Phenotypic and microbial influences on dairy heifer fertility and calf gut microbial development

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ABSTRACT (Academic)

Pregnancy loss and calf death can cost dairy producers more than $230 million annually. While methods involving nutrition, climate, and health management to mitigate pregnancy loss and calf death have been developed, one potential influence that has not been well examined is the reproductive microbiome. I hypothesized that the microbiome of the reproductive tract would influence heifer fertility and calf gut microbial development. The objectives of this dissertation were: 1) to examine differences in phenotypes related to reproductive physiology in virgin Holstein heifers based on outcome of first insemination, 2) to characterize the uterine microbiome of virgin Holstein heifers before insemination and examine associations between uterine microbial composition and fertility related phenotypes, insemination outcome, and season of breeding, and 3) to characterize the various maternal and calf fecal microbiomes and predicted metagenomes during peri-partum and post-partum periods and examine the influence of the maternal microbiome on calf gut development during the pre-weaning phase.

In the first experiment, virgin Holstein heifers (n = 52) were enrolled over 12 periods, on period per month. On -3 d before insemination, heifers were weighed and the uterus was flushed. Flush pH and glucose concentration were measured. Blood was collected from coccygeal vessels on d -3, 15, 18, 21, 24, 27, and 30 relative to insemination and serum progesterone concentration was measured. Ultrasound measurements of dominant follicle diameter and corpus luteum volume. Insemination outcome was determined on d 30 using ultrasound and pregnancy was checked on d 42, 56, 70, and 84. Heifers were clustered based on outcome of insemination at d 30 (not pregnant, NP30, n = 24; pregnant, PS30, n = 28), d 84 (not pregnant, NP84, n = 24;

pregnant but lost before d 84, PL84, n = 2; successfully pregnant through d 84, PS84, n = 26). Differences in phenotypes were assessed based on insemination outcome at d 30 and d 84. Weight was greater in NP30 heifer than PS30 heifers. Progesterone was greater in PS30 and PS84 heifers than NP30 or NP84 heifers on d -3 and 18 to 30 and CL volume was greater in PS30 and PS84 heifers than NP30 and NP84 heifers on d 21 and 30. To summarize, traits related to pregnancy maintenance were different in virgin Holstein heifers based on first insemination outcomes and might be able to be used to predict heifer reproductive performance.

Uterine flushes were examined in a subset of heifers (n = 28) based on insemination outcome and period. This subset was also clustered based on season (spring, n = 3; summer, n = 12; fall, n = 8; winter, n = 5). From this subset of heifers, DNA was extracted from uterine flush and 16S amplicons of the V4 region underwent 250 paired-end sequencing via Illumina NovaSeq 6000. Filtered reads were clustered into operational taxonomic units using a 97% similarity and assigned taxonomy using the SSURNA Silva reference version 132. Alpha and beta diversity were measured and differences in alpha and beta diversity measurements were analyzed based on insemination outcome at d 30 or d 84 and season of breeding. Differential abundance analyses were performed at the phylum and genus taxonomic ranks based on insemination outcome at d 30 or d 84 and season of breeding. Bacterial richness was reduced in PL84 heifers than NP84 and PS84 heifers and reduced in heifers bred in spring than those bred in other seasons. Bacterial community structure was different based on insemination outcome at d 30 and d 84 using unweighted Unifrac distances and was different based on season of breeding using weighted Unifrac distances. We observed an increase of Bacteroidetes in PS30 and PS84 heifers compared to NP30 and NP84 heifers. *Ureaplasma* and *Ruminococcus* had an increased abundance in PS30 and PS84 heifers than NP30 and NP84 heifers, while *Afipia* and *Gardnerella* had an increased

abundance in NP30 and NP84 heifers than PS30 and PS84 heifers. *Prevotella* and *Ruminococcus* had a reduced abundance in summer bred heifers than winter bred heifers. Proteobacteria had a moderate negative correlation with -3 d progesterone (r_p = -0.42) and Actinobacteria had a moderate negative correlation with fetal growth rate (r_p = -0.66). Uterine microbiome of virgin Holstein heifers differed based on insemination outcomes and season of breeding and might be a new phenotype to indicate heifer fertility.

In the second experiment, multiparous Holstein cows (n = 12) were placed in individual box stalls 14 d before expected calving. Sterile swabs were used to collect samples from the posterior vagina of the dam approximately 24 h before calving, dam feces, dam oral cavity, and colostrum within 1 h after calving, and cotyledonary placenta within 6 h after calving. Calves (n = 12; bulls = 8, heifers = 4) were isolated immediately after parturition to prevent environmental contamination. Colostrum was fed to calves using a clean bottle that was assigned to the calf for the duration of the study. Calves were individually housed for 60 d until weaning. Sterile swabs were used to collect calf fecal samples at birth, 24 h, 7 d, 42 d, and 60 d of age. A subset of calf-dam pairs (n = 6; bulls = 3, heifers = 3) were selected and DNA was extracted from all samples. Amplicons covering V4-V5 16S rDNA regions were generated using extracted DNA and sequenced using 300 bp paired end sequencing via Illumina MiSeq. Sequences were aligned into operational taxonomic units using the 97% Greengenes reference database. Spearman correlations were performed between maternal and calf fecal microbiomes. Negative binomial regression models were created for genera in calf fecal samples at each time point using genera in maternal microbiomes. Metagenomes were predicted, collapsed into gene pathways and differences in predicted metagenomes were analyzed within STAMP (Statistical Analysis of Metagenomic Profiles). We determined that Bacteroidetes dominated the calf fecal microbiome

at all time points (relative abundance ≥ 42.55%) except for 24 h post-calving, where Proteobacteria were the dominant phylum (relative abundance = 85.10%). Colostrum and placenta had low diversity within samples and clustered independently from fecal samples. Each maternal microbiome was a significant predictor for calf fecal microbiome during at least 2 time points. Genes for infectious disease and neurodegenerative disease were greater in colostrum and 24 h calf fecal samples compared to other samples. Results indicated that no one maternal microbiome was a major influence on calf fecal microbiome inoculation and development. Instead, calf fecal microbial development stems from various maternal microbial sources.

Overall, the reproductive microbiome was predictive of heifer pregnancy outcomes and calf fecal microbial development. The virgin heifer uterine microbiome could be used to predict fertility and adaptation to heat stress, but further research including a larger group of pregnancy loss is needed. Maternal microbiomes from the reproductive tract, colostrum, oral cavity, and feces could all be used to predict calf microbial development, but more research including other maternal microbiomes and environmental microbial contributions is needed. However, the results from this dissertation indicate reproductive microbiome composition is a trait that might be predictive of dairy cattle performance.

GENERAL AUDIENCE ABSTRACT

The ability of a cow to become pregnant and a calf to thrive after birth are crucial to successful dairy farm operations. Recent evidence in humans has shown bacteria in the reproductive tract can influence maternal fertility and the bacterial community of newborns, an indicator of early health. This same relationship might exist in dairy cattle. I propose that specific traits related to fertility and the bacterial community in the reproductive tract of dairy cattle influences their ability to become pregnant and influences the bacterial community developing in calves after their birth.

In my first experiment, I collected samples of uterine fluid from cattle that had never been pregnant before the first time they would be bred. I also collected blood samples before and after breeding to measure hormone levels as well as measurements of portions of reproductive tract using an ultrasound. Using a specific portion of DNA that is similar across all bacteria, I identified the bacterial community in the collected uterine fluid. Cattle were grouped based on breeding outcome (not pregnant, pregnant but lost, or kept pregnancy) and season of breeding. Differences in various traits and bacterial communities were examined based on breeding outcome and season. I found that traits like hormone levels in the blood and size of structures on the reproductive tract, and uterine bacterial community all differed based on breeding outcome. We also found that uterine bacterial community also differed based on season of breeding. These results could be used to predict if a cow will become pregnant before they are ever bred, but more research is needed.

In our second experiment, we collected samples from the reproductive tract, milk, mouth, and feces of cows immediately after they gave birth. We then collected samples from their calves right at birth as well as at various time points during their early life. Using the same section of

DNA used during the first experiment, we identified the bacterial community composition from the various maternal and calf samples. We then identified correlations between maternal and calf bacteria and used a mathematical model to see if the maternal bacteria could predict bacteria in the calf. We found that the various maternal bacteria could predict calf bacteria throughout the calves early life. While an experiment using a larger group of cows and calves is needed, our results indicate that the maternal bacteria could be used to predict calf bacteria and may help determine which calves are more likely to become sick than others.

Overall, we found that the bacteria in the reproductive tract could be used to predict ability to become pregnant and calf bacterial development. The incorporation of this bacterial community as a trait on farms could help reduce pregnancy loss and calf illness, but further research examining how the bacteria interact with the animal is needed.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

IVF: *in vitro* fertilization
LPS: lipopolysaccharide
TLR: toll-like receptor
CL: corpus luteum
NK: natural killer
MHC: major histocompatibility complex
Th: T-helper
IDO: indoleamine 2,3 dioxygenase
AHR: aryl hydrocarbon receptors
ROS: reactive oxygen species
Ets-1: E26 transformation specific oncogene homolog 1
NF-kB: nuclear factor kappa-light-chain-enhancer of activated B
VEGF: vascular endothelial growth factor
IGF-1: insulin-like growth factor 1
GWAS: genome wide association study
SNP: single nucleotide polymorphism
GPTA: genomic predicted transmitting ability
DPR: daughter pregnancy rate
P4: progesterone
TAI: timed artificial insemination
BCS: body condition score
PS30: pregnancy successful at d 30
NP30: not pregnant at d 30
NP84: not pregnant at d 84
PL84: pregnant at d 30, but lost before d 84
PS84: pregnancy successful through d 84
CV: coefficient of variation
CIDR: controlled internal drug release
OTU: operational taxonomic unit
MUSCLE: multiple sequence comparison by log expectation
UCG: uncultured genus-level group
PAGs: pregnancy associated glycoproteins
IgG: immunoglobulin G
ELISA: enzyme-linked immunosorbent assay
PERMANOVA: permutational analysis of variance
PICRUSt: phylogenetic investigation of communities by reconstruction of unobservable states
KEGG: Kyoto Encyclopedia of Genes and Genomes
STAMP: Statistical Analysis of Metagenome Profiles

CHAPTER 1: Literature Review: Potential mechanisms of interaction between bacteria and the reproductive tract of dairy cattle

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1.1 ABSTRACT

While bacterial presence has been characterized throughout the reproductive tracts of multiple species, how these bacteria may be interacting with the host has yet to be described. Previous reviews have described how pathogenic bacteria interact with the reproductive tract to cause infections like metritis. This review aims to provide a summary of the knowledge related to the pathogenic and non-pathogenic bacteria in various locations of the bovine reproductive tract and the possible mechanisms of host-microbe interaction during gametogenesis and early pregnancy. Lactic acid bacteria like *Lactobacilllus* seem to be beneficial in multiple areas of the reproductive tract, as they have been associated with increased oocyte quality when in follicular fluid and secrete reactive oxygen species that are beneficial during placental angiogenesis. However, other bacteria, like *Enterococcus, Staphylococcus,* and *Streptococcus*, may modulate T helper cells that inhibit maternal recognition of pregnancy. Available data on the reproductive microbiome focuses on variations in microbial communities and their associations with reproductive performance. However, research on these host-microbiome interactions may provide more insight on how bacteria affect fertility.

Keywords: reproduction, microbiome, host-microbe interaction.

1.2 INTRODUCTION

In the late 1600s Anton van Leeuwenhoek discovered and characterized microscopic organisms, opening the door to our understanding of bacteria, protozoa, and fungi. Research from Louis Pasteur and Robert Koch in the 1800s added an understanding of microorganisms as sources of human infection and disease. In those early studies, microorganisms were isolated from infected animals and inoculated into healthy individuals that subsequently developed disease, thus demonstrating that microorganisms can be pathogenic. Bacteria were almost exclusively considered harmful until 1958. At that time, Ben Eiseman performed fecal enemas, an early form of fecal transplants, to successfully treat humans that had *Clostidium difficile* infections (Eiseman et al., 1958). Since then, whether a microorganism is considered pathogenic or commensal depends on its interactions with its host environment.

Research in dairy cattle has demonstrated how bacteria can function in both a synergistic, commensal capacity and a harmful, pathogenic capacity, depending on the location within the host. While some bacteria may not cause an inflammatory response in one location of the dairy cow, that same species can be detrimental in a different location. For instance, cows rely on metabolic functions of microbiota in the rumen in order to efficiently utilize nutrients from their diets while simultaneously providing an ideal environment for bacteria to survive. However, the presence of similar bacteria, like *Escherichia coli*, in the mammary gland or uterus causes inflammation, infection, and overall reduction in cow performance. Because of how sensitive the uterus is to infection, especially after calving, lack of infection is commonly seen as evidence the upper reproductive is sterile.

Recent advancements in technology to detect bacteria in the gut have provided evidence contradicting the conventional wisdom of the sterile uterus. Instead of relying on culture-based

techniques to isolate bacteria, sequencing of the highly conserved 16S ribosomal RNA region of bacterial DNA has provided the opportunity to identify microbiomes, the total bacterial community of a particular location, without needing specific culture parameters. This method has led to the discovery of a microbiome in the uterus of virgin heifers and pregnant cows, one that apparently does not negatively impact the dam or the pregnancy (Moore et al., 2017). The postpartum uterine microbiome of beef cattle was recently documented to vary based on pregnancy outcome (e.g., pregnant or not pregnant) (Ault et al., 2019a,b). In humans, a *Lactobacillus*-dominated uterus is associated with increased success of in vitro fertilization (**IVF**) (Moreno et al., 2016). These observations in cows and humans demonstrate that a reproductive microbiome exists and that it may impact reproductive performance. Understanding how bacteria interact with the reproductive tract could lead to the development of new techniques to aid in dairy cattle reproductive performance. Therefore, the purpose of this review is to delineate which commensal bacteria might be associated with reproductive traits in dairy cattle and propose possible mechanisms these bacteria utilize to influence oocyte development, fertilization, and pregnancy recognition.

1.3: CURRENT KNOWLEDGE OF PATHOGENIC INTERACTIONS

1.3.1 Pathogenic Infections and Pathogenesis

Much of the current knowledge on bacterial interactions with the dairy cows uterus is limited to pathogenic bacteria. Metritis, or bacterial infection of the uterus, occurs soon after parturition, with the act of giving birth allowing for pathogens like *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necromorphum*, and *Bacteroides* spp. to enter the uterus and secrete pyolysin, leukotoxin, or lipopolysaccharide (**LPS**; Jeon and Galvao, 2018). Pyolysin, secreted by *T. pyogenes*, is a cholesterol-dependent, pore-forming exotoxin that binds to the cholesterol

domain of endometrial cell membranes, allowing bacteria to enter cells and cause tissue damage (Sheldon et al., 2009). Leukotoxin, secreted by *F. necromorphum,* induces apoptotic cell death of leukocytes and can lead to necrosis of the endometrium at concentrations ≥ 625 U/mL (Narayanan et al., 2002). The endotoxin LPS, found with cell membranes of *E. coli* and *Bacteroides* spp., primarily induces immune response by binding to and activating Toll-like receptor-4 (**TLR-4**), and leading to the recruitment of immune cells via release of cytokines and chemokines (Sheldon et al., 2009). These cytokines and chemokines attract macrophages and polymorphonuclear neutrophils, but it is noted that their function is typically depressed during the early post-partum period (Sheldon et al., 2009). Endometritis, a less severe uterine infection, occurs about 20 d postpartum (Sheldon et al., 2008). Clinical cases are due to pathogens similar to metritis, but subclinical cases have been associated with the absence of typical pathogens and an increase of "commensal" bacteria, like *Lactobacillus* or *Acinetobacter* (Wang et al., 2018). Subclinical cases are also associated with persistence of polymorphonuclear neutrophils in the uterus 21 d after parturition (Sheldon et al., 2009). Approximately 25 – 40% of animals will develop metritis within 2 wk postpartum and 20% of animals will develop clinical endometritis 3 wk postpartum or later (Sheldon et al., 2008). While commensal or non-pathogenic bacteria dominate the uterus for the majority of the time, fertility issues still persist in the dairy cow. Understanding how these commensal bacteria interact with the reproductive tract is critical to discovering the roles the microbiome plays during reproduction.

