is limited. Understanding the microbiome and the related mechanisms allows for the identification of targeted probiotic and supplementation strategies to better prevent pathogen-associated disease (Ruiz et al., 2016). Microbial interventions could be used as an effective strategy to prevent or reduce the severity of diseases associated with altered microbial colonization during early life (Tamburini et al., 2016).

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CHAPTER 2

SOURCES OF VARIATION IN THE MICROBIOME OF PRE-WEANED DAIRY CALVES

ABSTRACT

Death loss of pre-weaned dairy heifers is a major challenge confronting U.S. dairy producers, creating economic losses of at least \$100 million each year. Neonatal calf diarrhea, the leading cause of neonatal calf mortality, is controlled through colostrum feeding to ensure the passive transfer of immunity. However, these methods are not always effective in preventing calf morbidity and mortality. Therefore, other factors must play an integral role in calf health, morbidity, and mortality; the gut microbiome of the calf is likely significant. The dam's vaginal, fecal, and oral microbiomes are believed to be the primary sources of the newborn calf's gut microbiome, but the long-term health effects of the colonization by these microbes are yet to be determined. The objective of this study was to characterize the dam's fecal, vaginal, and oral microbiomes and the calf fecal microbiome. Our hypothesis stated that the dam's vaginal, fecal, and oral microbiomes will influence the early microbiome of the calf.

Multiparous Holstein cows (n = 6) were sampled and observed during the final weeks of pregnancy. Vaginal swab samples were collected from cows ~24 h prior to parturition. Oral, rectal, and vaginal swab samples were collected from cows immediately following parturition. Oral and rectal swabs were collected from calves (n = 6) at birth (h 0), h 24, d 7, d 42, and during the post-weaning period (d 60). Metagenomic 16S rDNA

data was converted to inferred amplicon sequence variants (ASVs) and clustered into operational taxonomic units (OTUs) to assess microbial diversity and relative abundance within fecal, vaginal, and oral microbiome samples. One-way ANOVA followed by Tukey's procedure for pair-wise comparisons were used to determine sample type differences. Significance was declared at $P \leq 0.05$. Using phylum count data, a negative binomial regression model was used to determine the effects of the dam's vaginal, fecal, and oral microbiomes on the calf fecal microbiome.

Metagenomic analysis revealed a diverse microbiome within the meconium of newborn calves, suggesting that microbial colonization of the neonatal calf GIT begins *in utero*. We identified 10 phyla associated with the calf meconium and fecal microbiome. The fecal community of the newborn calf is composed of Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Cyanobacteria, Verrucomicrobia, Spirochaetes, Tenericutes, Fibrobacteres, and Lentisphaerae. Principle-coordinate analysis revealed a strong relationship between the vaginal microbiome of the dam and the meconium microbiome of the calf. The dam vaginal microbiome and the calf meconium microbiome were highly similar, displaying significant differences ($P = 0.05$) in only two phyla, Bacteroidetes and Fusobacteria. The abundances of three phyla, Bacteroidetes, Proteobacteria, and Verrucomicrobia, differed significantly ($P = 0.05$) between the dam fecal microbiome and the meconium microbiome. The dam fecal microbiome was highly similar to the calf fecal microbiome post-weaning (d 60), making the fecal microbiome of the dam a strong indicator of the microbial composition of calf feces. Overall, the calf meconium and fecal microbiome is influenced by a combination of the maternal vagina, oral, and fecal microbiomes.

Further studies will be needed to identify the transference mechanisms of maternal microbes to offspring and the associated host-microbial interactions.

MATERIALS AND METHODS

Ethical Approval

This experiment was conducted at the Virginia Tech Dairy Research Complex in Blacksburg, VA, under the review and approval of the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee, 17-187-DASC.

Animals and Observation Units

Multiparous pregnant Holstein cows (n = 12) were selected from the Virginia Tech Dairy Center (Blacksburg, VA) to observe microbial composition within target body sites and in the calves they delivered during the study.

Newborn Processing

Calves (n = 12; bulls = 8, heifers = 4) were separated from their dams immediately post-birth and calf navels were dipped in a 7% solution of iodine. Calves were transferred into a large plastic tub to prevent contact with the environment. Colostrum was collected using a stainless steel portable bucket milking machine and all calves received one 4 L feeding of their dam's colostrum within 1 h post-birth. All milking equipment was cleaned using soap and hot water after each milking. Colostrum was required to meet a quality requirement of ≥ 18 brix using a brix refractometer (VEE GEE Scientific, Vernon Hills, IL). All colostrum met quality requirements, with measurements ranging from 22 to 30 brix. Calves were assigned to a new bottle and nipple at the first colostrum feeding, specific to each individual throughout the entirety of the study. All calves were relocated from the maternity pen to an individual hutch after

the first colostrum feeding. Calves received one 4 L feeding at 12 h post-birth of their dam's transition milk, collected from the dam 12 h post-birth using a stainless steel portable bucket milking machine. All calves were vaccinated with INFORCE™ 3 (Bovine Respiratory Syncytial Virus Vaccine; 2 ml intranasally; Zoetis Inc., Kalamazoo, MI).

Housing and Feeding

Approximately three weeks prior to expected parturition, pregnant cows were moved to group housing with access to a close-up dry cow TMR. Cows were fed twice daily at 0900 h and 1900 h and were provided *ad libitum* access to water. At 12 to 14 d prior to parturition, cows were moved from group maternity housing to individual box stalls and closely monitored. Box stalls were bedded with sawdust and re-bedded after each calving, to avoid contamination between dams. At 7 d prior to estimated parturition, the Moocall calving alert system (Moocall Ltd., Dublin, Ireland) was placed on the dam's tail to monitor calving.

Calves were housed at the Virginia Tech Dairy Center in individual sawdust bedded plastic hutches (outside dimensions: 219 cm L x 121 cm W x 139 cm H) with metal hog panels providing a 2.5 m² fenced area. Calves were placed in hutches after their initial colostrum feeding and remained there until the end of the study.

Calves were fed 4 L of a 27.0% CP, 20.0% fat milk replacer (Cow's Match® ColdFront® Medicated (67 mg/kg lasalocid sodium), Land O'Lakes® Animal Milk Products Co., Shoreview, MN) twice daily at 0600 h and 1800 h. Milk replacer was made by mixing 567.0 g of milk replacer powder per 3.8 L of water heated to 43°C. Calves were fed using bottles and nipples specifically assigned to the individual to avoid cross-

contamination. Calves were supervised during feeding and were allowed as much time as needed to consume the milk replacer. Bottles and nipples were thoroughly cleaned immediately after each feeding using soap and hot water. Calves were fed 22% CP starter grain (Intensity 22% Textured Calf Starter Medicated, Cargill Animal Nutrition, Minneapolis, MN) starting at 4 weeks of age and were allowed *ad libitum* access to water beginning at 1 d of age. Starter and water refusals were recorded at each feeding (twice daily). Weaning was initiated at 42 d of age and all calves were weaned by 60 d of age. Calves were weighed once weekly approximately 1 h before evening feeding.

Calf Health

Calves were assigned a fecal score twice daily throughout the trial, using the scoring system of the School of Veterinary Medicine at University of Wisconsin-Madison (McGuirk and Peek, 2014). All calves remained healthy throughout the study and no calves received antibiotic treatment.