Post-partum infections can have long term effects, as even after symptoms have subsided, the uterine microbiome differs between healthy and metritic cows for up to 7 wks post-partum (Knudson et al., 2016). Long-term influences of the microbiome could influence fertility in subsequent breedings, as evidence in beef cattle has demonstrated differences in the microbial

composition between pregnant and open animals -2 d before insemination (Ault et al, 2019a). A decrease in genera like *Corynebacterium*, *Staphylococcus*, and *Prevotella* at -2 d was associated with cows that later became pregnant (Ault et al., 2019b). These differences in the microbial composition between cows that will or will not become pregnant is helpful in understanding the extent uterine infection influences future fertility. Further research on how bacteria interact with the reproductive tract is needed in order to fully utilize the microbiome to benefit fertility.

1.3.2 Pathogens and Follicle Development

Postpartum uterine infections not only influence the reproductive tract shortly after birth, but also affect ovarian follicles during following estrous cycles. Proper dominant follicle development and follicular fluid production are essential for production of high-quality oocytes for fertilization. Sheldon et al. (2002) observed that when uterine infections occurred, dominant follicles in subsequent estrous cycles were smaller and grew at slower rates compared to healthy animals. In turn, this led to a smaller corpus luteum (**CL**) diameter and reduced production of estradiol and progesterone (Sheldon et al., 2002; Williams et al., 2007). Reduced progesterone production may be beneficial in clearing infection, as progesterone can inhibit uterine eicosanoid production, reducing the immune response necessary for clearing infection (Rawson et al., 1953; Hawk et al., 1964; Szekeres-Bartho et al., 2001). However, this reduces the likelihood of successfully maintaining a pregnancy. This inflammatory response is the main cause for poor follicular development. Increased inflammation in the uterus increases LPS concentration in follicular fluid (Herath et al., 2009). Granulosa cells in ovarian follicles, which are responsible for estradiol production, contain TLR-4, the natural receptor for LPS (Herath et al., 2007). Binding of LPS to TLR-4 induces inflammation, reduces follicular steroidogenesis, and increases rates of meiotic arrest in the oocyte [**Figure 1.1A** (Herath et al., 2007; Bromfield and Sheldon,

2012)]. Therefore, bacteria present anywhere in the reproductive tract could be inducing an inflammatory response and lead to lower quality oocytes.

1.3.3 Pathogens, the Uterine Body, and Insemination

Another factor required for pregnancy success is a healthy, functioning endometrium. In ruminants like cattle and sheep, the endometrium consists of aglandular caruncular areas that contain dense stroma and intercaruncular areas that contain uterine glands. These uterine glands secrete substances required for embryo survival and development (Gray et al., 2001a). A uterine gland knock-out sheep has been developed, where the adult uterus does not contain any glands (Gray et al., 2001b). These adult knockout sheep were unable to sustain pregnancy and consistently experienced pregnancy loss (Gray et al., 2002). Uterine glands also interact with sperm during insemination. After insemination, some sperm enter uterine glands and induce an acute inflammatory response via the TLR-2 pathway (Ihshan et al., 2020). This response is important in removing excess sperm and preparing the uterus for embryo implantation. Damage to glands may prevent this response, leading to pregnancy failure. Defects or damage to endometrial glands could negatively impact fertility, both immediately after damage and in future pregnancies.

While tissue damage and inflammation are normal after parturition, uterine bacterial infections have the ability to further damage glands in the dairy cow's uterus. Endometrial glands in cows that experienced metritis had predominantly cuboidal epithelial cells, compared to the columnar cells in healthy cows (Sicsic et al., 2018). While these cuboidal cells may be able to secrete nutrients necessary for pregnancy, they may not function as efficiently as the typical columnar epithelia. Those that experienced septic metritis, or foul smelling vaginal discharge alongside high fever and other signs of infection, experienced some destruction of endometrial

glands (Sicsic et al., 2018). The specific bacteria responsible for this damage have not been identified, but it could be due to pyolisin secretion by *T. pyogenesis*, which forms pores in endometrial cell membranes [**Figure 1.1B**; (Carneiro et al., 2016)]. Depending on the extent of infection, this damage could have a long-term influence on fertility if the uterine glands are unable to recover.

1.3.4 Pathogens, the Placenta, and Abortions

The uterus is sensitive to subtle changes during gestation and bacterial infiltration can cause pregnancy loss. While not as common due to vaccine development, *Brucella abortus* is a pathogen known to cause abortions. Typically, *B. abortus* is ingested, where it grows in the lymph nodes before spreading to the udder and pregnant uterus via blood vessels (Anderson, 2007). It then invades trophoblasts in the placenta, causing inflammation of the placenta, necrosis of trophoblasts, and fetal death within 72 h [**Figure 1.1C**; (Anderson, 2007)]. Other pathogens, like *Listeria monocytogenes*, *Salmonella dublin*, and *Campylobacter* spp., function similarly, but time between infection and pregnancy loss can be up to three months (Anderson, 2007). However, opportunistic bacteria are associated with 25 to 50% of pregnancy losses (Anderson, 2007). These opportunistic pathogens are typically found at low quantities in mucosal membranes, but disruptions to homeostatic conditions can lead to dysbiosis. Opportunistic bacteria associated with uterine disease and abortion have been found in endometrium and placentomes of pregnant cows using fluorescence *in-situ* hybridization (Karstrup et al., 2017). However, because there were no signs of inflammation in observed animals, it is possible bacteria normally existed in the uterus and placenta at a low abundance. These bacteria could influence the microenvironment of the placenta in a non-pathogenic

manner, but further research is needed examining bacteria in the absence of inflammation or clinical infection.

1.4: NON-PATHOGENIC BACTERIA AND POTENTIAL INTERACTIONS

1.4.1 Non-Pathogenic Bacteria, Oocyte Development, and Ovulation

Successful fertilization of an oocyte relies on the reproductive capabilities of both the bull and the dam. While a microbiome has been detected in the testes, vesicular fluid and ejaculate of male humans, mice, and cattle (Moretti et al., 2009; Javurek et al., 2016; Alfano et al., 2018), this review will focus on the microbiome within the female. Literature suggests that bacteria present within the female urogenital tract influence key mechanisms involved in development of the ova, capacitation of sperm in the female, and fertilization of the ova. As discussed next, it appears that bacteria are capable of altering the environment for the sperm, oocyte, and embryo, therefore influencing principal mechanisms required to support a pregnancy.

The environment in which the oocyte develops plays a key role in oocyte quality and its ability to be fertilized. Granulosa and theca cells within the follicular antrum create follicular fluid, which supports oocyte development (Fortune, 1994). Bacteria in follicular fluid could affect follicular fluid composition. In humans, *Lactobacillus* spp., *Actinomyces* spp., *Bifidobacterium* spp., and *Propionibacterium* spp. have been cultured from follicular fluid of ovaries but were not cultured from the vagina (Pelzer et al., 2013), demonstrating that live bacteria exist in the follicular fluid that are not contaminants from the vagina. Of these bacteria, only *Lactobacillus* spp. were positively associated with embryo transfer success during IVF, while other species were negatively associated with IVF outcomes (Pelzer et al., 2013). In the dairy cow, increased follicular fluid concentrations of flavonoids and phenolics have been

associated with increased oocyte competency, while increased eicosanoid and docosanoid concentrations have been associated with lower quality oocytes (Guerreiro et al., 2018). Flavonoids and phenolics have an antioxidant property, which help protect the oocyte against oxidative stress and its negative impact on oocyte quality (Martins et al., 2016). Eicosanoids and docosanoids are intermediates in inflammation pathways, serving as markers for inflammation processes such as oxidative stress that have a negative impact on fertility (Jabbour et al., 2009). Within the gastrointestinal tract, *Lactobaciilus* spp. have been shown to produce phenolics as well as suppress the production of eicosanoids (Fiander et al., 2005; Rodríguez et al., 2009). It is possible that the species identified in human follicular fluid can perform either of these functions, thereby enhancing fertility (**Figure 1.1D**). To our knowledge, bovine follicular fluid microbiome is not yet studied in depth. Employment of non-culture-based techniques of bovine follicular fluid samples, metabolomic screenings, or both could reveal new biomarkers for fertility.

Another important aspect to consider is the amount of bacteria necessary to influence the reproductive tract. Bacterial density in the bovine reproductive tract also influences the level of host response. Cows with a higher density of bacteria during post-partum infection had smaller follicles and corpora lutea as well as reduced circulating estradiol and progesterone (Williams et al., 2007). While commensal bacteria have been identified throughout the dairy cow reproductive tract, is their density level enough to influence reproductive performance without causing dysbiosis?

1.4.2 Non-pathogenic Bacteria, Insemination, and Fertilization

Once sperm are placed within the female reproductive tract, they begin a process of final maturation called capacitation. During this process, sperm rely on the influx of bicarbonate

(HCO_3^-) and Ca^{2+} through the plasma membrane to activate soluble adenylyl cyclase, cyclic adenosine monophosphate, and protein kinase A (Ickowicz et al., 2012). Activation of these enzymes then leads to the activation of the phosphoinositide 3-kinase pathway, which is responsible for exciting motility of sperm required for fertilization (Ickowicz et al., 2012). This sperm hyperactivated motility, characterized by asymmetrical beating of flagella, also enhances penetration through the zona pellucida for fertilization (Ickowicz et al., 2012).

An increase in extracellular HCO_3^- and Ca^{2+} increases the ability for sperm to undergo capacitation once in the female, and conversely a decrease prevents sperm from reaching full maturity (Visconti et al., 2011). Bacteria that have been identified in the uterus could influence this gradient for the sperm. For instance, in humans, a *Lactobacillus* spp. dominated uterus (≥ 90% relative abundance) is more likely to become and stay pregnant compared to a uterus not dominated by *Lactobacillus* spp. (Moreno et al., 2016). *Lactobacillus delbrueckii* has been commonly identified in the human vagina and is able to utilize carbonic anhydrase to convert H_2O and CO_2 into carbonic acid, which quickly disassociates into HCO_3^- and diffuses out of the cell (Li et al. 2015). Thus, *Lactobacillus* spp. present in the uterus may conceivably increase the amount of HCO_3^- in the reproductive tract, thus facilitating the capacitation process (**Figure 1.1E)**. While the phyla *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* were detected in the virgin heifer uterus (Moore et al., 2017), functional assessment is still lacking and we do not yet know if, or how, uterine bacteria positively affect the capacitation process in dairy cattle.

1.4.3 Inflammatory Response During Early Pregnancy and Placentation

The conceptus implements mechanisms in early pregnancy to prevent the dam from treating it like a foreign body. During early pregnancy, interactions between the uterus and the conceptus are broadly defined as anti-inflammatory. Cytotoxic T cells and natural killer (**NK**)

cells reside within the endometrium; these cells utilize major histocompatibility complex (**MHC**) class I to detect if antigens in the uterus are self or non-self (Bessoles et al., 2014). If the cell produces MHC class I with peptides recognized as the host's, NK cells are not triggered to kill said cell (Bessoles et al., 2014). However, the conceptus does not produce these "self" MHC class I molecules during early pregnancy and is susceptible to triggering these NK cells. Natural killer cells seem to contribute towards pregnancy and placentation, as they modify maternal arteries to aid in placental angiogenesis and produce T-helper (**Th**) 2 cytokines during early pregnancy (Mori et al., 2016). What could be modulating the function of the NK cells to aide in pregnancy rather than hinder it? While not fully understood, it is possible that increased production of indoleamine 2,3, dioxygenase (**IDO**) caused by the presence of the bovine embryo regulates the function of NK and cytotoxic T cells to suppress their inflammatory functions. The gene for IDO1, an immune response regulator that decreases inflammation, is stimulated by interferon tau and has increased expression at the implantation site in mice (von Rango et al., 2007). Increased IDO1 levels lead to increased activation of aryl hydrocarbon receptors (**AHR**). These AHR promote production of peroxisome proliferator-activated receptor-γ, which decrease pro-inflammatory cytokine production and improves immune tolerance, leading to recognition of the conceptus as "self" cells (Vacca et al., 2010).

Of the bacteria found in the uterus, *Lactobacillus* spp. have been positively associated with increased success of IVF in humans and so is a potential mediator of IDO1 production and AHR activation. However, research provides conflicting information on how the different *Lactobacillus* spp. may be influencing early pregnancy success. Within the mouse gastrointestinal tract, presence of *L. reuteri* inhibits the activity of IDO1 through its production of reactive oxygen species (**ROS**), leading to an overall increase in immune response by the

mouse (Marin et al., 2017). *Lactobacillus reuteri* could be inhibiting IDO1 production at the site of implantation, reducing immune tolerance that aides in successful establishment of pregnancy. However, *L. reuteri* also produce indole-3-lactic acid, an AHR agonist, which converts $CD4^+$ T cells into $CD4^+CD8\alpha\alpha^+$ T regulator cells [**Treg**, (Cervantes-Barragan et al., 2017)]. This suppresses immune response and reduces inflammation (Cervantes-Barragan et al., 2017). Production of AHR agonists in the uterus by *L. reuteri* could increase the number of epithelial Treg cells that aide in immunosuppression and improve the ability of cow to become pregnant. Other species of lactobacilli have shown to affect the bovine endometrial production of inflammatory cytokines *in vitro*. Gärtner et al. (2015) co-cultured bovine endometrial epithelial cells with four different *Lactobacillus* spp. *Lactobacillus vaginalis* and *L. buchneri* did not affect expression of pro-inflammatory cytokines *IL1A, IL6,* and *IL8*, while *L. amylovorus* and *L. ruminis* increased expression of all genes with increased concentration of bacteria.

Of these pro-inflammatory cytokines, IL6 seems to be important for pregnancy recognition and embryo development. Interleukin 6 can act in both a pro-inflammatory and anti-inflammatory manner, depending on the signaling method. If IL6 binds to a membrane bound receptor, the Janus kinase/signal transducer and activator of transcription proteins pathway induces production of yes-associated protein for cell proliferation, tissue regeneration, and anti-inflammatory activities (Rose-John et al., 2006). If IL6 binds to soluble receptors, a complex forms that can bind to nonspecific surface receptors and induce an inflammatory response (Rose-John et al., 2006). The bovine embryo has increased inner cell mass numbers and blastocyst development when cultured with IL6, so it seems the embryo may primarily utilize the membrane bound receptor (Wooldridge and Ealy, 2019). In humans, increased inner cell mass is associated with a decrease in early embryonic loss; therefore, increased IL6 production induced by bacteria could

be aiding in early pregnancy (Richter et al., 2001). Conversely, it also is a key cytokine during the early phases of graft-versus-host disease, where blocking IL6 receptors reduced the magnitude of inflammation (Belle et al., 2017). In order to fully understand IL6's role during implantation and the effect bacteria may have on pregnancy success, research involving IL6 in the presence of both the embryo and endometrium is needed.