Sample Collection

Sterile flocked swabs (Puritan; Guilford, ME) were used to collect fecal and oral microbiome samples. Vaginal swab samples were collected from all pregnant cows ~24 h prior to estimated parturition. Vaginal samples were collected from the posterior vaginal wall using a clean glove and palpation sleeve for each collection. Vaginal samples were placed in cyrotubes and snap frozen in -200°C liquid nitrogen immediately after collection and stored at -80°C until analysis.

Fecal swab samples were collected rectally from cows immediately after parturition, using a clean glove and palpation sleeve for each collection. Fecal samples

were placed in cyrotubes and snap frozen in -200°C liquid nitrogen immediately after collection and stored until analysis.

Oral swab samples were collected from the left and right inner buccal wall from cows immediately after parturition, using a clean glove and palpation sleeve for each collection. Oral swab samples were placed in cyrotubes and snap frozen in -200°C liquid nitrogen immediately after collection and stored at -80°C until analysis.

Sterile flocked swabs were used to collect microbiome samples from the oral and rectal cavities from all calves at birth (0 h), 24 h, 7 d, 42 d, and 60 d. Swab samples were placed in cyrotubes and snap frozen in -200°C liquid nitrogen immediately after collection and stored at -80°C until analysis.

Ten ml of blood was collected from each calf via jugular venipuncture into Monoject blood tubes with no additive approximately 24 h after birth, approximately 12 h after the 2nd feeding (transition milk). Samples were refrigerated (4°C) for approximately 12 h, then centrifuged at $2200 \times g$ for 20 min at 4°C to isolate serum.

DNA Extraction and Quality Testing

The QIAamp® BiOstic Bacteremia DNA Kit (QIAGEN, Germantown, MD) was used to extract DNA from all vaginal, fecal, and oral swab samples. Samples were extracted using the QIAamp® BiOstic Bacteremia DNA Kit manufacturer's protocol with one modification. In step 11, prior to centrifugation, 4 μL of RNase A (Illumina, San Diego, CA) was added and incubated at room temperature for 3 minutes to rid the sample of any RNA contamination. Quantity and quality of DNA were examined using the Qubit® 2.0 Fluorometer (Life Technologies™, Grand Island, NY) with the Qubit® dsDNA HS Assay Kit. The NanoPhotometer® (Implen, Inc., Westlake Villiage, CA) was

used before sequencing to confirm that the 260/280 value was approximately 1.8, indicating pure DNA. Samples were run on an electrophoresis gel with 2.66 μ L loading dye (sybergreen, monensin blue, and glycerol) to 4 μ L sample.

Library Preparation and Sequencing

Samples were submitted to the Virginia Bioinformatics Institute Genomics Research Laboratory (Blacksburg, VA) for DNA Seq library purification and 16S Illumina paired-end MiSeq, 300 cycle sequencing. 16S rDNA amplicons covering variable regions V4 to V5 were generated using primers 515F – 806R (reverse barcoded: FWD: GTGCCAGCMGCCGCGGTAA; REV: GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2012). Amplicons were pooled and purified using a Pippin Prep 1.5% gel cassette (Sage Science, Inc., Beverly, MA). 16s rDNA sequence data was converted to inferred amplicon sequence variants (ASVs) and clustered into operational taxonomic units (OTUs) using the DADA2 (version 1.10) package in R version 3.4.4 (R Core Team, 2018). The DADA2 (1.10) package is used to remove substitutions and chimeric errors in amplicon data. The DADA2 package infers sample sequences within a collection of amplicon sequence reads. Reads were clustered and assigned OTUs using the Greengenes (Greengenes, Berkeley, CA) reference genome.

Statistical Analysis

Statistical analysis of metagenomic data was with R version 3.6.1 (R Core Team, 2019). The phyloseq package was used for all graphics, microbial diversity, and relative abundancy analyses using the following libraries: *ggplot2*, *vegan*, *treemap*, *dplyr*. One-way ANOVA followed by Tukey's procedure for pair-wise comparisons were used to determine sample type differences. Significance was declared at $P \leq 0.05$.

Using count data by phylum, the effect of the dam's vaginal, fecal, and oral microbiomes on the calf fecal microbiome were evaluated with R version 3.6.1 (R Core Team, 2019) with the following negative binomial regression model:

$$\ln \mu = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \ln t$$

Where

$\ln \mu$ = Natural log of calf fecal bacterial count

β_0 = Intercept

β_1 = Expected change in $\ln \mu$ if x_1 changes by 1

x_1 = Dam fecal bacterial count

β_2 = Expected change in $\ln \mu$ if x_2 changes by 1

x_2 = Dam oral bacterial count

β_3 = Expected change in $\ln \mu$ if x_3 changes by 1

x_3 = Dam vaginal bacterial count

$\ln t$ = Natural log of time.

Count data was collected at the phylum level. The dependent variable included the count of each bacterium within a phylum in individual calf fecal samples. Predictors were declared significant at $P \leq 0.05$.

RESULTS

Animal characteristics

Beginning this study, we aimed to enroll 12 cow-calf pairs, but the mis-estimation of calving events led to incomplete sample sets from eight of the cow-calf pairs. Six cow-calf pairs were removed from the study because they were missing two or more key pre-calving samples, leaving this study to include six cow-calf pairs, with three female and

three male calves. Two cow-calf pairs yielded nearly complete sample sets, only missing pre-birth vaginal samples, while the four remaining cow-calf pairs yielded complete sample sets.

Male calves (n = 3) had an average daily gain (ADG) of 0.76 kg per day, while female (n = 3) calves had an ADG of 0.65 kg per day (*Table 1*).

Dam associated microbiota

Nine phyla comprised the vaginal community of the dams' (n = 4) 24 hours prior to parturition (*Figure 1*): Firmicutes, Proteobacteria, Tenericutes, Bacteroidetes, Verrucomicrobia, Actinobacteria, Cyanobacteria, Lentisphaerae, and Spirochaetes (*Figure 1*). *Mogibacterium* and *Streptococcus* were the two most abundant genera in the pre-birth vaginal microbiome (*Figure 1*).

No core vaginal microbiome of pregnant Holstein cows was obvious because high individual variation was observed between the subjects. Two dams' vaginal microbiomes were dominated by Firmicutes, while the remaining two dams' vaginal microbiomes were dominated by Proteobacteria and Tenericutes (*Figure 2*).

The dams' oral (n = 6) and fecal (n = 6) microbiomes at birth were comprised of the same nine phyla as observed in the vagina. The oral community was dominated by Proteobacteria, while the fecal community was dominated by Bacteroidetes and Firmicutes (*Figure 1*).

Calf associated microbiota

The results of this study indicate that calves are born with a diverse bacterial population within the GIT. The fecal microbiome of pre-weaned dairy calves (n = 6) was dominated by 10 phyla. We identified Bacteroidetes, Firmicutes, Proteobacteria,

Actinobacteria, Cyanobacteria, Verrucomicrobia, Spirochaetes, Tenericutes, Fibrobacteres, and Lentisphaerae in the meconium of newborn calves collected within one hour after birth.

At h 24 of age, the fecal gut microbiota shifted from Bacteroidetes and Proteobacteria dominated to being heavily dominated by Proteobacteria. The calf fecal microbiome at 1 day of age (h 24), was comprised of only Proteobacteria and Firmicutes (**Figure 1**). Little individual variation was observed in the calf fecal microbiome at 24 hours of age (**Figure 1**).

At d 7, the abundance of Proteobacteria significantly decreased ($P = 0.05$) (**Table 2**), while Firmicutes and Bacteroidetes increased. Firmicutes and Bacteroidetes continued to increase throughout (d 42) and post-weaning (d 60; post-weaning). Proteobacteria, Bacteroidetes, and Firmicutes were the main phyla detected in feces on days 42 and 60 (**Figure 1**).

As the calves aged, the fecal microbiome began to resemble the fecal microbiome of an adult (**Figure 1**). High abundances of *Bifidobacterium*, *Lactobacillus*, and *Butyricoccus* were detected in the feces calves on d 7 (**Figure 3**). *Roseburia*, *Sutterella*, *Parabacteroides*, *Sharpea*, *Treponema*, and *Phascolarctobacterium* were the most abundant genera in the calf fecal microbiome post-weaning (d 60) (**Figure 3**).

Relationship between dam microbiota and calf microbiome

High individual variation was observed in the vaginal microbiomes of the four dams observed in this study (**Figure 2**). A strong relationship was observed between the vaginal microbiome of dam 1 and the fecal microbiome of her calf at birth (**Figure 4**). In this case, both the vaginal microbiome of the dam and the fecal microbiome of the calf at

birth were dominated by Proteobacteria, Bacteroidetes, and Firmicutes (*Figure 4*). Direct correlations between dam and offspring microbiomes were less obvious in the remaining three cow-calf pairs.

A negative binomial regression model was used to identify dam microbiomes that could be significant predictors of the bacterial composition of the calf fecal microbiome at birth (*Table 3*). Of the four phyla that were above the limit of detection used for this model, which includes Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, none of the dam's oral, fecal, or vaginal microbiomes were found to be significant predictors at the phylum level (*Table 3*).

Principle-coordinate analysis revealed a strong relationship between the vaginal microbiome of the dam and the meconium microbiome of the calf at birth (*Figure 5*). Principle-coordinate analysis allows for the visualization of individual data points described by multiple inter-correlated quantitative variables. This analysis identified principal components along which the data variation is maximal, thus allowing us to see important relationships that were not revealed by the regression model.

When comparing the dam vaginal microbiome and the calf meconium microbiome, significant differences ($P = 0.05$) were detected in only two phyla, Bacteroidetes and Fusobacteria (*Table 2*). The calf meconium microbiome more closely resembled the dam's vaginal microbiome than the dam's fecal microbiome (*Figure 6*). Abundances of all phyla in the calf meconium and dam feces were not significantly different ($P = 0.05$), with the exceptions of 3 phyla, Bacteroidetes, Proteobacteria, and Verrucomicrobia (*Table 2*). High similarities were detected between the fecal microbiome of the dam and the fecal microbiome of the weaned calf (d 60) (*Figure 6*).

Comparison of these samples revealed a significant difference between only one phylum, Verrucomicrobia, revealing that maternal feces is a strong indicator of the bacterial composition of calf feces post-weaning (**Table 2**). Significant differences in the abundances of Bacteroidetes and Proteobacteria were detected between the dams' oral microbiome and the calf meconium microbiome (**Table 2**). Overall, the calf meconium and fecal microbiome was influenced by a combination of the maternal vagina, oral, and fecal microbiomes (**Figure 5**).

DISCUSSION

The goal of this study was to characterize the oral, fecal, and vaginal microbiomes from cows and the fecal microbiomes of their calves. The hypothesis stated that the dam's vaginal, fecal, and oral microbiomes will influence the early microbiome of the calf and that this is related to health and growth outcomes of pre-weaned calves.

Maternal influence on the calf microbiome

The present study revealed a diverse microbiome present in the meconium and feces of calves that was influenced by a combination of the maternal vaginal, fecal, and oral microbiomes. The transference of maternal microbes to offspring during early labor and birth has been well documented in humans and mice, but the timing of the establishment of the intestinal microbiome is unknown, especially in dairy cattle (Romano-Keeler and Weitkamp, 2015). The presence of a rich meconium microbiome in newborn calves suggests that microbial contributions may be from a combination of exposure *in utero* and during labor, such as from the placenta and allatonic fluid and/or the vaginal secretions or feces from the dam (Guzman et al., 2015).

The human fecal microbiome of vaginally-delivered infants closely resembles the maternal fecal microbiome (Dominguez-Bello et al., 2010, Bäckhed et al., 2015).

Although the maternal fecal microbiome closely resembles that of the post-weaned calf, our study revealed the maternal fecal microbiome as less influential to the calf meconium microbiome than the maternal vaginal microbiome (**Figure 5**). Research in humans suggests that amniotic fluid is swallowed by the fetus during the second trimester of pregnancy (Gilbert and Brace, 1993) and the ingestion of this fluid is thought to serve as a source of microbes of the fetal gut (Guzman et al., 2015), possibly suggesting that allatonic fluid could act as the source of the microbiome found in the meconium of newborn calves. Proteobacteria, Bacteroidetes, and Firmicutes dominate the pregnant cow endometrial microbiota, another possible *in utero* source of microbes to the neonatal calf (Karstrup et al., 2017, Alipour et al., 2018).

This study revealed Proteobacteria, Firmicutes, and Bacteroidetes as the dominant phyla in the Holstein cow vagina ~24 hours before parturition. The vaginal microbiome of pregnant Nellore cattle has previously been characterized and was dominated by the same three phyla (Laguardia-Nascimento et al., 2015a). High amounts of individual variation were observed between the vaginal microbiomes of the dams observed in this study (**Figure 2**) (**Figure 3**). High individual variation (more than 50%) has also been observed in the vaginal microbiome of pregnant Nellore cows (Laguardia-Nascimento et al., 2015a). Further studies involving a greater number of cows will be necessary to potentially identify a core vaginal microbiome of pregnant cows of a specific breed.

The vaginal microbiome of the dam is influential to the pre-weaned calf GIT microbiome. In alignment with the three dominant phyla previously identified in the

pregnant Nellore vaginal microbiome (Laguardia-Nascimento et al., 2015a), Firmicutes, Bacteroidetes, and Proteobacteria have also been identified as the dominant phyla of the pre-weaned calf GIT (Malmuthuge et al., 2014). Similarities between the vaginal microbiome of the cow and fecal microbiome of the calf suggest that the maternal vaginal microbiome plays a key role in influencing the microbial composition the calf GIT.

The bacterial community of the calf meconium was dominated by Proteobacteria, Firmicutes, and Bacteroides. Characterization of the calf meconium microbiome revealed Proteobacteria and Firmicutes, specifically *Citrobacter* spp., *Lactococcus* spp., *Leuconostoc* spp., and *Lactobacillus* spp., as the first main colonizers of the GIT (Mayer et al., 2012). These primary colonizers of the calf GIT were also identified in the dam's fecal, vaginal, and oral communities. 16S rDNA data has indicated the presence of a diverse microbiota, composed of Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes in neonatal rectal meconium and mucosa (Alipour et al., 2018).