1.4.4: *Bacteria and Placental Attachment and Function*

Once implantation has successfully occurred and placentomes have formed, energy delivered to the fetus is primarily used for growth. Fetal growth is regulated by the placenta, with magnitude of vasculature of the placenta positively correlated with fetal weight (Echternkamp, 1993). The availability of certain nutrients affects placental growth and for angiogenesis. A microbiome unique to the placenta has been identified in both humans and dairy cows in the absence of an inflammatory response (Aargard et al., 2014; Moore et al., 2016). The bacteria present in the placenta have the capability to influence placental growth rate, angiogenesis, or transport of nutrients to the fetus.

Though the microbiome in the placenta has been detected, it is not as well characterized. In the dairy cow, research on the "non-pathogenic" microbiome in the reproductive tract is limited. Moore et al. (2016) observed that both the placentome (the site of placental attachment) and the intercotyledonary placenta in the pregnant dairy cow were dominated by the bacterial phyla *Firmicutes* (35 to 42% of the total microbiome) and *Bacteroidetes* (23 to 33% of the total microbiome). Results mirrored bacterial phyla present in the endometrium of the same cows (Moore et al., 2016). However, in that study, observations were limited and the category of "phylum" was too large of a taxa to make any meaningful conclusions regarding the functions of identified bacteria. Aargard et al. (2014) discovered a microbiome within the placenta of

humans, comprised primarily of *Proteobacteria* and *Actinobacteria* phyla members and a greater level of *Tenericutes* when compared to other locations in the body. Also, *E. coli* species and *Bacteroides* spp. were abundant in the human placenta microbiome (Aargard et al., 2014). However, this study did not characterize other locations of the reproductive tract, so the placental microbiome may not be completely distinct from the uterus, for instance. If the microbiome of the placenta and uterus are similar, *Lactobacillus* spp. could positively influence the placenta and its growth, as they have been positively associated with pregnancy outcomes (Moreno et al., 2016). More research into the placental microbiome of dairy cattle and reproductive outcomes seems warranted. That said, the importance of a placental microbiome has been questioned. While Aargard et al. (2014) characterized a human placental microbiome, findings have not, to our knowledge, been replicated. This suggests either a low abundance microbiome in the placenta or contamination (Leiby et al., 2018). It is possible that a true, distinct placental microbiome does not exist, but that the placenta is instead another tissue in which bacteria in the uterus can proliferate. Bacterial species, such as *Fusobacteria necromorphum*, *Trueparella pyogenes*, and *Porphyromonas levii*, and other unspecified bacteria have been identified in the placentomes and endometrium of dairy cattle using fluorescent in situ hybridization (Karstrup et al., 2017). This provides evidence that even if the previously identified placental microbiome was due to reagent contamination, there are still bacteria within the bovine placenta that may influence placental angiogenesis.

Increased vasculature of the placenta causes greater fetal growth rate and improves placental ability to transport nutrients from dam to fetal calf. If angiogenesis is negatively impacted, there is a negative impact on the size of the fetus and could even lead to a termination of the pregnancy (Kang et al., 2014). Reactive oxygen species, such as H_2O_2 and superoxide, are

naturally produced by the dam when under metabolic stress through the oxidative phosphorylation pathway in the mitochondria (Starkov, 2008). Reactive oxygen species help control cellular development and function when adequately counterbalanced by antioxidants such as glutathione peroxidase (Iwaoka and Tomoda, 1994). During the first trimester of mammalian pregnancy, there is a dramatic increase in placental oxygen level when blood flow is established in the intervillous space, leading to increased oxidative stress and ROS production (Jauniaux et al., 2000). This spike in ROS throughout gestation could play a role in placental angiogenesis. Increased angiogenesis during periods of oxidative stress is most likely a response to meet the tissue's need for oxygen; by increasing vasculature in tissue, transport of oxygen and nutrients to the desired location is increased.

While not as well defined in the placenta, interactions between ROS and transcription factors that regulate angiogenesis and cellular differentiation have been studied in other blood vessels in cattle and humans (Pereira et al., 2014). Transcription factors such as E26 transformation specific oncogene homolog 1 (**Ets-1**) and nuclear factor kappa-light-chain-enhancer of activated B (**NF-κB**) increase rate of angiogenesis and trophoblast invasion. These transcription factors are also upregulated in the presence of ROS (Shono et al., 1996; Oikawa et al., 2001). When Ets-1 production is increased, it increases the production of vascular endothelial growth factor (**VEGF**), a protein that then increases angiogenesis (Hashiya et al., 2004). It is also thought that because Ets-1 is positively correlated with trophoblast invasion for placental attachment, increased Ets-1 production increases expression of matrix metalloproteinase 9 and urokinase-type plasminogen activator, which both are known to be important for trophoblast invasion during placental attachment (Dittmer, 2003). Increased production of NF-κB occurs when intracellular ROS production increases, leading to an upregulation of downstream

angiogenic factors (Gloire et al., 2006). Interactions between NF-κB and ROS also aid in the restructuring or formation of tubules for angiogenesis. When cells are exposed to H_2O_2, NF-κB has an increased capacity to bind to DNA and activate production of IL6 and IL8, which influence cellular differentiation and growth (Shono et al., 1996; Bonavia et al., 2011). Changes in the regulation of these transcription factors and their interactions with ROS could influence angiogenesis in the placenta and subsequently affect fetal growth. Several bacterial genera, including *Lactobacillus, Streptococcus,* and *Enterococcus*, have demonstrated an ability to produce H_2O_2 using NADH oxidase (Marty-Teysset et al., 2000; McLeod and Gordon, 1922). These genera have all been found in the oviduct, uterus, and vaginal microbiomes, with notable species including *L. delbruecki, L. crispatus* and *L. jenseniii* (Antonio et al., 1999). If these species are present and produce appreciable quantities of H_2O_2 at the site of placental attachment in dairy cattle, resultant exposure of placental and endometrial cells to ROS could conceivably increase production of transcription factors Ets-1 and NF-κB. The increased angiogenesis due to production of these factors would improve likelihood of a successful pregnancy (**Figure 1.1F**). If this is the case, there would be a positive association, functional relationship, or both between certain placental bacterial species and fetal growth rate. However, this might only occur at a lower bacterial density, with small increases in bacteria shifting the uterus into dysbiosis and allowing opportunistic bacteria to cause infection or pregnancy loss.

1.5: CONCLUSION

Commensal bacteria have been isolated from healthy female reproductive tracts of a variety of animals. In many, but not all instances, presence of these bacteria has been linked to favorable reproductive outcomes. In comparison to other animals, considerably less is known about presence and function of bacteria within healthy reproductive tracts of female dairy cattle.

We outlined pathogenesis of bacteria that induce uterine infections and potential ways that commensal bacteria may interact with reproductive host tissues in dairy cattle (**Figure 1.1**) and we proposed future avenues of research. Uterine pathogens like *E. coli, T. pyogenes*, and *F. necromorphum* can cause damage at the infection site and have negative influences during subsequent ovulations. *Lactobacillus* could be secreting flavonoids while mitigating the secretion of eicosanoids in follicular fluid to improve oocyte quality, disrupting the ion gradient necessary for successful sperm capacitation. Alternatively, they may be producing H_2O_2 and increasing production of the primary angiogenic factor VEGF in the placenta. Further investigation of relationships between the reproductive tract's microbiome and specific reproductive performance traits in the dairy cow are needed to fully understand the role the reproductive microbiome plays at all points in pregnancy. It also important to understand the bacterial density necessary to induce a physiologic response. Once these mechanisms are more thoroughly understood, strategies can be developed to utilize the microbiome to benefit fertility in the dairy industry**.**

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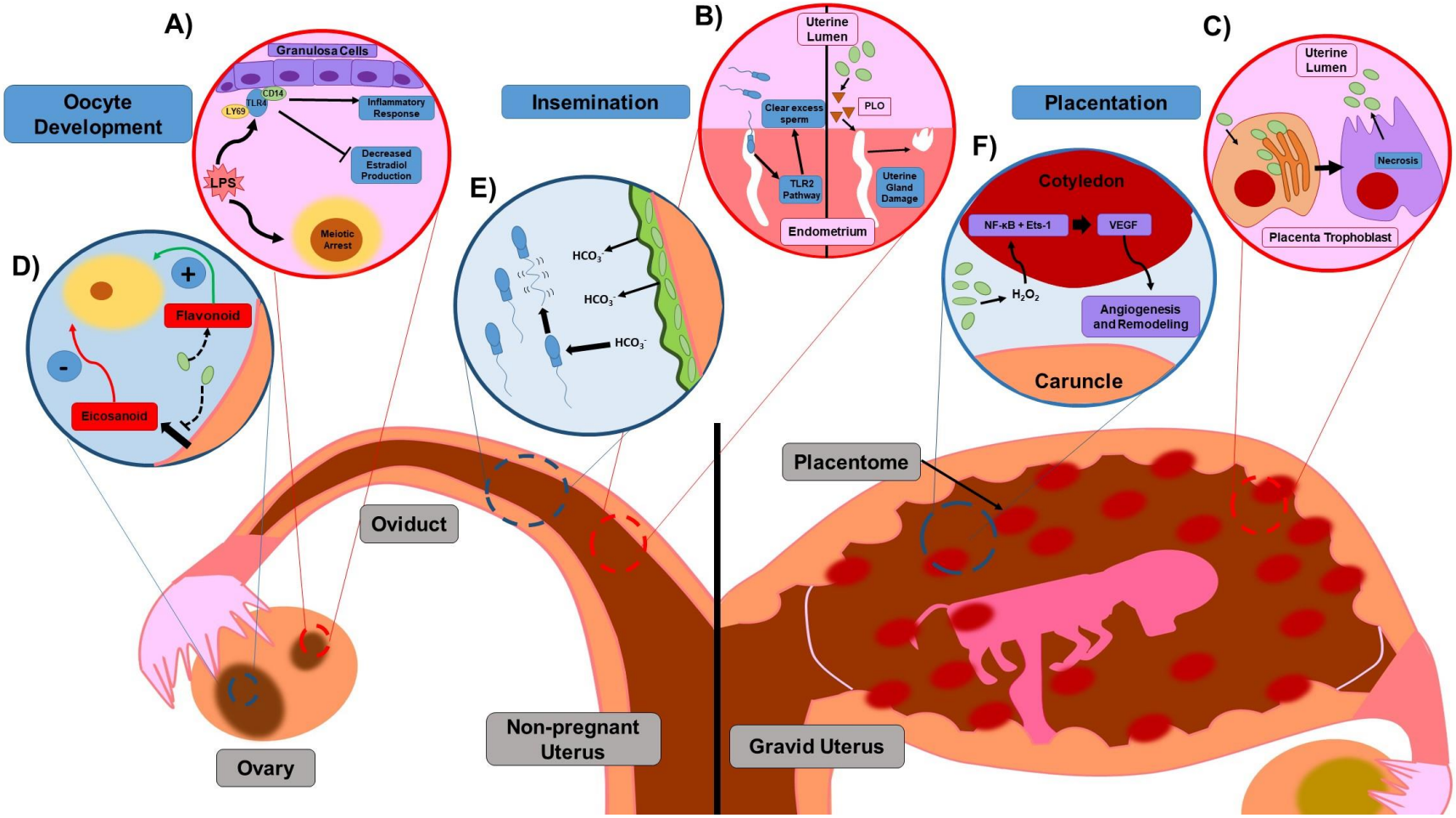
1.7: FIGURES

Figure 1.1 Summary figure of the pathogenic (A-C) and possible non-pathogenic (D-F) interactions the microbiome of the reproductive tract may have with the host. A) *Oocyte Development*: Lipopolysaccharide (LPS) from *Escherichia coli* in the uterus increase LPS in dominant follicles, causing meiotic arrest in the oocyte and binding to receptors on granulosa cells. This induces an inflammatory response and decreases estradiol production. B) *Insemination*: Normally, some sperm will enter uterine glands after insemination, inducing a response that prepares the uterus for the fertilized embryo. Uterine glands are damaged by pyolysin (PLO) from *Truperella pyogenes*, which could hinder the response needed to clear excess sperm and allow embryo attachment. C) *Placentation*: After being ingested, *Brucella*, *Listeria*, or *Campylobacter* travel through blood stream to placentomes in the uterus. They then invade placental trophoblasts and replicate, causing necrosis of the trophoblast and spreading the pathogen throughout the uterus. D) *Oocyte Development*: *Lactobacillus* identified in the follicular fluid could be secreting flavonoids, which have been associated with increased oocyte competency, while also mitigating the secretion of eicosanoids, which have been associated with decreased oocyte quality. E) *Capacitation*: *L. delbrueckii* within the uterus and vagina have the ability to produce HCO_3^- ion. This may enhance the ion gradient necessary for sperm capacitation, increasing the amount of fully capacitated sperm. F) *Placentation*: The rate of angiogenesis is increased in the presence of reactive oxygen species (i.e. H_2O_2), which increase the expression of transcription factors *Ets-1* and *NF-κB* and increase production of the primary angiogenic factor VEGF. *Lactobacillus*, *Streptococcus*, and *Enterococcus* found in the uterus have been associated with increased pregnancy success and produce H_2O_2, which could increase the rate of angiogenesis and increase placentation.

Figure 1. 1


A)
Oocyte Development
Granulosa Cells
CD14
TLR4
LY69
Inflammatory Response
LPS
Decreased Estradiol Production
Meiotic Arrest
B)
Insemination
Uterine Lumen
Clear excess sperm
PLO
TLR2 Pathway
Uterine Gland Damage
Endometrium
C)
Placentation
Uterine Lumen
Necrosis
Placenta Trophoblast
D)
+
Flavonoid
-
Eicosanoid
E)
HCO3-
HCO3-
HCO3-
F)
Cotyledon
NF-κB + Ets-1
VEGF
H2O2
Angiogenesis and Remodeling
Caruncle
Placentome
Oviduct
Ovary
Non-pregnant Uterus
Gravid Uterus

CHAPTER 2: Virgin Holstein heifer fertility phenotypes vary based on first insemination outcome

2.1 ABSTRACT

While 90% of inseminations in dairy cows and heifers result in fertilized embryo, less than half will be successfully maintained through gestation. Phenotypes related to fertility and pregnancy loss, like progesterone profiles and corpus luteum (CL) volume, have been associated fertility traits in cows, but not virgin heifers. The objective of this study was to determine which fertility phenotypes differ in virgin Holstein heifers based on first insemination outcomes. We hypothesized that have high progesterone concentrations, greater uterine glucose concentrations, and positive GDPR PTA in heifers would be associated with successful pregnancy during first insemination. Virgin Holstein heifers (n = 52) were placed on a Double Ovsync synchronization protocol. Heifers were weighed and body condition score was collected on d -3 relative to insemination. The uterus of heifers were flushed using a sterile saline flush on d -3 and pH of flush was measured. Flush glucose concentration was measured using gas chromatography mass spectrometry. Serum was collected from coccygeal vessels on d -3, 15, 18, 21, 24, 27, and 30 relative to insemination and serum progesterone concentration was measured using a commercially available chemiluminescence assay. Ultrasound was used to collect dominant follicle diameter on d -3 and CL volume on d -3, 15, 21, and 30 relative to insemination. Heifers were separated based on outcome of insemination on d 30 (Not pregnant, NP30, n = 24; Pregnancy success, PS30, n = 28). Pregnancy in PS30 heifers was checked via ultrasound on d 42, 56, 70 and 84 in case loss occurred. Two PS30 heifers lost pregnancy between 30 and 84 d and heifers were clustered into groups based on d 84 outcome (not pregnant, NP84, n = 24;

pregnant but lost before 84 d, PL84, n = 2; successfully pregnant though 84 d, PS84, n = 26). Ability of dominant follicle diameter, uterine pH, GDPR PTA, and flush glucose concentration to predict insemination outcomes was analyzed using a logistic regression with age, weight, and BCS on d -3 included as covariates. Differences in progesterone concentration and CL volume were analyzed using a mixed ANOVA with repeated measures with the main effects of insemination outcome at d 30 or d 84, time, and insemination outcome × time interaction. Dominant follicle diameter, GDPR PTA, uterine pH, and flush glucose concentration did not differ based on insemination outcome at d 30 or d 84. Progesterone concentration was greater in PS30 and PS84 heifers on d -3 and d 18 – 24 than NP30 or NP84 heifers. Corpus luteum volume was greater in PS30 and PS84 heifers than NP30 or NP84 heifers on d 21 and 30. Our results indicate that progesterone concentration before insemination was associated with insemination outcomes, but further research is needed.