Maternal colostrum is likely to influence the community of microorganisms within the neonatal calf gut microbiome. Although the colostrum microbiome was not considered in the present study, it serves as a source of immunoglobulins, proteins, growth factors, enzymes, and a rich microbiome that influences the gut microbial establishment in young ruminants (Malmuthuge et al., 2015b). Previous characterization of the bovine colostrum microbiome revealed Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Tenericutes as the dominant phyla (Lima et al., 2017). The colostrum microbiome contains valuable probiotic bacteria, such as members of the *Bifidobacterium* and *Lactobacillus* genera (Lindner et al., 2011). The colostrum microbiome could be a contributing source of high amounts of Proteobacteria and

Firmicutes seen in the feces of calves at 24 hours of age and supply microorganisms that begin to shift the neonatal fecal microbiome toward maturation. (These data from the current study were outside of the scope of this thesis, therefore not presented here).

The core microbiome of the 3-week old calf gut is comprised of Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Cyanobacteria, Elusimicrobia, Fibrobacteres, Fusobacteria, Lentisphaerae, Spirochaetes, Synergistetes, Tenericutes, and Verrucomicrobia (Malmuthuge et al., 2014). The phyla identified by Malmuthuge et al., align with the phyla identified in this study, except that we did not observe Fusobacteria, Synergistetes, and Elusimicrobia. These differences in phyla could be explained by differences in sampling method and location. Malmuthuge et al. (2014), identified Fusobacteria, Synergistetes, and Elusimicrobia in the tissue of the cecum while our study focused on the meconium and fecal microbiome. Although the dam microbiomes were not identified as significant predictors of the calf fecal microbiome (**Table 3**), these data do suggest a link between the oral, vaginal, fecal, and reproductive tract microbiota of the dam and the calf meconium and fecal microbiome. Further examinations of the maternal reproductive tract microbiome are necessary to fully investigate the link and exact transference mechanisms of microbes to offspring, beginning at conception.

In-depth understanding of the maternal sources and transference mechanisms of bacteria to the neonatal calf GIT will allow for adapted maternity and calf management protocols to improve calf health throughout early life. The transference of maternal microbiota to the neonatal GIT is vital for the development of intestinal epithelium, mucosal layer (Sharma et al., 1995), and overall immune efficacy (Malmuthuge and Guan, 2017), highlighting the importance early life gut inoculation by maternal microbes.

Development of calf fecal microbiota throughout weaning

There is a diverse microbiome present within calf feces prior to birth. Our findings align with data from Dill McFarland et al. (2017), who identified fecal-associated bacterial communities of Holstein cows from two weeks to two years of age. These fecal-associated communities were dominated by Firmicutes and Bacteroidetes with smaller contributions from Actinobacteria, Proteobacteria, Spirochaetes, Tenericutes, and Fibrobacteres (Dill-McFarland et al., 2017).

Dramatic changes occur in the neonatal calf fecal microbiome between birth and 24 hours of age. In the current study, the fecal microbiota at 24 hours of age was comprised of only Proteobacteria and Firmicutes, indicating a lower diversity than of meconium collected at birth. This has been seen previously in a recent study investigating the composition of the perinatal intestinal microbiome in Holstein and Ayrshire calves (Alipour et al., 2018). Others have observed the neonatal gut microbiota as an unstable community due to its rapid variation and colonization by facultative anaerobes, specifically Proteobacteria (Shin et al., 2015). Proteobacteria play an important role in preparing the neonatal gut microbiota for successive colonization by strict anaerobes by consuming oxygen, altering pH, lowering redox potential, and producing carbon dioxide, and nutrients (Wilson, 2005, Chow and Lee, 2006, Shin et al., 2015). Proteobacteria have been observed as a dominant phylum in many environmental niches including, soil (Lauber et al., 2009), plants (Redford and Fierer, 2009), freshwater (Pascual et al., 2014), seawater (Teeling et al., 2012), and the atmosphere (Whon et al., 2012, Shin et al., 2015), suggesting that the high prevalence of Proteobacteria in the fecal microbiome of calves at 24 h of age could be a result of the calf's first environmental exposure. This

suggests significant environmental effects on calf gut microbiome within a short period of time.

The present study demonstrates that as the calf matures, the bacterial composition of the fecal microbiome becomes more similar to the fecal microbiome of a mature cow. Similarities between the dam fecal microbiome and weaned calf (60 d) fecal microbiome are noted by high amounts of Spirochaetes, which is unique to these communities (*Figure 1*).

The neonatal calf microbiome is likely influenced by the birth environment. The neonatal calf is born with a diverse microbiome that is immediately subject to rapid changes due to exposure to the environment (Alipour et al., 2018). A limitation of our study are the unknown microbial communities within the birth environment (calving pen). All cows calved in stalls bedded with clean shavings 3 to 4 days prior to birth, but the degree of fecal, aerial or other bacterial contamination in that short pre-calving window certainly varied. In a study characterizing the bacterial communities present in the birth environment, calf body habitat microbiomes were different from maternal microbiomes, but did not differ from the calving pen floor microbiome (Malmuthuge, 2016). These results suggest that the calf gut microbiome is influenced by bacterial communities from both the dam and the calving environment.

Influence of host genetics on cow and calf microbiota

Host genetics play a role in determining the composition of the host's microbiome. Differences observed between the meconium microbiome of humans and calves suggest species-specific microbial colonization at birth (Malmuthuge, 2016, Hall et al., 2017) . Major differences in the gut microbiome are observed between different

species, while many similarities can be observed in animals that are phylogenetically related. For instance, *Lactobacillus*, *Bifidobacterium*, *Enterobacteriaceae*, and *Enterococcaceae* dominate the fecal microbiome of human infants during the first 24 hours of life (Dominguez-Bello et al., 2010) while Malmuthuge et al. (2016) reported high abundances of *Propionibacterium*, *Pseudomonas*, *Prevotella*, and *Ralstonia* in the calf small intestine at birth, and the calf fecal microbiota is dominated by Firmicutes during the first weeks of life (Oikonomou et al., 2013, Malmuthuge, 2016).

Although hosts belonging to similar species are similar in their microbiomes, variation in the microbiome is commonly seen among individuals within a species due to genetic differences (The Human Microbiome Project et al., 2012, Goodrich et al., 2016). High individual variation has been previously reported in the vaginal microbiome of Nellore cattle (Laguardia-Nascimento et al., 2015a). Metagenomic analysis revealed individual characteristics as a greater source of variation in the vaginal microbiome than age, pregnancy status, or hormonal maturity (Laguardia-Nascimento et al., 2015a). Host genetics influence the chemical and physical landscapes occupied by the microbiome, possibly contributing to the high amounts of individual variation seen in the vaginal microbiome between subjects (Laguardia-Nascimento et al., 2015a, Hall et al., 2017).

Several studies have revealed that high individual variation is common in the human microbiome, even in individuals with similar functional profiles (Lozupone et al., 2012, Bonder et al., 2016). Data from the Human Microbiome Project revealed that most individual variation in the human gut microbiome is not well explained by phenotypic measures and can only be related to individual genetic characteristics. Relationships among host properties including age, body mass index (BMI), and ethnicity, accounted

for little variation in the human microbiome, suggesting that this variation is due to host genetics (The Human Microbiome Project et al., 2012).