Keywords: virgin heifer, phenotypes, fertility

2.2 INTRODUCTION

While 90% of inseminations in dairy cattle result in fertilized embryo, only 40-55% of inseminated cows will produce a calf (Diskin et al., 2011). Cows that continue to have poor reproductive performance, like requiring greater number of inseminations per conception or increased days open, cost approximately $261 more per cow per year than those with superior reproductive performance (Inchaisri et al., 2010). Early detection of cows that may have poor lifetime reproductive performance can aid in making reproductive management decisions based on fertility, improving overall herd reproductive performance at a greater rate, and reducing potential economic loss.

Dairy cow fertility is a complex trait influenced by a combination of phenotypic factors, including nutritional status, environmental factors, ovarian function, and hormonal balance. Cows will enter negative energy balance in the days prior to parturition and during early lactation due to the increased energetic demands for fetal growth and milk synthesis (Taylor et al., 2004). In order to meet this increased energetic demand, cows will utilize their lipid stores and increase hepatic production of glucose (Bauman and Currie, 1980; Lucy et al., 2001). However, cows will enter a period of insulin resistance after calving, which prevents glucose storage and results in low blood glucose concentrations (Bauman and Currie, 1980). This insulin resistance decreases insulin-like growth factor (**IGF**)-1 production, which prevents cows from returning to a normal estrous cycle, delaying first ovulation, inhibiting oocyte development, and reducing conception rates even after the animal is out of negative energy balance (Beam and Butler, 1999; Taylor et al., 2004; Wathes et al., 2007). A decrease in IGF-1 also decreases steroidogenesis by granulosa cells in the ovary, decreasing circulating estradiol and progesterone concentrations (Spicer and Aad, 2007; Mani et al., 2010). Estradiol and progesterone regulate ovarian follicular development and their decreased production is detrimental to oocyte

development, ultimately causing poor reproductive performance (Spicer and Aad, 2007; Mani et al., 2010). These factors regulating cow fertility also play a role in heifer fertility. Prepubertal heifers with increased blood glucose concentrations achieved puberty earlier (Fiol et al., 2017). Additionally, prepubertal IGF-1 in dairy heifers has been positively correlated with reproductive performance during first lactation (Taylor et al., 2004). Nulliparous heifers that have poor reproductive performance will also have poor fertility later in life (Wathes et al., 2014). Examining other factors related to reproductive performance in heifers might allow for an earlier and more accurate selection for lifetime fertility.

The bovine uterine environment may also affect reproductive performance. Research on optimum *in vitro* bovine embryo culture conditions indicates that metabolite concentration is important for embryonic survival. While not required until d 4 of development, addition of glucose to culture media at a concentration of 1.5 m*M* improves embryonic development (Kim et al., 1993). However, increased concentrations of glucose to 5 – 6 m*M*, similar to those in blood plasma, can actually inhibit blastocyst development (Matsuyama et al., 1993). Ability to regulate uterine luminal glucose concentration could be related to reproductive performance and concentration of glucose in the bovine uterine lumen before insemination could influence outcome of insemination. Another factor of the uterine environment that may regulate embryonic cellular mechanisms is pH of the uterine lumen. Heifers with reduced uterine pH also have decreased first service conception rates (Elrod and Butler, 1993). In embryo culture, decreased media pH reduced cleavage rate and development to blastocyst stage (Ocon and Hansen, 2003). Bovine uterine pH before insemination might be associated with insemination outcome.

The majority of studies examining fertility focus on multiparous cows, but factors influencing fertility seem to affect nulliparous heifers differently. Net Merit utilizes heifer

conception rate to differentiate between heifer and cow fertility, but heifer conception rate has a low heritability and is not currently genomically predicted. Recent genome wide association studies (**GWAS**) have identified single nucleotide polymorphisms (**SNP**) specifically associated with heifer fertility, but they have yet to be used to determine genomic predicted transmitting ability [(**GPTA**) (Kiser et al., 2019)]. However, daughter pregnancy rate (**DPR**) GPTA is currently used in genomic selection and may be indicative of heifer reproductive performance. Other phenotypes related to reproductive physiology differ between nulliparous heifers and multiparous cows. Heifers have greater concentrations of circulating progesterone (**P4**) and lower luteal tissue volume than lactating cows (Sartori et al., 2004). The influence of these factors on first insemination outcomes may also be different than what has been observed in multiparous cows. Therefore, the objective of this study was to determine which fertility phenotypes differ in virgin Holstein heifers based on first insemination outcomes. I hypothesized that increased progesterone concentrations, greater uterine glucose concentrations, and positive GDPR PTA in heifers would be associated with successful pregnancy from first insemination.

2.3 MATERIALS AND METHODS

2.3.1 *Animal Use and Sample Collection*

Animal procedures were approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (protocol #18-114-DASC). Virgin Holstein heifers (n = 52) were enrolled over a one-year period (March 2019 to March 2020) once they were at least 322 kg and 335 d of age. Heifers were enrolled in 12 periods (1 period/month) with 3 to 10 heifers/period. Heifers grazed pasture and were supplemented with a total mixed ration throughout the duration of the experiment (**Supplemental Table S2.1**).

Heifers were placed on a Double OvSynch protocol -27 d relative to timed artificial insemination (**TAI**). Briefly, 100 μg of GnRH (Factrel, Zoetis, Parsippany-Troy Hills, NJ) were administered intramuscularly at the neck on -27 d, -17 d, -10 d, and -16 h relative to insemination. Approximately 25 mg of $PGF_{2\alpha}$ (Lutalyse, Zoetis, Parsippany-Troy Hills, NJ) was administered intramuscularly at the neck on -20 d and -3 d relative to TAI. On -3 d, an estrotect alert patch (Rockway Inc., Spring Valley, WI) was placed on the tailhead of heifers to monitor signs of estrus. Patches were inspected -1 d before TAI. Heifers with patches > 50% colored in the morning were bred in the afternoon of -1 d. Otherwise, heifers were administered the last GnRH injection and bred according to TAI protocol. Heifers were bred using conventional semen from one of five bulls selected for high fertility by one of two breeding technicians.

2.3.2 *Uterine Flushing and Blood Collection*

Immediately before $PGF_{2\alpha}$ injection on -3 d, heifers were brought into a chute for uterine flushing. Body weights were obtained and body condition score (**BCS**) was assigned using the average from the same 2 individuals for all heifers. Body condition score ranged from 1 to 5 on a 0.25-point scale, with 1 being lean and 5 being obese. Vulva, interior of the vestibule, and surrounding area were disinfected using a 2% chlorhexidine gluconate solution (Aspen, Liberty, MO). A sterile, vortech silicone catheter (14 - 16 Fr and 23” long; Agtech Inc., Manhattan, KS) was maneuvered just past the cervix using a sterile, stainless-steel stylet (Agtech Inc., Manhattan, KS). Once catheter was just past the cervix, the balloon was inflated using 10 cc of air. Approximately 240 mL of sterile 0.9% saline solution was used to flush the uterus in 60 mL increments. Return flush solution was collected from catheters using 60 mL catheter-tip syringes (Becton, Dickinson, and Company, Franklin Lakes, NJ) and transferred to sterile, 50 mL conical tubes (Fisher Scientific, Waltham, MA). Return flush volume was measured and tubes were

immediately placed on ice for transport to the laboratory. In the laboratory, 10 mL flush aliquots were made using 15 mL conical tubes (Fisher Scientific, Waltham, MA) and stored at -80 °C. Flush pH was measured using 5 mL of flush on a Fisher Science Education Laboratory Benchtop pH meter (Fisher Scientific, Waltham, MA). One heifer could not have pH measured due to insufficient flush return for both pH measurement and additional analyses.

On -3, 15, 18, 21, 24, 27, and 30 d relative to TAI, 10 mL of blood were collected from coccygeal vessels using Monoject collection tubes with no additive (Covidien, Dublin, Ireland) for serum isolation. Blood for serum isolation was allowed to clot at room temperature for 4 h before being centrifuged at 2,000 × *g* for 15 min at 4 °C. Serum was isolated and stored at -20 °C.

2.3.3 *Ultrasound Measurements and Insemination Outcome Assignment*

On d -3, 15, 21, and 30 relative to TAI, measurements of ovarian follicles and corpora lutea (**CL**) were collected using a transrectal ultrasound (IBEX PRO; E. I. Medical Imaging, Loveland, CO). Vertical and horizontal diameter measurements of follicles and CL were made at the widest cross section. Vertical and horizontal diameters of inner CL pocket, if present, were measured at the widest cross section. Dominant follicle diameter was calculated by averaging vertical and horizontal diameter measurements obtained on -3 d before insemination. Corpus luteum volume (*V*) was calculated using the formula:

$$V = \frac{{}^{4}/_{3} \times ((d_{V1} - d_{V2}) \times (d_{H1} - d_{H2})) \times {}^{(d_{V1} + d_{H1})}/_{2}}{2}$$

where, d_{V1} and d_{H1} are the outer vertical and horizontal CL diameters, respectively, and d_{V2} and d_{H2} are the inner vertical and horizontal CL pocket diameters, respectively, if a pocket of fluid in the CL was identified.

Pregnancy status was assessed on 30 d relative to TAI using a transrectal ultrasound (IBEX PRO; E. I. Medical Imaging, Loveland, CO). No more sampling or measurements occurred in non-pregnant heifers. Pregnancy status was assessed again in pregnant heifers using ultrasound on 42, 56, 70, and 84 d relative to TAI. If pregnancy was lost after 30 d, measurements were collected on day of ultrasound examination when loss was observed, and no more measurements were collected from the heifer. Heifers were initially clustered into two categories based on outcome of first insemination at 30 d: pregnant (**PS30**, n = 28) and not pregnant (**NP30**, n = 24). Two heifers lost pregnancy between 30 d and 84 d. Heifers were then clustered into three categories based on first insemination outcome at 84 d: not pregnant (**NP84**, n = 24), pregnant and lost before 84 d (**PL84**, n = 2), and successfully pregnant through 84 d (**PS84**, n = 26).

2.3.4 *Progesterone*

Serum progesterone concentration on -3, 15, 18, 21, 24, 27, and 30 d relative to TAI was measured in duplicate using a commercially available chemiluminescence assay (Immulite 2000 XPi Immunoassay System, Siemens Healthcare, CA, USA) according to manufacturer instructions. In short, a progesterone antibody-coated polystyrene bead was placed in 500 μL of isolated serum in a reaction tube. Serum was incubated with an alkaline phosphatase-labeled reagent and reaction mixture was separated from the bead. Reaction tubes and bead were washed separately four times and beads were placed back in reaction tubes. A dioxetane substrate was then added to react with bead-bound alkaline phosphatase label to produce light. Quantity of emitted light was measured and used to quantify progesterone concentration. All samples were run in a single assay. Inter-assay coefficients of variation were determined using low, moderate, and high concentration progesterone standards provided with the assay and run in quadruplicate.

Inter-assay coefficients of variation were 10.30%, 4.82%, and 5.33% for low, moderate, and high standards.

2.3.5 *Glucose Concentration*

A subset of heifers was selected for uterine flush glucose concentration analysis based on insemination outcome and controlled for flush period (NP30 = 16, PS30 = 12; NP84 = 16, PL84 = 2, PS84 =10). Glucose concentration of uterine flush was measured using gas chromatography mass spectrometry. Uterine flush was thawed and a D-glucose ^{13}C6 tracer was added to 300 µL of sample at a concentration of 4 µg/mL and 1 mL of methanol added before freezing -80 °C. At a later date, samples were thawed and nitrilation was performed by adding 100 µL of hydroxylamine hydrochloride (2.1 mg/100 µL pyridine solution) to the sample and heating to 90 °C for 30 min. Then, acetylation was performed by adding 75 µL of acetic anhydride and heating to 90 °C for 60 min. Reaction was allowed to cool and 1.5 mL water and 300 µL methylene chloride were added to the reaction mixture. Reaction mixed was then centrifuged 200 x G for 5 min at room temperature. Lower methylene chloride layer was isolated and dried using a Reacti-Vap with a nitrogen gas stream at ~5 PSI on a heating block at 50 °C. Dried sample was re-dissolved in 50 µL ethyl acetate. Gas chromatography mass spectrometry analysis was performed on a Rts-5MS column (Restex, Bellefonte, PA) using a mass window of 45 – 600 m/z. Glucose concentration was quantified using a calibration curve of standards ranging from 0 to 6.98 m*M*. Uterine flush samples were read in duplicate.

2.3.6 *Statistical Analyses*

Ability for phenotypes measured once (dominant follicle diameter, uterine pH, GDPR PTA, and flush glucose concentration) to predict insemination outcomes was analyzed using a logistic regression analysis with the stats package version 4.0.2 in R version 4.0.2 (R Team,

2020). The dependent variable was insemination outcome at 30 d (NP30 or PS30) for one model and insemination outcome at 84 d (NP84, PL84, or PS84) for a second model. Uterine flush glucose concentration was adjusted based on flush return volume. Weight, age, and BCS on d -3 were included as covariates in both models. A *P*-value ≤ 0.05 was considered significant. Association of phenotypes with repeated measures (circulating progesterone concentration and CL volume) with first insemination outcomes was analyzed using mixed ANOVA with repeated measures using the package rstatix version 0.5.0 in R version 4.0.2 (R Team, 2019). For the 30-d insemination outcome model, main effects were insemination outcome at 30 d, day, and insemination outcome × day interaction. For the 84-d insemination outcome model, main effects were insemination outcome at 84 d, day, and insemination outcome × day interaction. Weight, age, and BCS at -3 d were included as covariates, initially. Body condition score was not significant and removed from the final models. A *P*-value ≤ 0.05 was considered significant. For insemination outcome at 84 d, phenotypes with repeated measures were analyzed at each time point using a post-hoc ANOVA with weight and age at -3 d included as covariates. A *P*-value ≤ 0.05 was considered significant. Pairwise comparisons between each insemination outcome group on each day were performed using a pairwise t-test for the main effects of insemination outcome on 30 d and insemination outcome on 84 d. A Bonferroni corrected *P*-value ≤ 0.05 was considered significant.

2.4 RESULTS

2.4.1 *Descriptive Statistics*

At breeding, heifers were 354.558 ± 3.90 kg and 394.384 ± 3.84 d of age (**Table 2.1**). Of the 52 heifers bred, 7 were bred based on observed heat on -1 d relative to scheduled TAI and 45 were bred based according to TAI protocol. Heifers initially clustered into two categories based

on pregnancy outcome of first insemination at 30 d: pregnant (PS30, n = 28) and not pregnant (NP30, n = 24). Two PS heifers lost pregnancy between 30 d and 84 d. Heifers were clustered into three groups based on insemination outcome at 84 d: pregnant (PS84, n = 26) pregnancy loss (PL84, n = 2), and not pregnant (NP84, n = 24).