Individual variation in within a host's microbiome is related to an individual's predisposition to metabolic and immune disorders. Further genome-wide association studies investigating the interrelationships between genetic variants and microbiome composition will help to understand the specific gene variants that have an effect on the composition of host microbiome in a manner that promotes disease (Hill et al., 2017). An enhanced understanding of the relationships between microbiome and disease predisposition will allow for specific health-related selection methods.

Table 1. Body weight and ADG of Holstein calves (n = 6)

Calf	Sex	Birth (h 0)	d 7	d 42	Weight (kg)		ADG	
					d 60	Weight gained at weaning (d 42)		Total weight gained (d 60)
1	M	45	46	78	91	33	46	0.76
2	F	45	48	69	87	24	42	0.70
3	F	49	54	69	87	20	38	0.64
4	M	45	49	68	90	23	45	0.75
5	F	49	51	66	86	17	37	0.61
6	M	44	42	65	89	21	46	0.76

¹ Values indicate weight in kilograms (kg)

Table 2. Relative abundance of bacterial phyla in microbiomes of cows and calves

Phylum	Dam vaginal pre-birth	Dam oral at birth	Dam fecal at birth	Calf fecal at birth	Calf fecal 24h	Calf fecal 7d	Calf fecal 42d	Calf fecal 60d	SEM	<i>P</i> value
<i>Actinobacteria</i>	0.9 ^{ab}	1.1 ^{ab}	1.7 ^{ab}	0.9 ^{ab}	3.8 ^a	4.3 ^b	0.8 ^{ab}	0.4 ^{ab}	0.91	0.07
<i>Bacteroidetes</i>	13.4 ^{ab}	19.6 ^{ab}	48.0 ^c	37.8 ^{bc}	0.0 ^a	45.1 ^c	47.0 ^c	48.4 ^c	6.54	0.00
<i>Cyanobacteria</i>	0.2 ^a	0.3 ^a	1.1 ^a	0.4 ^a	0.0 ^a	0.0 ^a	0.6 ^a	0.2 ^a	0.32	0.35
<i>Elusimicrobia</i>	0.0 ^a	0.0 ^a	0.1 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.2 ^a	0.07	0.51
<i>Fibrobacteres</i>	0.0 ^a	0.0 ^a	0.1 ^a	0.1 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.05	0.46
<i>Firmicutes</i>	41.0 ^a	12.5 ^a	39.6 ^a	17.8 ^a	15.0 ^a	27.9 ^a	27.9 ^a	31.0 ^a	6.35	0.03
<i>Fusobacteria</i>	0.9 ^{ab}	0.2 ^a	0.0 ^a	0.0 ^a	0.0 ^a	3.1 ^b	0.2 ^a	0.0 ^a	0.58	0.01
<i>Lentisphaerae</i>	0.1 ^a	0.1 ^a	0.2 ^a	0.1 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.06	0.24
<i>Planctomycetes</i>	0.1 ^a	0.0 ^a	0.1 ^a	0.1 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.02	0.01
<i>Proteobacteria</i>	27.7 ^{ab}	63.6 ^{bc}	3.5 ^a	40.6 ^{ab}	85.0 ^c	19.6 ^a	22.6 ^{ab}	12.2 ^a	9.26	0.00
<i>Spirochaetes</i>	0.1 ^a	0.3 ^a	2.2 ^a	0.5 ^a	0.0 ^a	0.0 ^a	0.0 ^a	7.0 ^a	2.15	0.30
<i>Tenericutes</i>	14.6 ^a	1.5 ^a	0.6 ^a	0.7 ^a	0.0 ^a	0.0 ^a	0.9 ^a	0.7 ^a	3.31	0.17
<i>Verrucomicrobia</i>	1.0 ^{ab}	0.6 ^{ab}	2.9 ^b	1.1 ^{ab}	0.0 ^a	0.0 ^a	0.0 ^a	0.0 ^a	0.64	0.03

^{a-c} Means within row with different superscripts differ ($P < 0.05$)

¹ Values indicate relative abundances calculated as the percentage of the total bacterial phyla.

² Tukey pairwise comparisons of sample type by bacterial phylum ($P = 0.05$).

Table 3. Dam microbiome samples as indicators of the calf fecal microbiome*

Phylum	Sample type	Estimate	P value
<i>Firmicutes</i>	Dam fecal	1.46E-06	0.853
	Dam oral	-2.95E-06	0.715
	Dam vaginal	-1.37E-05	0.237
<i>Bacteroidetes</i>	Dam fecal	5.25E-07	0.984
	Dam oral	3.10E-07	0.983
	Dam vaginal	-1.24E-05	0.964
<i>Proteobacteria</i>	Dam fecal	-6.12E-06	0.886
	Dam oral	-5.13E-07	0.626
	Dam vaginal	7.90E-08	0.912
<i>Actinobacteria</i>	Dam fecal	-6.04E-05	0.787
	Dam oral	3.60E-04	0.257
	Dam vaginal	6.96E-04	0.553

*Parameter estimates for Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria were fit according to a negative binomial regression model. Convergence was not met for the four models.

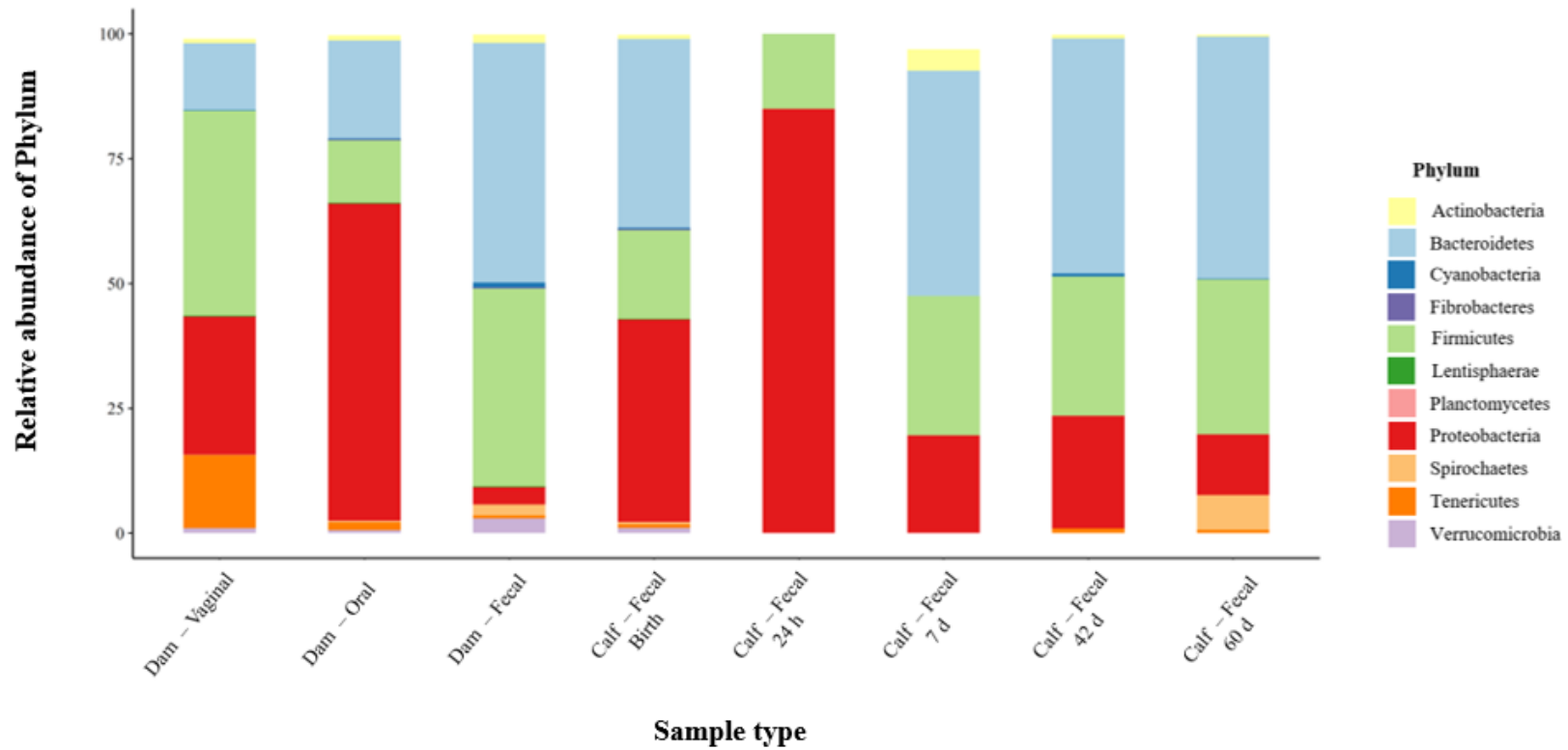


Figure 1. Average relative abundance of bacterial phylum in each sample type. Stacked bar plots showing: dam vaginal pre-birth (n = 4), dam oral at birth (n = 6), dam fecal at birth (n = 6), calf fecal at birth (n = 6), calf fecal at 24 h (n = 6), calf fecal at 7 d (n = 6), calf fecal at 42 d (n = 6), calf fecal at 0 d (n = 6).

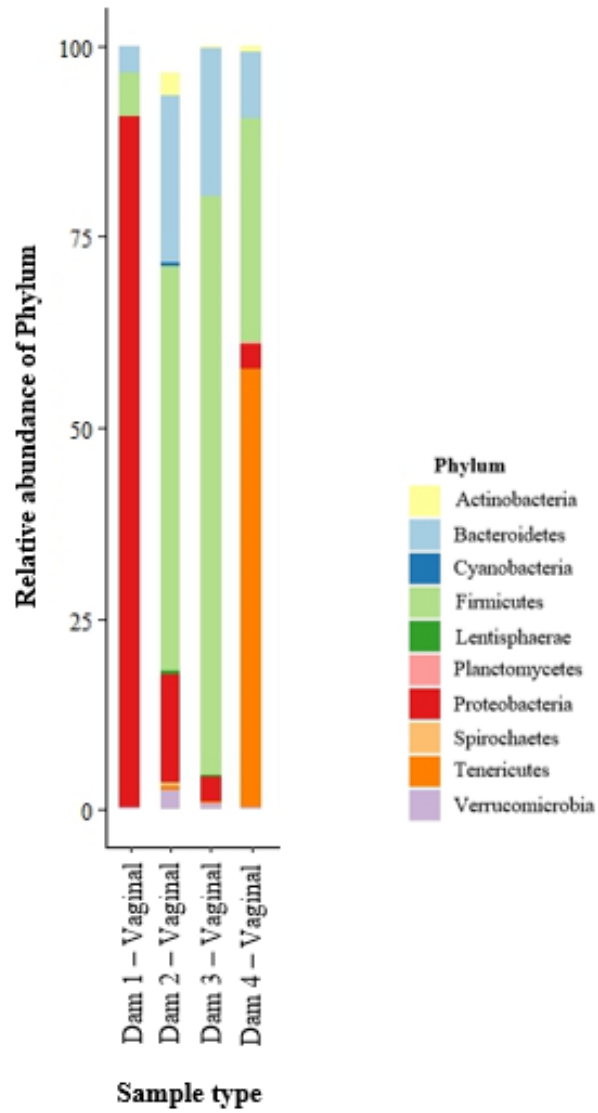


Figure 2. Individual variation within the cow vaginal microbiome. Stacked bar plot showing relative abundance of bacterial phylum within dam vaginal samples (n = 4).

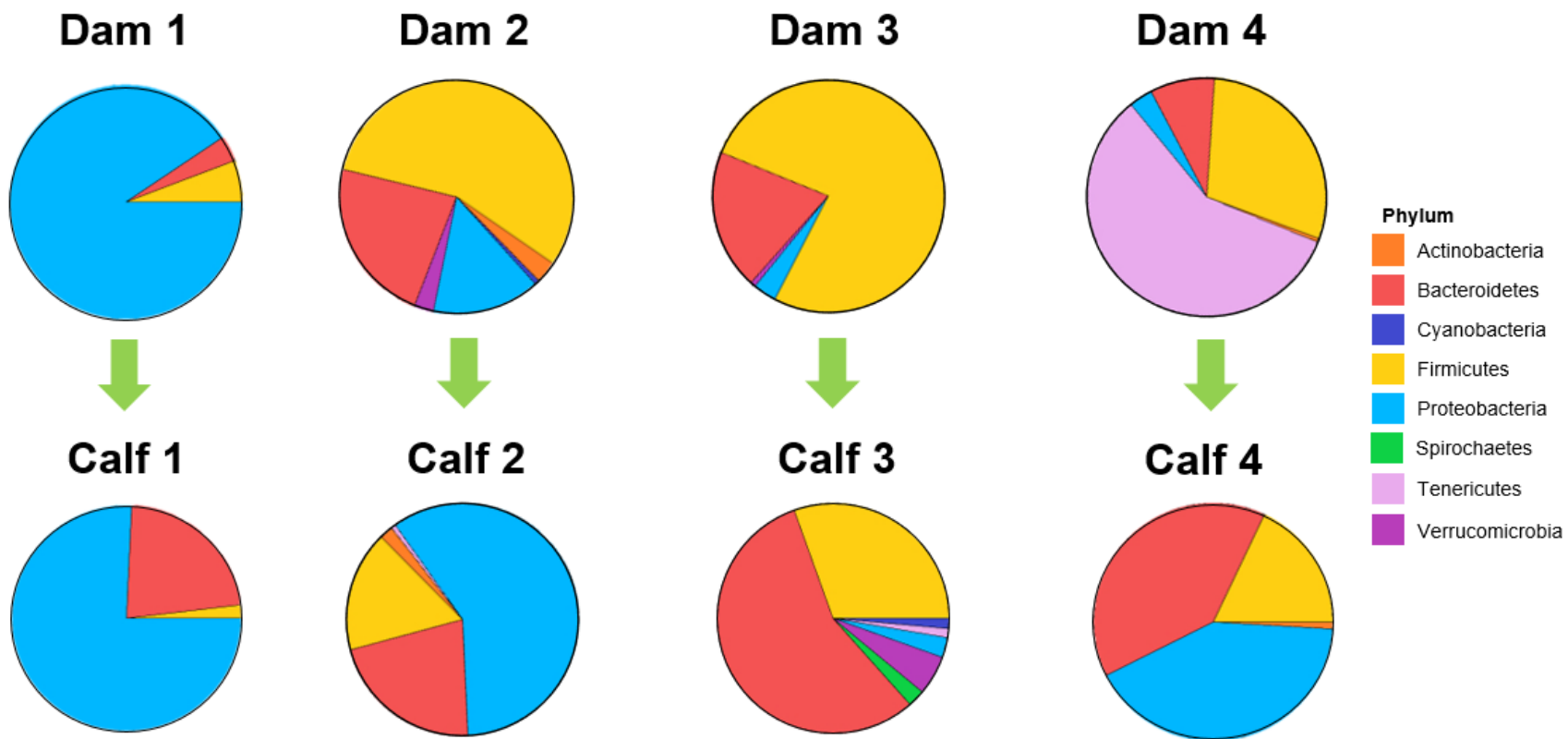


Figure 3. Bacterial composition of the dam vaginal and corresponding calf fecal microbiome at phylum level