2.4.2 *Serum Progesterone*

I analyzed difference in serum P4 concentration based on insemination outcome on d 30 and d 84, time, and their interaction. For both d 30 and d 84 insemination outcomes, NP heifers had reduced serum P4 concentrations than PS heifers on d -3 and 18 – 30, while PL84 heifers were not different from PS84 or NP84 heifers (**Figure 2.1**). For insemination outcome at 30 d and 84 d, a mixed ANOVA with repeated measures determined a difference based on insemination outcome ($P < 0.001$), day ($P < 0.001$) and insemination outcome × day ($P \leq 0.008$). A one way post hoc ANOVA determined a difference in serum P4 concentration on d -3 and 18-30 due to insemination outcome on d 84 ($P \leq 0.022$).

2.4.3 *Corpus Luteum Volume*

I analyzed the difference in CL volume based on insemination outcome on d 30 and d 84, time, and their interaction. A mixed ANOVA with repeated measures found CL volume was different based on insemination outcome at 30 d ($P < 0.001$) and day ($P = 0.008$), but not their interaction ($P = 0.694$). Pairwise comparisons based on insemination outcome at 30 d found CL volume was greater in PS30 heifer than NP30 heifers on d 15 – 30 [($P \leq 0.033$) (**Figure 2.2A**)]. Corpus luteum volume was different based on insemination outcome at 84 d ($P < 0.001$) and day ($P = 0.009$), but not their interaction ($P = 0.938$). Post-hoc ANOVA at each time point determined CL volume was different based on insemination outcome on d 21 and 30 ($P \leq 0.001$). Pairwise comparisons based on insemination outcome at 84 d determined CL volume was greater

in PS84 heifers than NP84 heifers on d 21 and 30 [($P \leq 0.004$) (**Figure 2.2B**)]. Corpus luteum volume was not different between PL84 heifers and NP84 or PS84 heifers at any time point ($P \geq 0.156$).

2.4.4 *Single Measurement Phenotypes*

Logistic regression models with the main effects of insemination outcome at 30 d or 84 d analyzed differences in phenotypes measured once. Dominant follicle diameter, uterine pH, GDPR PTA, and flush glucose concentration did not differ based insemination outcome at 30 d or 84 d [($P \geq 0.65$) (**Table 2.2**)].

2.5 DISCUSSION

The current study aimed to determine which phenotypes were different in virgin Holstein heifers based on first insemination outcome at 30 d and 84 d. I found that circulating P4 concentration differed based on insemination outcome, with P4 on -3 d before insemination and 18 – 30 d after insemination greater in PS30 and PS84 heifers than NP30 and NP84 heifers. Corpus luteum volume was also different based on insemination outcomes. Other phenotypes, like dominant follicle diameter, uterine pH, GDPR PTA, and uterine fluid glucose concentration were not different based on insemination outcome. Overall, traits related to pregnancy maintenance were different based on insemination outcomes.

Factors related to ovarian-derived endocrine profiles may improve reproductive performance faster than current fertility traits. For example, researchers utilizing a GWAS in Holstein heifers found anti-Müllerian hormone, which regulates ovarian follicle growth, had an estimated genomic heritability of 0.36 (Nawaz et al., 2018). This has a greater heritability than traits traditionally used for selection, like calving to first service interval [(h = 0.05) (Pryce and Veerkamp, 2001)]. While heritability was not estimated, a GWAS found 44 single nucleotide

polymorphisms associated with normal or atypical progesterone profiles in lactating cows (Nyman et al., 2019). Progesterone profiles and production might be linked to the genome as well. I observed an increase in progesterone on -3 d in PS30 and PS84 heifers compared to NP30 and NP84 heifers. One possible explanation of this is that progesterone regulates ovarian follicular waves and follicle dominance (Martins et al., 2018). In lactating cows, decreased circulating progesterone allowed multiple follicles to become dominant and ovulate, but fewer successful pregnancies (Lopez et al., 2005; Martins et al., 2018). A similar mechanism could be occurring in heifers in this experiment, but we did not measure follicular waves or follicle growth. Future research estimating progesterone production heritability and examining progesterone's influence on heifer follicle dominance is needed.

Evidence in non-Holstein breeds of dairy cattle have indicated heifer fertility is a different trait than primiparous or multiparous cow fertility. Heritabilities for interval from first service to conception, number of inseminations per conception, and nonreturn rate at 56 d after first service were lower in virgin Brown Swiss heifers (h2 = 0.017, 0.020, and 0.016, respectively) than primiparous (h2 = 0.039, 0.046, and 0.017, respectively) or multiparous cows [(h2 = 0.029, 0.045, and 0.026, respectively) (Tiezzi et al., 2012)]. Differences in fertility traits between heifers and cows indicate that heifer fertility may need to be a separate trait used in predicting reproductive performance. Heifer conception rate is measured in the United States and used in the Net Merit index, but it has a low heritability. Only recently have loci associated with heifer conception rate been identified and validated in Holstein cattle, which could mean it could be improved with genomic selection (Kiser et al., 2019). My results did not find differences in GDPR based on first insemination outcomes in virgin heifers, but there may be an association with a genomically predicted heifer conception rate. Current models to predict fertility and

insemination outcome use traits like parity, days in milk, milk yield, body condition score, number of breeding services, and health events (Rutten et al., 2016). Models using milk P4 and endocrine related traits like commencement of luteal activity have been successful in predicting insemination outcomes and more accurate than previous models in lactating cows (Friggens et al., 2005; Tenghe et al., 2016). Incorporation of traits like serum P4 concentration and CL activity into heifer fertility prediction models could aid in early selection for lifetime fertility.

Other factors not measured in this study could be indicative of heifer fertility and future reproductive performance. Reproductive tract scoring is indicative of fertility and pregnancy loss in beef heifers (Holm et al., 2015). Similar to BCS, reproductive tract scoring is subjective and tracts are scored on a five-point scale based on ovarian structures and size of uterine horns, with one being immature and 5 being mature and cycling (Anderson et al., 1991). Tracts were scored via transrectal palpation and ultrasonography (Holm et al., 2016). A similar scoring system has been developed in dairy cattle and cows that had smaller uterine diameter and volume 72 h before insemination are more likely to become pregnant (Baez et al., 2016; Young et al., 2017). Tract scores of dairy heifers have been measured through transrectal palpation and did predict pubertal status (Stevenson, 2008). Uterine horn length, uterine volume, and tract scores could indicate dairy heifer reproductive performance. Another potential indicator of dairy heifer fertility is biomarkers in the blood. Genes associated with hormonal feedback in the gonadal-hypothalamic-pituitary axis were differentially expressed by white blood cells in beef heifer that did or did not become pregnant (Dickinson et al., 2018). Metabolite proinflammatory markers had increased concentration in blood plasma of infertile beef heifers compared to fertile heifers (Phillips et al., 2018). A similar relationship might exist between the white blood cell

transcriptome or plasma metabolites and insemination outcome in dairy heifers, but research confirming or refuting this is needed.

Method of flush collection and storage before freezing could affect glucose concentrations. According to previous literature, uterine fluid glucose concertation typically ranges from 3.78 to 4.54 m*M* (Hugentobler et al., 2008). This is greater than the uterine flush glucose concentration I observed, which ranged from 0.16 to 0.35 m*M*. However, the previous study collected uterine fluid thought surgical procedures over a period of 3 h, while I used a saline flush to collect uterine fluid. This act of flushing could have influenced the glucose concentration I observed. Additionally, my flush samples were placed on ice before transportation to the laboratory for slow freezing at -80°C without addition of a bacteriostatic agent. Bacteria in the flush could have catabolized some glucose in the flush before freezing without a bacteriostat to prevent bacterial growth. Future research should either snap freeze flush samples with liquid nitrogen once collected or add a bacteriostat in order to prevent metabolite loss before freezing.

Elements of heifer reproductive management, like synchronization protocol, could have influenced insemination outcome and traits used for genetic selection. I used Double Ovsync in my experiment to increase the chance of heifers' ovaries being similar and to reduce potential variation due to reproductive management. The Virginia Tech Dairy Complex used a 5-d controlled internal drug release (**CIDR**) Cosync protocol to breed heifers before and after our experiment (**Supplemental Table S2.2**). They had slightly lower 1st service conception rates, but this may be primarily due to my heifers being older and having more time to experience a full estrus. Heifer breeding protocols vary in the United States, but use of certain ovulation synchronization protocols improved reproductive performance and economically beneficial when

compared to breeding based on observed heat (Silva et al., 2015). Heifers bred using the OvSync protocol tend to have poorer pregnancy rates (35.1%) than heifers given PGF2α and bred using estrus detection (74.4%) or even lactating cows [(37.8%) (Pursley et al., 1997)]. In contract, heifers bred using the Double Ovsync protocol had greater pregnancy rates (65.2%) than lactating cows [(37.5%) (Souza et al., 2008)]. Protocols using CIDR devices are also used in dairy and beef heifer reproductive management. Protocols using CIDR typically require fewer injections and less time managing heifers than OvSync, but they have slightly lower pregnancy rates than heifers bred based on heat [(63% and 70%, respectively) (Colazo and Mapletoft, 2017)]. The heifer breeding strategy chosen and compliance with reproductive management could influence traits like number of inseminations per conception or 56 d non-return rate. This makes heifer fertility difficult to predict through genetic selection. Future research examining response to ovulation synchronization protocol based on genotype could help account for variation in reproductive management when developing traits for future genomic selection.

2.6 CONCLUSION

Overall, phenotypes related to pregnancy maintenance, like P4 profile and CL volume, were different in virgin heifers based on first insemination outcome. I hypothesized that heifers that have high progesterone concentrations, greater uterine glucose concentrations, and positive GDPR PTA would be more likely to have a successful pregnancy with first insemination, but only progesterone concentration had an effect on insemination outcome. Dominant follicle diameter, uterine pH, GDPR PTA, and uterine glucose concentrations were not predictive of first insemination outcome. My results support previous literature indicating heifer fertility may be a different trait than cow fertility. Further research examining heritability of traits with less ties to reproductive management may lead to improved genomic selection for heifer fertility.

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2.8 TABLES

Table 2.1 Descriptive statistics for virgin Holstein heifers (n = 52) at -3 d relative to insemination. Values are presented as least square means ± standard error of the mean

	Mean	SEM	Minimum	Maximum
Body weight on d -3, kg	354	3.87	319	444
Age on d -3, d	394	3.87	335	462
Body condition score on d -3[1]	3.00	0.04	2.5	3.5

[1]Body condition score was assessed on a scale from 1 (lean) to 5 (obese) at 0.25 increments

Table 2.2 Results from logistic regression analysis for the influence of virgin Holstien heifer phenotypes related to fertility on insemination outcomes at 30 d[1] anf 84 d[2]. Values are presented as least square means ± standard error of the mean

Dependent variable	Uterine pH at d -3	Follicle diameter at d -3, mm	GDPR PTA	Uterine fluid glucose at d -3, m*M*[4]
Insemination outcome, 30 d				
NP30	7.15 ± 0.06	11 ± 0.57	0.69 ± 0.31	0.16 ± 0.05
PS30	7.05 ± 0.05	11 ± 0.52	0.54 ± 0.26	0.13 ± 0.06
P-value[3]	0.97	0.65	0.96	0.62
Insemination outcome, 84 d				
NP84	7.15 ± 0.06	11 ± 0.57	0.69 ± 0.31	0.16 ± 0.05
PS84	7.04 ± 0.05	12 ± 0.54	0.64 ± 0.27	0.09 ± 0.06
PL84	7.10 ± 0.27	10 ± 1.92	-0.35 ± 0.83	0.35 ± 0.14
P-value[3]	0.97	0.69	0.97	0.76

[1]Insemination outcome groups at d 30: not pregnant (NP30, n = 24) and pregnant (PS30, n = 28).
[2]Insemination outcome groups at d 84: not pregnant (NP84, n = 24), pregnant but lost before 84 d of gestation (PL84, n = 2), pregnant and successfully kept through 84 d of gestation (PS84, n = 26).
[3]A *P*-value ≤ 0.05 was considered significant.
[4]Subset of heifers were chosen based on insemination outcome and balanced for season: NP30 = 16, PS30 = 12; NP84 = 16, PL84 = 2, PS84 =10.

2.9 FIGURES

Figure 2.1. Serum progesterone concentration in virgin Holstein heifers before and after insemination based on **A**) insemination outcome at 30 d: not pregnant (NP30, n = 24) and pregnant (PS30, n = 28) and **B**) insemination outcome at 84 d: not pregnant (NP84, n = 24), pregnancy lost (PL84, n = 2), and pregnancy successful through 84 d (PS84, n = 26). Concentration is represented by least square means ± standard error of the mean. A Bonferroni corrected *P*-value ≤ 0.05 was considered significant (denoted by *). For insemination outcome at 30 d, $P \leq 0.05$ is denoted by *, $P \leq 0.01$ is denoted by **, and $P \leq 0.001$ is denoted by a ***. For insemination outcome at 84 d, difference in progesterone at one time point is denoted by a and b above bars. Progesterone was greater in PS30 heifers than NP30 heifers and greater in PS84 than NP84 heifers on d -3 and from d 18 to 30 relative to insemination.

Figure 2.2 Corpus luteum (CL) volume in Holstein heifers on 15, 21, and 30 d after first insemination. Heifers are clustered based on **A**) insemination outcome at 30 d: not pregnant (NP30, n = 24) and pregnant (PS30, n = 28) and **B**) insemination outcome at 84 d: not pregnant (NP84, n = 24), pregnancy lost (PL84, n = 2), and pregnancy successful through 84 d (PS84, n = 26). Volume is represented by least square means ± standard error of the mean. A Bonferroni corrected *P*-value ≤ 0.05 was considered significant (denoted by *). For insemination outcome at 30 d, $P \leq 0.05$ is denoted by *, $P \leq 0.01$ is denoted by **, and $P \leq 0.001$ is denoted by a ***. For insemination outcome at 84 d, difference in CL volume at one time point is denoted by a and b above bars. Corpus luteum volume was greater in PS30 heifers than NP30 heifers on d 15, 21, and 30 and was greater in PS84 heifers than NP84 heifers on d 21 and 30.

Figure 2. 1

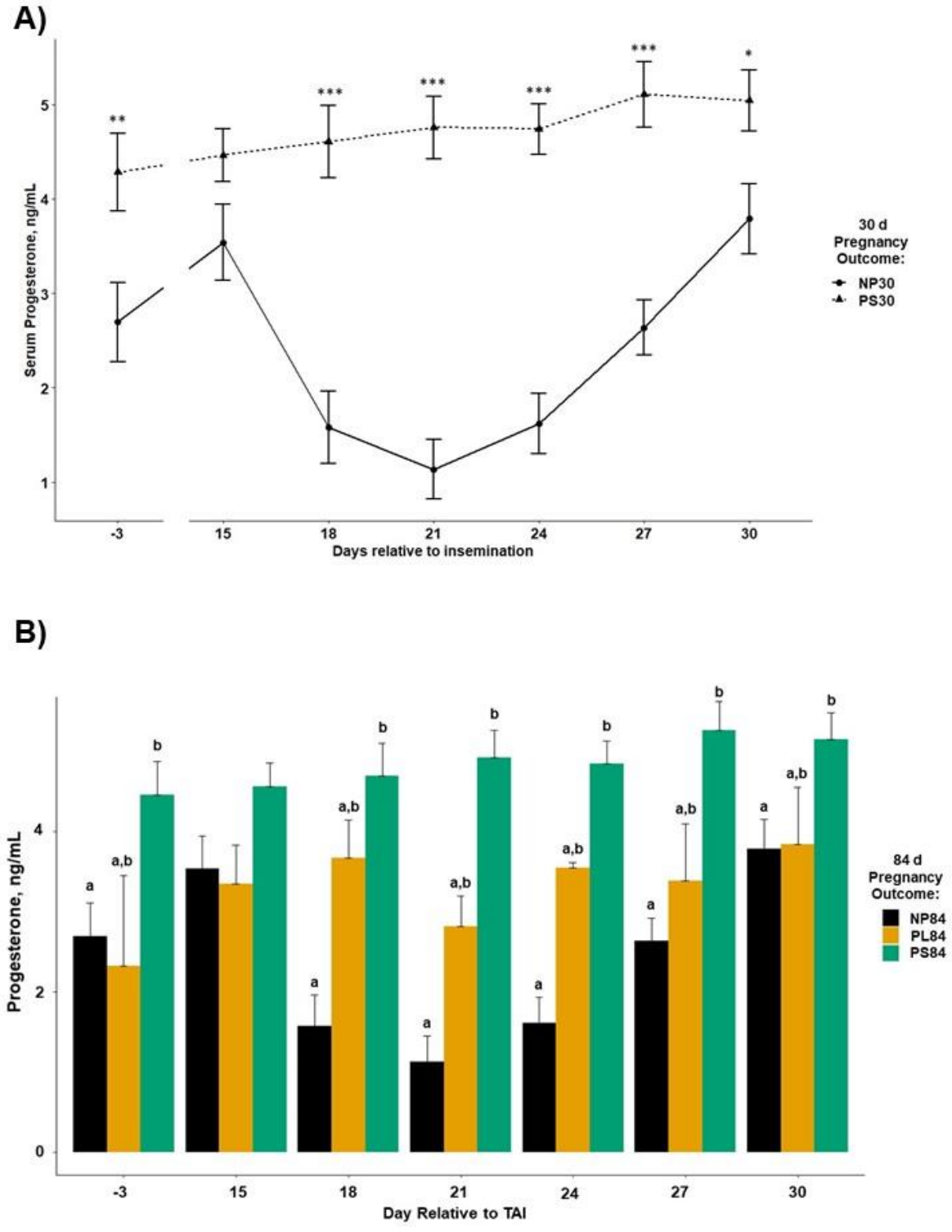
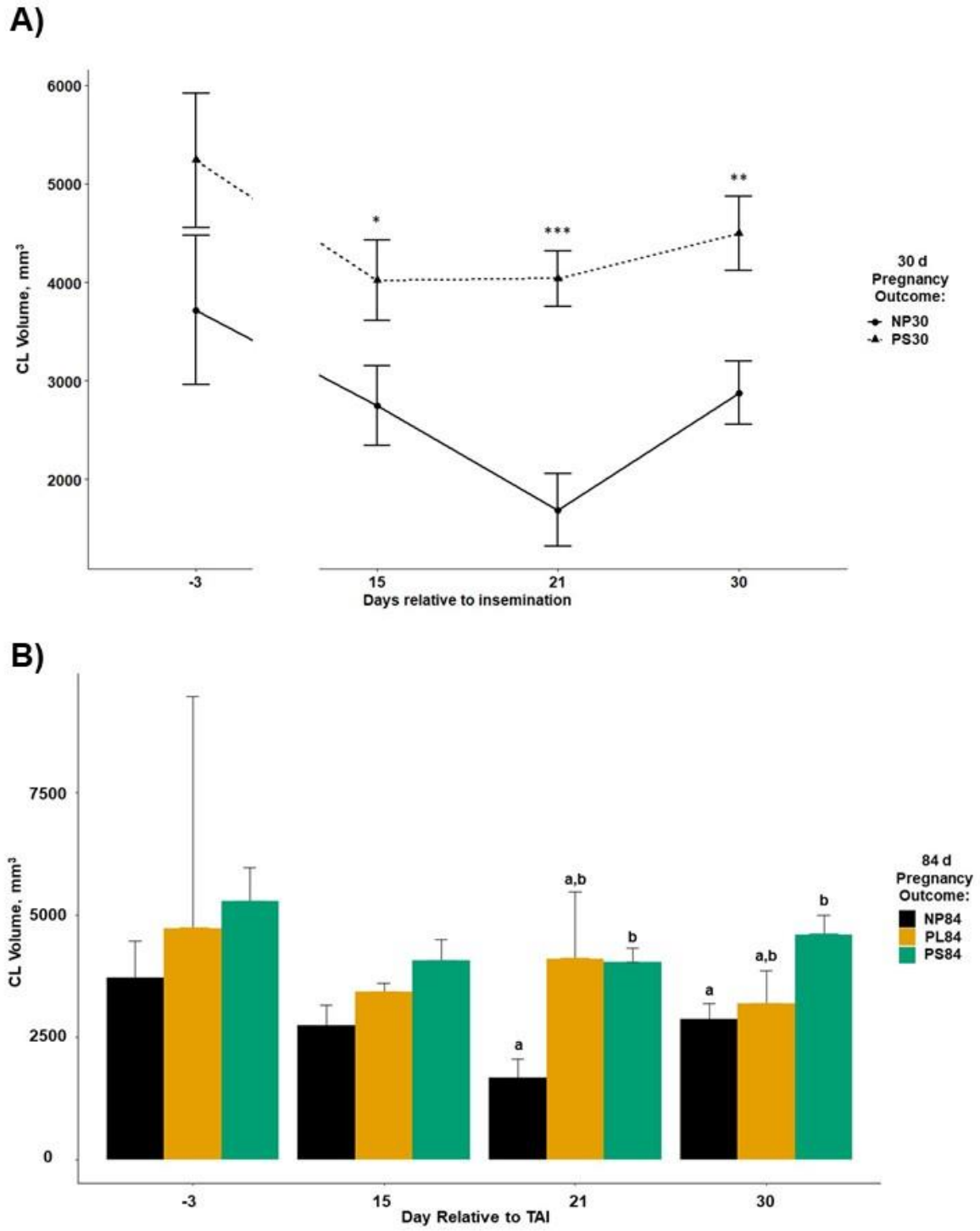

A)
Serum Progesterone, ng/mL
Days relative to insemination
30 d Pregnancy Outcome:
NP30
PS30
B)
Progesterone, ng/mL
Day Relative to TAI
84 d Pregnancy Outcome:
NP84
PL84
PS84

Figure 2. 2


A)
6000
5000
4000
3000
2000
CL Volume, mm³
*
***
**
-3
15
21
30
Days relative to insemination
30 d
Pregnancy
Outcome:
NP30
PS30
B)
7500
5000
2500
0
CL Volume, mm³
a
a,b
b
a
a,b
b
-3
15
21
30
Day Relative to TAI
84 d
Pregnancy
Outcome:
NP84
PL84
PS84

2.10 SUPPLEMENTAL INFORMATION

Supplemental Table S2.1 Mixed ration fed to virgin heifers while they were also on pasture throughout the duration of the experiment.

Component	Dry Matter, %	kgs per animal, as fed
Corn silage	30.00	6.80
Western alfalfa	90.00	1.76
Heifer concentrate mix[1]	89.32	1.52

[1]Purchased from Exchange Milling Company (Rocky Mount, VA)

Supplemental Table S2.2 Summary of heifer reproductive performance within the Virginia Tech dairy herd before, during, and after the experiment. Summary statistics were collected from the Heifer Tracker function in PCDART (DRMS, Raleigh, NC) and are presented as averages over the stated period.

Effect	**Before Experiment**[1]	**During Experiment**[2]	**After Experiment**[1]
Age at 1st service, d	371	423	363
Age at conception, d	409	456	403
Projected calving age, mo	22.7	24.2	22.5
1st service conception rate, %	40	46	37

[1]Heifers were placed on 5 d Cosync protocol (100 μg gonadotropin releasing hormone (GnRH; Factrel, Zoetis) injected intramuscularly at the neck and controlled internal drug release (CIDR) inserted on -8 d relatie to insemination. CIDR removed on d -3 and 25 mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Lutalyse, Zoetis, Parsippany-Troy Hills, NJ) injected intramuscularly at the neck. GnRH injected and heifer bred on d 0.)

[2]Heifers were placed on Double Ovsync protocol (100 μg of GnRH were administered intramuscularly at the neck on -27 d, -17 d, -10 d, and -16 h relative to insemination. Approximately 25 mg of PGF2α was administered intramuscularly at the neck on -20 d and -3 d relative to insemination.)

CHAPTER 3: Virgin Holstein heifer uterine microbiome before insemination varies with insemination outcome and season

3.1 ABSTRACT

Poor reproductive performance in dairy heifers can lead to economic loss due to increased days open, increased number of inseminations, and increased time before a heifer begins lactation. While some factors like climate or hormonal imbalance are known to affect fertility, the influence of the uterine microbiome on fertility has yet to be studied in dairy heifers. Therefore, the objectives of this study were to characterize the uterine microbiome of virgin Holstein heifers before first insemination and associate microbial profiles with variations in progesterone, fetal growth, uterine pH, outcome of insemination, and season. Over the course of one-year, virgin Holstein heifers (n = 52) were placed on a Double Ovsync synchronization protocol. On d -3 relative to timed artificial insemination (TAI), the uterus of heifers was flushed with 240 mL of sterile 0.9% saline solution and pH of flush was measured. Blood was collected from coccygeal vessels on d -3, 15, 18, 21, 24, 27, and 30 relative to TAI and serum was isolated to measure progesterone concentration. Transrectal ultrasound was used to check pregnancy status on d 30, 42, 56, 70, and 84 relative to TAI. Heifers were initially clustered based on outcome of insemination on d 30: not pregnant (NP30, n = 24), or pregnant (PS30, n = 28). Heifers were also clustered based on insemination outcome on d 84: not pregnant (NP84, n = 24), pregnant but lost before d 84 (PL84, n = 2), and successfully pregnant through d 84 (PS84, n = 26). Crown rump length was measured on d 30, 42, and 56 relative to TAI and daily fetal growth rate was calculated. A subset of heifers (n = 28) was selected for 16S sequencing based on insemination outcome and balanced for month of flushing (NP30 = 16, PS30 = 12; NP84 = 16, PS84 = 10, PL84 = 2). Total DNA was extracted from uterine flush and was used to created

16S rDNA amplicon libraries for the V4 region. Amplicons underwent 250 paired-end sequencing via Illumina NovaSeq 6000. Sequenced reads were filtered using a Phred score ≥ 30 and chimeric reads were removed. Filtered reads were clustered into operational taxonomic units using a 97% similarity and taxonomy was assigned using the SSURNA Silva reference version 132. Alpha diversity was measured using the Chao1 index and Phylogenetic diversity. Beta diversity was measured using unweighted and weighted Unifrac distances. Differential abundance analyses at the phylum and genus ranks were performed based on the main effects of insemination outcome on d 30 or d 84 and season. Pearson correlations were performed between taxa relative abundance and uterine pH, fetal growth rate, and -3 d circulating progesterone concentration. Chao1 species richness was lower in PL84 heifers than NP84 or PS84 heifers and lower in Spring heifers compared to other seasons. Beta diversity was different based on insemination outcome on d 30, d 84, and season. *Ureaplasma* and *Ruminococcus* had an increased abundance in PS30 and PS84 heifers than NP30 and NP84 heifers, while *Afipia* and *Gardnerella* had an increased abundance in NP30 and NP84 heifers than PS30 and PS84 heifers. *Prevotella* and *Ruminococcus* had a lower abundance in Summer bred heifers than Winter bred heifers. Proteobacteria had a moderate negative correlation with -3 d progesterone (r_p = -0.42) and Actinobacteria had a moderate negative correlation with fetal growth rate (r_p = -0.66). Overall, there was a difference in the uterine microbiome of virgin Holstein heifers before insemination based on insemination outcomes and season. The uterine microbiome could be used to predict reproductive performance in dairy heifers, but further research investigating variations due to reproductive management is needed.

Keywords: heifer, uterine microbiome, pregnancy

3.2 INTRODUCTION

Dairy cows with poor reproductive performance (increased days open, number of services, and calving interval) cost an additional $261 per cow per year when compared those with superior reproductive performance (Inchaisri et al., 2010). Producers incur this economic loss due to additional inseminations, increased days open, and decreased milk production (De Vries, 2006). Failure to conceive can be caused by a multitude of factors, including heat stress, bacterial infections, or hormonal imbalance. While strategies have been developed to mitigate heat stress, prevent or treat bacterial infections, and manage cows to restore a hormonal balance during early lactation, these come at additional costs. Additionally, even with these strategies in place, 21 d pregnancy rates can still vary from 18 – 32 % (Lima et al., 2009; Ferguson and Skidmore, 2013). This brings into question what additional factors may influence reproductive performance and if these factors affect those already known to cause pregnancy. Thoroughly understanding all factors that influence fertility will lead to the development of methods that prevent future reproductive failure.

Some factors that influence reproductive performance include hormonal imbalance, environmental conditions, and bacterial dysbiosis. Progesterone (**P4**) produced by the corpus luteum (**CL**) is responsible for pregnany maintenance and aids in ovarian follicular recruitment and development (Wiltbank et al., 2014). Decreased P4 concentrations during the estrous cycles surrounding estrus have been associated with decreased embryo survival rates (Disken et al., 2011; Stronge et al., 2005). Number and size of luteal cells in the CL and metabolism of circulating P4 by the liver drive circulating P4 concentration (Diaz et al., 2002; Wiltbank et al, 2006). Lower metabolic demand in heifers compared to lactating cows, and therefore slower metabolism of circulating P4, partially explains why heifers are more likely to become pregnant than cows and lose fewer pregnancies. However, heat stress influences the metabolism of both

cows and heifers, increasing respiratory rates, decreasing basal glucose levels, decreasing embryo size and birth weights (Biggers et al., 1987; Collier et al., 1982; Itoh et al., 1998; Wheelock et al., 2010). Heat stress occurs in the summer months, but it can occur during the spring months and residual effects are seen up to two months post-heat stress (Wolfensen et al., 1997). Poor reproductive performance is more likely to occur in the summer months (García-Ispierto et al., 2006; Zobel et al., 2013). Post-partum heat stress also is associated with an increased incidence of metritis, or bacterial infection of the uterus (DuBois and Williams, 1980; Gautam et al., 2010). Metritis increases days open by 16 d, costs $162.30/case, and increases likelihood of pregnancy loss in subsequent breedings (Mahnani et al, 2015; Mercadante et al., 2016). While influence of these factors on pregnancy loss has been examined, causes of poor reproductive performance are still not fully understood and other elements may play a role in maintaining pregnancy.

One underlying mechanism that may influence fertility is the interrelationship between the bovine uterus and the uterine microbiome. While previously thought of as sterile before parturition, the upper reproductive tract contains a resident microbiome before first breeding (Moore et al., 2017). In humans, changes in the uterine microbiome have been associated with pregnancy outcomes; increased *Lactobacillus* was associated with pregnancy success (Moreno et al., 2016). The uterine microbiome of multiparous beef cows before insemination was also associated with pregnancy outcomes, with *Corynebacterium*, *Staphylococcus*, and *Prevotella* having increased abundance in cows that did not become pregnant compared to those that did (Ault et al., 2019a). *Bacteroides*, *Ureaplasma*, and *Fusobacterium* had a greater abundance in the uterus of multiparous dairy cows at 35 days in milk in cows that were not pregnant by 200 days in milk than those that were pregnant (Machado et al., 2012). However, the influence of the

uterine microbiome on pregnancy has yet to be studied. It is possible that the dispersal of commensal and pathogenic bacterial profiles within the uterine microenvironment could influence pregnancy recognition in the dairy heifer. Heifer circulating P4 concentration influences uterine fluid energy metabolite profile and aids in conceptus elongation and fetal growth (Clemente et al., 2009; Simintiras et al., 2019). While the effect of uterine pH on the microbiome has not been studied, human vaginal pH influences vaginal microbiome composition (Boris and Barbés, 2000). The uterine microbiome may be related to phenotypes like circulating P4, fetal growth, or uterine pH. Reproductive function and uterine environment are also altered during periods of heat stress (Geisert et al., 1988; Kobayashi et al., 2013). Seasons with a greater occurrence of heat stress could alter the uterine microbiome as well. Understanding how the uterine microbiome intersects with reproductive function, fertility-related phenotypes, and season of breeding could lead to its use as a tool to improve fertility in dairy cows and reduce pregnancy loss. Therefore, the objectives of this study were to characterize the uterine microbiome of virgin Holstein heifers before first insemination and associate variations in the microbiome with variations in P4, fetal growth, uterine pH, and outcome of insemination. I hypothesized that increases in phyla like Bacteroidetes and Proteobacteria within the uterus would be associated with a decrease in P4, uterine pH, and fetal growth rate and ultimately, negative pregnancy outcomes.