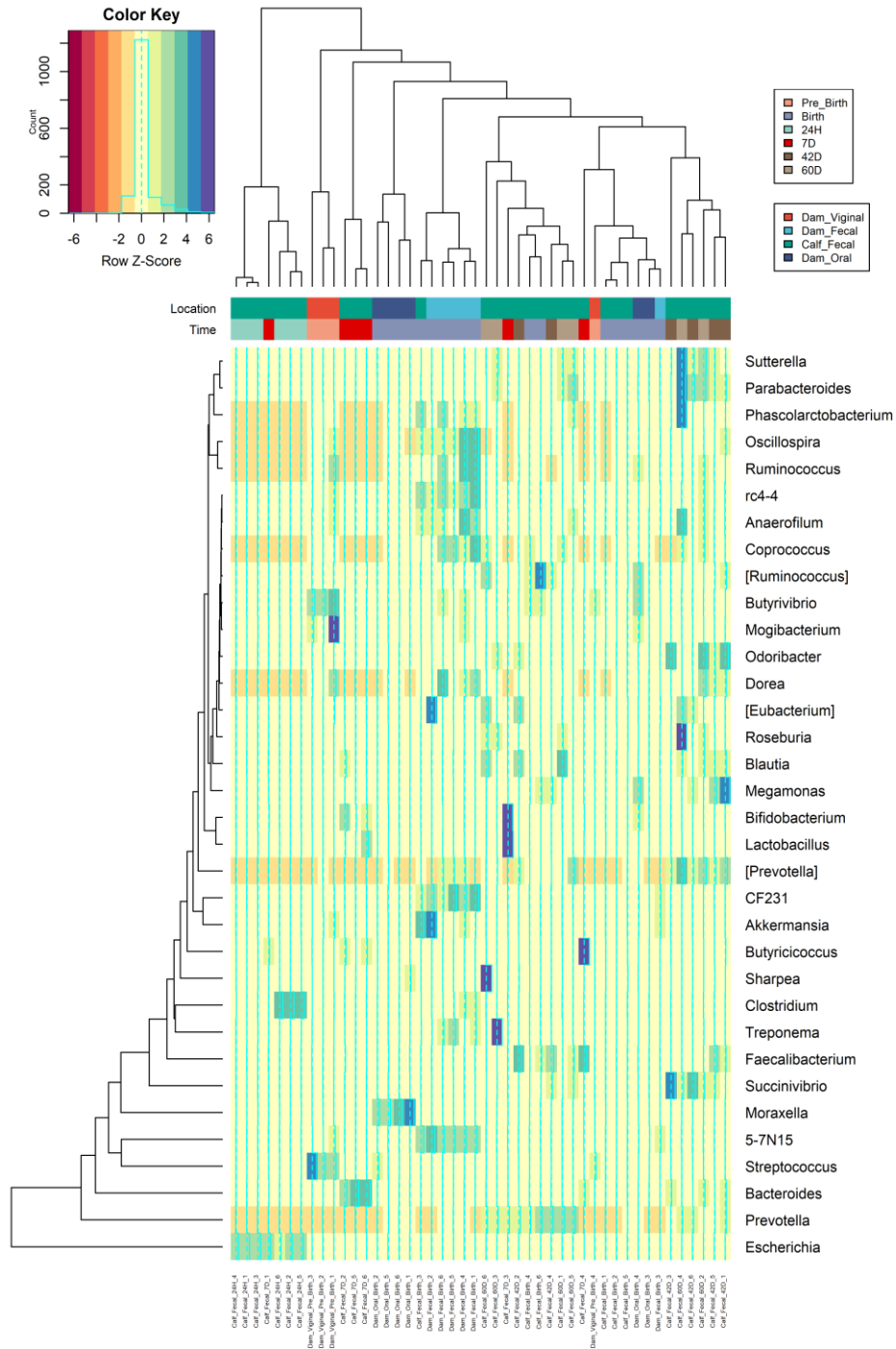


Figure 4. Relative abundance of bacterial genera present in cow and calf microbiomes. Heatmap color (dark red to dark blue) displays the relative abundance of each taxon across samples. Each sample is identified at the bottom of the heatmap by the sample name. Colors on top of each heatmap represent the time point to which each sample belongs.

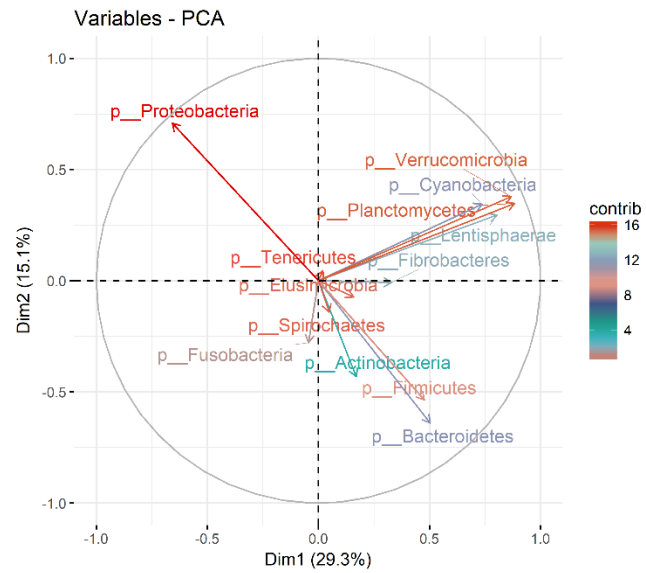
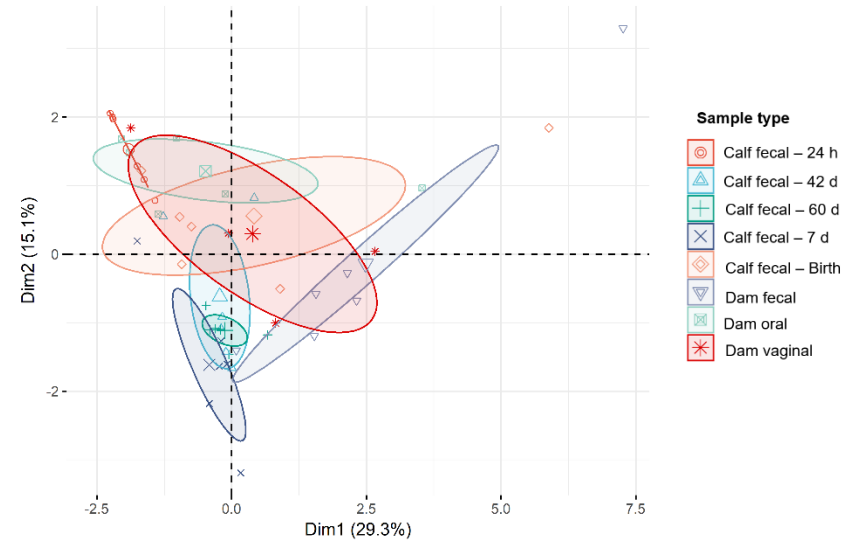
A**B** Individuals - PCA

Figure 5. Similarity of cow and calf samples. Principal component analyses (PCA) of Beta diversity at the phylum level. Variable contributions are labeled by color (A), where arrow direction indicates quality of representation on the factor map. Arrow length indicates variable contribution to the principal components (A). Points represent individual samples from the different sample locations and were colored by sample type.

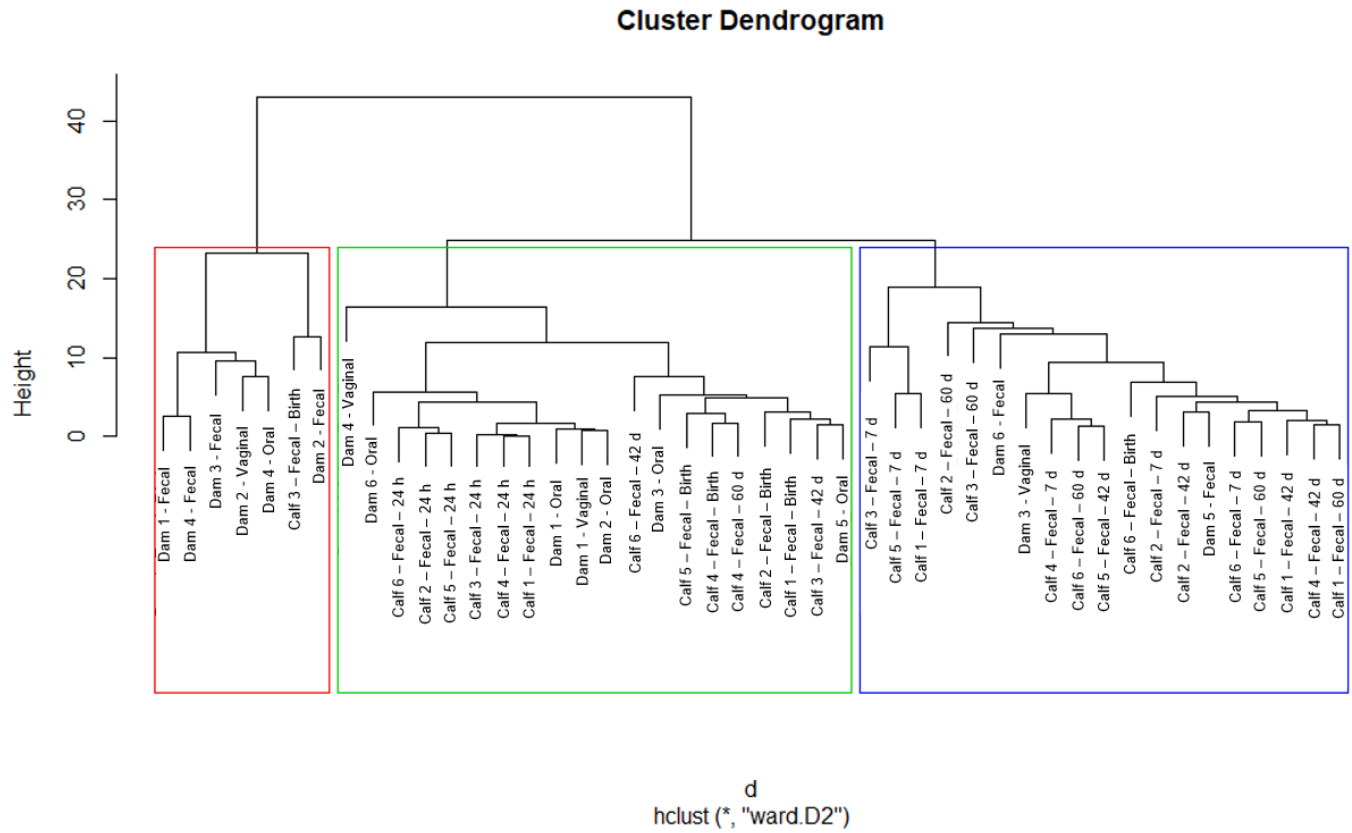


Figure 6. Hierarchical relationship between individual dam and calf samples. Sample relationships shown as a cluster dendrogram at phylum level of dam and calf microbiota at different anatomical locations and time points. The length of the scale bar represents a distance of 0.10.

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CHAPTER 3

CONCLUSION AND IMPLICATIONS

Summary and Future Studies

Despite the extensive use of management protocols aimed at optimizing calf health, neonatal diarrhea remains a major cause of calf mortality and morbidity (USDA, 2007, Malmuthuge, 2016). Pre-weaned dairy heifers are highly susceptible to many viral and bacterial enteric infections within the first few weeks of life and neonatal calf diarrhea accounts for more than 50% of pre-weaned dairy heifer deaths. Enteric pathogens such as *Escherichia coli*, *Cryptosporidium parvum*, and rotavirus are some of the most common causes of NCD and mortality (Meganck et al., 2015). More than half of all neonatal diarrheas appear during the first week of life, with an additional 15% occurring during the second week (Uetake, 2013). In cases of infection in dairy calves, disease is often managed through the use of antibiotics, however, the use of antibiotics in young ruminants is not always effective and does not come without adverse effects to the neonatal gut microbiome (Tamburini et al., 2016). With the growing concern of antibiotic resistance there has been a strong push to reduce the use of antibiotics in animal production, presenting the need to identify alternate treatment strategies. Modification of the gut microbiota could be utilized as an effective alternative method for disease management, providing a new strategy to suppress calf mortality (Willing et al., 2018).

This study investigated the maternal sources of bacteria to the neonatal calf and the composition of bacterial communities of the Holstein dam and calf. Our hypothesis was that the dam's vaginal, fecal, and oral microbiomes will influence the early microbiome of the calf. The microbiota of pre-weaned calves is dominated by 10

bacterial phyla. Three phyla, Proteobacteria, Firmicutes, and Bifidobacteria dominated the fecal and vaginal microbiomes of the cow. Similarities between the vaginal and fecal microbiome of the dam and the fecal microbiome of the calf indicate that the transference of maternal microbes prior to and during birth play a role in the inoculation of the neonatal calf gut microbiome. Further examinations are necessary to gain a complete understanding of the maternal reproductive tract microbiome and the microbial colonization patterns that occur *in utero*.

Although microbial communities of the same sample location and time point were similar, we observed individual variation between each of the subjects. Individual variation within the microbiome could be attributable to genetic or environmental factors. Further examinations of the maternal, environmental, and genetic effects on the microbiome of neonatal calves are necessary to gain a complete understanding of the development of the gut microbiome. Microbial interventions applied to the intestinal microbiome of neonatal calves could be used as a targeted strategy to improve immune function, health, and growth of dairy calves. An in-depth understanding of host-microbial interactions could lead to the development of disease management protocols through manipulation of the gut microbiome (Malmuthuge and Guan, 2017).

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