3.3 MATERIALS AND METHODS

3.3.1 *Animal Use and Sample Collection*

Animal procedures were approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (protocol #18-114-DASC). To reduce underlying environmental and physiological influences on the uterine microbiome, virgin

Holstein heifers (n = 52) were enrolled in the study once they reached 322 kg and 335 d of age over the course of one year in accordance with Virginia Tech's Dairy Complex breeding requirements. Heifers were enrolled in 12 periods (1 period/month) at a maximum of 10 heifers/period. Heifers were placed on a Double OvSynch protocol -27 d relative to timed artificial insemination (**TAI**). We used the Double OvSync protocol to avoid potentially altering the microbiome with a controlled internal drug release (**CIDR**) while also effectively synchronizing heifers (Souza et al., 2008). Briefly, 100 μg of GnRH (Factrel, Zoetis, Parsippany-Troy Hills, NJ) were administered intramuscularly at the neck on -27 d, -17 d, -10 d, and -16 h relative to insemination. Approximately 25 mg of $PGF_{2\alpha}$ (Lutalyse, Zoetis, Parsippany-Troy Hills, NJ) was administered intramuscularly at the neck on -20 d and -3 d relative to TAI. On -3 d, an estrotect alert patch (Rockway Inc., Spring Valley, WI) was placed on heifers after $PGF_{2\alpha}$ to monitor signs of estrus. Patches were inspected -1 d before TAI. Heifers with patches > 50% colored in the morning were bred in the afternoon of -1 d. Otherwise, heifers were administered the last GnRH injection and bred according to TAI protocol. Heifers were bred using conventional semen from one of five Holstein bulls selected for high fertility by one of two breeding technicians.

3.3.2 *Uterine Flushing and Blood Collection*

Immediately before $PGF_{2\alpha}$ injection on -3 d, heifers were brought into a chute for uterine flushing. Body weights were recorded and body condition score (**BCS**) was assigned using the average from the same two individuals for all heifers. Body condition score ranged from 1 to 5 on a 0.25-point scale, with 1 being extremely lean and 5 being obese. Vulva, interior of the vestibule, and surrounding area were disinfected using a 2% chlorhexidine gluconate solution (Aspen, Liberty, MO). A sterile, vortech silicone catheter (14 - 16 Fr and 23" long; Agtech Inc.,

Manhattan, KS) was maneuvered just past the cervix using a sterile, stainless-steel stylet (Agtech Inc., Manhattan, KS). Once the catheter was just past the cervix, the balloon was inflated using 10 cc of air. Approximately 240 mL of sterile 0.9% saline solution was used to flush the uterus in 60 mL increments. Return flush solution was collected from catheters using 60 mL catheter-tip syringes (Becton, Dickinson, and Company, Franklin Lakes, NJ) and transferred to sterile, 50 mL conical tubes (Fisher Scientific, Waltham, MA). Return flush volume was measured and tubes were immediately placed on ice for transport to the laboratory. In the laboratory, 10 mL flush aliquots were made using sterile 15 mL conical tubes (Fisher Scientific, Waltham, MA) and stored at -80 °C. Flush pH was measured using 5 mL of flush on a Fisher Science Education Laboratory Benchtop pH meter (Fisher Scientific, Waltham, MA). One heifer could not have pH measured due to insufficient flush return for both microbiome analysis and pH measurement.

On -3, 15, 18, 21, 24, 27, and 30 d relative to TAI, 10 mL of blood were collected from coccygeal vessels using Monoject collection tubes with no additive (Covidien, Dublin, Ireland) for serum isolation. Blood was allowed to clot at room temperature for 4 h before being centrifuged at 2,000 × G for 15 min at 4 °C. Serum was isolated and stored at -20 °C. Serum P4 concentration was measured in duplicate using a commercially available chemiluminescence assay (Immulite 2000 XPi Immunoassay System, Siemens Healthcare, CA, USA)..

3.3.3 *Ultrasound Measurements and Insemination Outcome Designation*

On d -3, 15, 21, and 30 relative to TAI, measurements of ovarian follicles and CL were collected using a transrectal ultrasound (IBEX PRO; E. I. Medical Imaging, Loveland, CO). Vertical and horizontal diameter measurements of follicles and CL were collected at the widest cross section. Vertical and horizontal diameters of inner CL pocket, if present, were collected at the widest cross section. Pregnancy status was checked on 30 d relative to TAI. Heifers were

clustered into two categories based on outcome of first insemination on d 30: not pregnant (**NP30**, n = 24) or pregnant (pregnancy successful, **PS30**, n = 28). No further measurements were collected from not pregnant heifers. If heifers were pregnant, pregnancy status was assessed on 42, 56, 70, and 84 d relative to TAI. If a heifer lost pregnancy before 84 d, measurements were collected on day los was observed and no further measurements wer collected from the heifer. Crown-rump length was measured on 30, 42, and 56 d relative to TAI using a transrectal ultrasound (IBEX PRO; E. I. Medical Imaging, Loveland, CO). Crown-rump length was measured from the crown of the skull of the fetus to the tailhead of thefetus. Daily fetal growth rate (mm/d) was calculated using crown-rump length measurements. Two heifers lost pregnancy between 30 d and 84 d. Heifers were clustered into three categories based on insemination outcome on d 84: not pregnant (**NP84**, n = 24), pregnant at d 30 but lost before d 84 (**PL84**, n = 2), and successfully pregnant through d 84 (**PS84**, n = 26).

3.3.4 *DNA Extraction and Sequencing*

A subset of heifers (n = 28) were selected based on breeding outcome (PS30 = 12, NP30 = 16; PS84 = 10, PL84 = 2, NP84 = 16). Heifers in insemination outcome groups were selected from the same periods to account for month of flushing. Heifers selected for sequencing were also clustered by season (**spring** = 3, **summer** = 12, **fall** = 8, **winter** = 5). Approximately 10 mL of uterine flush were pelleted via centrifugation at 4,500 × *g* for 30 min at 4 °C. Pellet was then transferred to a sterile, 2 mL microcentrifuge tube and centrifuged again at 18,000 × *g* for 30 min at 4 °C. Then, DNA was extracted using QIAGEN DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. Extracted DNA were incubated with RNase A (Promega, Madison, WI) for 30 min at 37 °C to remove RNA contamination.

Extracted DNA quantity and quality were estimated using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) before submission to Novogene (Novogene Co. Ltd., Beijing, China) for sequencing with 260/280 ranging from 1.8 to 2.0. At Novogene, DNA quality and quantity were validated using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA) before library construction. Amplicon libraries of the V4 region of 16S rDNA (Primers: 515F - GTGCCAGCMGCCGCGGTAA, 806R – GGACTACHVGGGTWTCTAAT) were created. Amplicons underwent 250 paired-end sequencing via Illumina NovaSeq 6000 (Illumina, San Diego, CA).

3.3.5 *Bioinformatics Analysis*

Bioinformatics analyses were performed in CLC Genomics workbench version 20.0.1 and Microbial Genomics Module version 20.1 (QIAGEN, Hilden, Germany). Raw sequencing reads were filtered using a Phred score ≥ 30 and chimeric reads were removed. Filtered reads were clustered into operational taxonomic units (**OTU**) using a 97% read similarity. Clustered reads were assigned taxonomy using the Small Sub-unit rRNA (SSUrRNA) database version 132 from Silva (Quast et al., 2013; Yilmaz et al., 2014) with a 0.8 threshold at each taxonomic rank. Operational taxonomic units with a combined read count < 10 across all samples were removed before further analyses.

A phylogenetic tree was constructed between identified OTU using multiple sequence comparison by log expectation (**MUSCLE**) version 3.8.31 (Edgar, 2004). Alpha diversity, bacterial diversity within a sample, was calculated using the Chao1 index (Chao, 1984) for bacterial richness and phylogenetic diversity using the phylogenetic tree constructed with MUSCLE. Differences in alpha diversity measures were analyzed at the rarefaction level of 11,695 using a Kruskal-Wallis H test across groups for the main effects of insemination outcome

at 30 d, insemination outcome at 84 d, and season. A Mann-Whitney U test for pairs of groups was also performed when analyzing insemination outcome at 84 d and season. A *P*-value ≤ 0.05 was considered significant.

Beta diversity, bacterial diversity between samples, was calculated using unweighted and weighted Unifrac distances (Luzopone and Knight, 2005). Beta diversity was displayed using a principle coordinate analysis. Difference in bacterial community structure based on insemination outcome at 30 d or 84 d and season was analyzed using a nonparametric multivariate ANOVA (Anderson, 2001) for each beta diversity measure. A *P*-value ≤ 0.05 was considered significant.

Differences in taxa at the taxonomic ranks phylum and genus were analyzed using a differential abundance analysis for the main effects of insemination outcome on d 30 or d 84 and season. A separate generalized linear model was constructed for each taxon and it was assumed taxa abundances followed a negative binomial distribution. A likelihood-ratio test was used for across group comparisons (McCarthy et al. 2012). A false discovery rate of 0.05 was assumed and a corrected *P*-value ≤ 0.05 was considered significant. A Wald test was used for pairwise comparisons (Chen et al., 2011). A Bonferroni corrected *P* – value ≤ 0.05 was considered significant.

3.3.6 *Correlation between Microbiome and Phenotypes*

Pearson correlations were performed between phylum relative abundance and uterine pH, fetal growth rate, and -3 d P4 concentration using cor.test function from the stats package in R version 3.6.1 (R Core Team, 2019). Pearson correlations were also performed between relative abundance of genera identified to vary based on insemination outcome and uterine pH, fetal growth rate, and -3 d P4 concentration using cor.test function from the stats package in R version

3.6.1 (R Core Team, 2019). Correlations with a *P*-value ≤ 0.05 were considered significant and correlations with a *P*-value between 0.05 and 0.10 were considered tendencies.

3.4 RESULTS

3.4.1 *Descriptive Statistics*

At breeding, heifers were 354 ± 3.90 kg and 394 ± 3.84 d old (**Table 3.1**). Of the 52 heifers bred, 7 were bred based on observed heat on -1 d relative to scheduled TAI and 45 were bred based according to TAI protocol. Heifers were clustered into two categories based on insemination outcome of first insemination on d 30: not pregnant (NP30, n = 24), and pregnant (PS30, n = 28). Heifers were clustered into three categories based on insemination outcome on d 84: not pregnant (NP84, n = 24), pregnant on d 30 and lost before d 84 (PL84, n = 2), and successfully pregnant through d 84 (PS84, n = 26).

3.4.2 *Uterine Microbiome*

An average of 72,063 ± 2,385 raw reads and 60,941 ± 2,375 filtered reads were sequenced across all samples (**Table 3.2**). A total of 8,158 OTU were identified across all samples (**Figure 3.1A-B, Figure 3.2A**). Firmicutes was the dominant phylum in all groups based on insemination outcome at 30 d and 84 d [(NP30 = 38.51%, PS30 = 37.62%; NP84 = 38.51%, PL84 = 41.96%, PS84 = 36.50%) (**Figure 3.1C**)]. Firmicutes was also the dominant phylum in heifers based on season [(spring = 35.42%, summer = 43.01%, fall – 31.96%, winter = 39.79%) (**Figure 3.2B**)]. The genus with the greatest abundance varied based on insemination outcome and season. *Bacteroides* was the most abundant in NP30 and NP84 heifers (NP30 = 3.89%, PS30 = 3.35%; NP84 = 3.89%, PL84 = 6.32%, PS84 = 2.59%), *Ruminococcaceae* uncultured genus-level group (**UCG**)-005 was the most abundant in PL84 heifers (NP84 = 3.02%, PL94 = 8.98%, PS84 = 3.00%), and *Ureaplasma* was the most abundant in PS30 and PS84 heifers (NP30 =

0.09%, PS30 = 5.43%; NP84 = 0.09%, PL84 = 0.00%, PS84 = 7.31%) (**Supplemental Table S3.1, S3.2**)]. *Ureaplasma* was most abundant in spring heifers (spring = 14.77%, summer = 0.92%, fall = 0.16%, winter = 0.04%), *Ruminococcaceae* UCG-010 was the most abundant in summer and winter heifers (spring = 3.30%, summer = 5.00%, fall = 2.76%, winter = 3.05%), and *Muribaculaceae* uncultured bacterium-042 was the most abundant in Fall heifers [(spring = 6.59%, summer = 2.03%, fall = 7.55%, winter = 1.77%) (**Supplemental Table S3.3**)].

Alpha diversity was measured using the Chao1 index and phylogenetic diversity. Chao1 diversity or phylogenetic diversity were not different based on insemination outcome at 30 d [($P \geq 0.600$) (**Figure 3.3A**, **3.3C**)]. Chao1 diversity was different based on insemination outcome at 84 d ($P = 0.050$), but phylogenetic diversity was not [($P = 0.100$) (**Figure 3.3B**, **3.3D**)]. Beta diversity was measured using weighted and unweighted Unifrac distances. Unweighted Unifrac distances were different based on insemination outcome at 30 d and 84 d ($P \leq 0.001$) (**Figure 3.4A, 3.4C**)]. Weighted Unifrac distances were not different based on insemination outcome at 30 d or 84 d [($P \geq 0.531$) (**Figure 3.4B, 3.4D**)]. Chao1 and phylogenetic diversity were different based on season [($P < 0.050$) (**Figure 3.5A-B**)]. Weighted Unifrac distances were different based on season ($P = 0.040$), but unweighted Unifrac distances were not [($P = 0.557$) (**Figure 3.5C-D**)].

A differential abundance analysis was performed at the taxonomic ranks phylum and genus based on insemination outcome at d 30 or d 84 and season. Bacteroidetes were more abundant in PS30 and PS84 heifers than NP30 and NP84 heifers, respectively [($P \leq 0.037$) (**Table 3.3, Table 3.4**)]. Tenericutes were more abundant in spring and summer heifers than fall heifers ($P \leq 0.025$), while Bacteroidetes were more abundant in fall heifers than summer heifers [($P = 0.031$) (**Table 3.5**)]. Of the 1,749 genera identified, 526 had a different abundance based

on insemination outcome at d 30, 524 had a different abundance based on insemination outcome at d 84, and 334 had a different abundance based on season (**Supplemental Tables S3.1-S3.3**). *Ureaplasma* had an increased abundance in PS30 compared to NP30 heifers and an increased abundance on PS84 heifers compared to PL84 and NP84 heifers [($P < 0.001$) (**Table 3.3, Table 3.4**)]. *Afipia* and *Gardnerella* had an increased abundance in NP30 and NP84 heifers compared to PS30 and PS84 heifers [($P < 0.001$) (**Table 3.3, Table 3.4**)]. *Ureaplasma* increased in abundance in spring heifers compared to fall and winter heifers ($P = 0.018$), *Mycoplasma* increased in abundance in summer heifers compared to fall heifers ($P = 0.034$), and *Ruminococcus* increased in abundance in winter heifers compared to summer heifers [($P < 0.001$) (**Table 3.5**)].

3.4.3 *Phenotypic Correlations*

Pearson correlations were performed between either phyla relative abundance or select genera relative abundance and -3 d P4 concentration, uterine pH, or fetal growth rate. There was a moderate negative correlation between Proteobacteria relative abundance and -3 d P4 concentration [($r_p = -0.424$, $P = 0.028$) (**Figure 3.6A**)]. No other taxa were significantly correlated with -3 d P4 concentration ($P \geq 0.116$) and no taxa were correlated with uterine pH ($P \geq 0.113$). Actinobacteria and Thermomicrobia had moderate negative correlations with fetal growth rate [($r_p = -0.658$, $P = 0.020$ and $r_p = -0.632$, $P = 0.028$, respectively) (**Figure 3.6B-C**)]. No other taxa were correlated with fetal growth rate ($P \geq 0.058$).

3.5 DISCUSSION

The objectives of this experiment were to characterize the uterine microbiome of virgin Holstein heifers before first insemination and associate variations in the microbiome with insemination outcomes, season of breeding, P4 concentrations, uterine pH, and fetal growth rate.

I found that the uterine microbiome before insemination was different based on outcome of insemination and season. Firmicutes, Proteobacteria, and Bacteroidetes were the dominant phyla across all heifers. Microbial community structure varied based on insemination outcome, but bacterial richness and phylogenetic diversity did not. Proteobacteria were negatively correlated with P4 concentration and Actinobacteria were negatively correlated with fetal growth rate. Overall, taxa within the uterine microbiome could be used to predict heifer fertility.

To my knowledge, this is the first paper examining the virgin Holstein heifer uterine microbiome in relation to fertility. While limited, there has been some research on the uterine microbiome in multiparous cows and its relationship to pregnancy. Ault and colleagues (2019a) found that at -2 d before insemination, the phyla Firmicutes, Bacteroidetes, and Proteobacteria dominated the uterine microbiome of multiparous beef cows. They also found that microbial community structure varies between pregnant and not pregnant beef cows when using unweighted Unifrac distances (Ault et al., 2019b). My research confirms similar results in virgin Holstein heifers. However, they found Actinobacteria, Alcaligenaceae, and *Corynebacteria* had an increased abundance in beef cows that did not become pregnant compared to those that did (Ault et al., 2019a). I did not observe these differences based on insemination outcome in dairy heifers, which could indicate a potential influence of parity or breed on the microbiome. Previous research on the uterine microbiome in nulliparous Holstein heifers has only evaluated the effects of using a CIDR with *in vitro* culture techniques and characterization with 16S rDNA amplicon sequencing of endometrial biopsies (Fischer-Tenhagen et al., 2012; Moore et al., 2017). My results and those from previous virgin heifer experiments all support the hypothesis that the uterus is not sterile in heifers at age of first breeding, challenging the previous dogma that the upper reproductive tract is sterile until after first parturition. However, sequencing results have

all been DNA-based, indicating that bacteria were there at one point, but not necessarily if they were still alive or how they were interacting with the reproductive tract. I can only use my results to speculate how bacteria were influencing insemination outcome. Future research will require bacterial RNA-based sequencing, or metatranscriptomic, approaches to evaluate host-microbiome interactions in the bovine reproductive tract before insemination.

Establishing the existence of a uterine microbiome in virgin heifers raises the question of what routes bacteria use to reach the uterus. The easiest route may be through direct transmission from the lower reproductive tract, where bacteria ascend from the vagina, through the cervix, and into the uterus. The vaginal microbiome is well characterized in cattle and has been associated with pregnancy status in beef heifers (Laguardia-Nascimento et al., 2015; Deng et al., 2019). While high concentrations of immunoglobulin A, cytokines, and antimicrobial peptides in cervical mucus prevent uterine infection, it is still possible vaginal bacteria could evade these to reach the uterus (Tsiligianni et al., 2003; Sheehan et al., 2006). I did not characterize the vaginal microbiome of these heifers, but elucidating the relationship between vaginal and uterine microbiomes could identify bacteria that may ascend the reproductive tract. Another route of transmission is through the blood, or the hematogenous route. Uterine pathogens, such as *Fusobacterium* and *Bartonella*, have demonstrated the ability to travel from the oral cavity to the placenta in mice and from the gastrointestinal tract to the uterus in dairy cows (Fardini et al., 2010; Jeon et al., 2017). Uterine microbial community structure is also more similar to the fecal microbiome than the vaginal microbiome in dairy cows (Jeon et al., 2017). I identified *Ruminococcus* and *Methylobacterium*, bacteria commonly found in the gastrointestinal tract, in the virgin heifer uterus. These bacteria may utilize the hematogenous route to reach the uterus,

but further research examining the microbiomes of feces, blood, rumen, and uterus in heifers concurrently is needed to support or refute this.

Bacteria that had an increased abundance in not pregnant heifers compared to successfully pregnant heifers might be damaging the reproductive tract. The genera *Afipia* and *Gardnerella* had a greater abundance in NP30 and NP84 heifers than in PS30 and PS84 heifers. *Afipia*, a genus within the family Bradyrhizobiaceae, is an opportunistic pathogen closely related to *Brucella* and *Bartonella* (Brenner et al., 1991; Moreno, 2002). *Brucella* spp. are known to cause abortions in ruminants by invading embryonic trophoblast cells and delaying production of proinflammatory cytokines (Carvalho Neta et al., 2008). Antibodies for *Brucella* spp. are cross-reactive with *Afipia clevelandensis*, indicating immune response by the cow to *Afipia* in the uterus may be similar to that of *Brucella* (Drancourt et al., 1997). *Bartonella* spp. are intracellular pathogens that travel through the blood in cattle and have been identified in the blood alongside other uterine pathogens shortly after calving (Gutiérrez et al., 2014; Jeon et al., 2017). While *Bartonella* was not associated with abortion or embryonic loss, its close phylogenetic relationship with *Afipia* could mean *Afipia* might reach the uterus hematogenously (Maillard et al., 2006). *Gardnerella*, bacteria typically found in the lower reproductive tract, have demonstrated the ability to damage ciliated cells in the bovine oviduct *in vitro* (Taylor-Robinson and Boustouller, 2011). Increased abundance of *Gardnerella* in the human uterus before *in vitro* fertilization was associated with not achieving pregnancy or having a miscarriage (Moreno et al., 2016). It is possible that once in the upper reproductive tract, *Afipia* and *Gardnerella* damage tissue and modulate immune response. Their increased abundance around insemination could decrease the likelihood of successful pregnancy. Research further examining the relationship

with these bacteria and heifer immune response could elucidate mechanisms of host-microbe interaction that lead to negative reproductive performance.

Increased abundance of certain bacteria in PS30 and PS84 heifers than NP30 and NP84 heifers could be preventing dysbiosis by pathogenic bacteria. Bacteroidetes and *Ureaplasma* had a greater abundance in PS heifers than NP heifers at both d 30 and d 84, but this contradicts previous literature on these taxa in the reproductive tract. Increases in Bacteroidetes and *Ureaplasma* in the vagina and uterus have been associated with an increased incidence of endometritis in lactating cows (Bicalho et al., 2017; Miranda-CasoLuengo et al., 2019). *Ureaplasma diversum* has been identified as a pathogen in dairy cattle, causing vulvitis, abortion, and infertility (Silva et al., 2016; Waites et al., 2005). However, our sequencing results were not able to identify the specific *Ureaplasma* species. In humans, various *Ureaplasma* species reside in the urogenital tract without causing infection (Juhász et al., 2011). The same could be said for the species we observed in heifers, but further classification is needed. Another taxon that had increased abundance in PS heifers than NP heifers was *Ruminococcus*. These have been previously detected throughout the reproductive tract and were negatively correlated with metritis pathogens (Jeon et al., 2015). One potential reason for the relationship between *Ruminococcus* and a "healthy" uterus is that they may be able to outcompete pathogenic bacteria. Instead of interacting directly with the uterus to aid in pregnancy success, *Ruminococcus* and even potentially *Ureaplasma* might utilize available metabolites more efficiently than pathogens, preventing dysbiosis and infection. Future research will need to elucidate if certain commensal uterine bacteria have a metabolic preference and if that can be utilized to prevent overgrowth of pathogenic bacteria.

Uterine microbiomes of heifers that lost pregnancy could be used to hypothesize which bacteria cause loss in heifers. I observed a greater abundance of Kiritimatiellaeota in PL84 heifers than NP84 heifers. Kiritimatiellaota is relatively new, established as a unique phylum in 2015 after initially grouped with Verrucomicrobia (Spring et al., 2015). They are halophilic and can catabolize complex glycoproteins to utilize for glycolysis (Spring et al., 2016). Bovine pregnancy-associated glycoproteins (**PAGs**) are glycoproteins exclusively produced by binucleate cells of the placenta (Wooding et al., 2005). The function of PAGs is not established, but due to their localization at the site of fetal-maternal placental attachment, they might aid in binding at this site as well as forming an immunological barrier (Wooding et al., 2005). It is possible that Kiritimatiellaeota catabolize PAGs, inhibiting placental attachment and causing pregnancy loss. I also observed a moderate negative correlation between Actinobacteria and fetal growth rate, which could be due to interference with placental attachment. Research examining the relationship between the microbiome and PAGs is needed to elucidate potential mechanisms related to fetal growth and loss. Even though the saline solution was sterilized and UV irradiated to degrade residual bacterial DNA before flushing, Kiritimatiellaeota is halophilic and their presence during sequencing could indicate they are a saline flush contaminant. Further research examining a greater number of pregnancy loss heifers and the microbiome potential sources of contamination could define bacteria exclusive to the uterus that cause loss.

More than half of pregnancy losses occur during summer months or periods of heat stress (García-Ispierto et al., 2006; Zobel et al., 2013). Uterine bacteria that vary based on season could be indicative of climate response or thermoregulatory ability. The uterus of dairy cattle is directly affected by heat stress, decreasing total uterine protein production and calcium content, increasing prostaglandin E_2 production, and potentially decreasing transport of the embryo

through the oviduct (Geisert et al., 1988; Kobayashi et al., 2013). Cows will activate their thermoregulatory system when heat-stressed to mitigate this response, but some cows can thermoregulate more efficiently than others depending on breed or coat type (Dikmen et al., 2008; Riley et al., 2012). Changes in the uterine microenvironment and changes in thermoregulatory ability could influence uterine microbiome composition. I observed an increase in *Anaerococcus* and *Gardnerella* and a decrease in *Prevotella* in Summer heifers compared to Winter heifers, all of which have been associated with vaginal and uterine disease in postpartum cows (Taylor-Robinson and Boustouller, 2011; Jeon and Galvao, 2018; Pascottini et al., 2020). Changes in these taxa might be indicative of how well heifers can cope with heat stress and prevent uterine dysbiosis. Further research examining uterine microbial composition within each season in relation to vaginal temperature, respiration rate, or other factors related to thermoregulation could provide insight on how to utilize the uterine microbiome to reflect cow physiology.

Some additional factors not controlled in this study may also influence the uterine microbiome. One assumption made in this study is the microbiome would not change greatly from -3 d to day of TAI, with -3 d giving the best indication of the microbiome during insemination without influencing its outcome. Previous research examining the uterine microbiome in multiparous beef cows indicated a difference in dominant phyla on d -21, -9, and -2 before insemination (Ault et al., 2019a). This indicates the uterine microbiome does change over time and the microbiome we observed on -3 d may not have been the same as that on day of insemination. Between -3 d and insemination, luteolysis occurs and circulating P4 decreases, allowing ovulation to occur. We observed a negative correlation between P4 and Proteobacteria, so it is possible abundance of this phylum increased during that time, altering microbial

composition. Additionally, synchronization method and insemination practices could influence the microbiome. While CIDR Co-synch is commonly used as a method of heifer ovulation synchronization, presence of a CIDR increases the abundance of metritis and vaginosis pathogens in the reproductive tract (Fischer-Tenhagen et al., 2012). The Double OvSync protocol was used to avoid potentially altering the microbiome while also effectively synchronizing heifers (Souza et al., 2008). Breeding technicians could unknowingly be introducing foreign bacteria into the uterus during AI if equipment is not handled carefully. While only two technicians were used to reduce potential environmental bacterial exposure, I did not examine the microbiome of insemination equipment. Research investigating the influence of synchronization protocol, act of insemination, and stage of the estrus cycle on the uterine microbiome is needed to evaluate their potential effects.

3.6 CONCLUSION

The objectives of this experiment were to characterize the virgin heifer uterine microbiome and identify relationships between the microbiome and first insemination outcome, season, and fertility-related phenotypes. Overall, the uterine microbiome in virgin Holstein heifers before insemination was different based on outcome of insemination and season. Difference in microbial community structure based on insemination outcome is primarily due to taxa that comprise a smaller portion of the microbiome, while difference based on season is due to taxa that comprise a larger portion of the microbiome. Increased abundance of *Afipia* and *Gardnerella* in NP30 and NP84 heifers could indicate damage to the reproductive tract, while the increased abundance of *Ureaplasma* and *Ruminococcus* in PS30 and PS84 heifers could indicate their ability to outcompete pathogenic bacteria. Changes in the microbiome based on season could indicate a heifer's thermoregulatory ability. Future research examining influence of stage

of estrous cycle, synchronization protocol, act of insemination, and uterine microenvironment on the microbiome could elucidate the extent of host-microbe interactions.

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3.8 TABLES

Table 3.1. Descriptive statistics for virgin Holstein heifers (n = 52) before first insemination.

	Mean	SEM	Minimum	Maximum
Weight at breeding, kg	354	3.90	319	445
Age at breeding, d	394	3.85	335	462
Body Condition Score	3.00	0.04	2.50	3.50
Uterine pH	7.13	0.03	6.58	7.73

Table 3.2. Results from 250 bp paired end sequencing of V4 region of 16S rDNA amplicons and operational taxonomic unit (OTU) clustering derived from uterine flush of virgin Holstein heifers (n = 28) based on insemination outcome at d 84.[1] Sequencing was performed on the Illumina Novoseq 6000 platform. Values are presented as least square means ± standard error of the mean.

	Raw Reads	Filtered Reads	Reads in OTU
NP84	71,138 ± 3,298	59,648 ± 3,418	30,344 ± 2,013
PL84	78,715 ± 7,324	70,463 ± 9,566	40,179 ± 8,650
PS84	72,213 ± 4,058	61,107 ± 3,449	28,832 ± 1,168

[1]Samples are separated based on outcome of first insemination at 84 d: not pregnant (NP84, n = 16), pregnant but lost before 84 d (PL84, n = 2), and successfully pregnant through 84 d (PS84, n = 10).

Table 3.3. Differential abundance analyses at the ranks phylum and genus within the uterine microbiome of virgin Holstein heifers were performed based on insemination outcome at d 30.[1]

	NP30, %[2] (n = 16)	**PS30, %**[2] (n = 12)	***P*-value**[3]
Phylum			
Bacteroidetes	16.84 ± 2.45	22.33 ± 5.12	0.006
Genus			
Ureaplasma	0.09 ± 0.03	5.44 ± 5.33	< 0.001
Lactobacillus	3.10 ± 0.67	0.95 ± 0.25	0.004
Xanthobacteraceae (Unknown Genus)	2.87 ± 2.02	0.00 ± 0.00	< 0.001
Afipia	1.95 ± 1.32	0.13 ± 0.06	< 0.001
Ruminococcus	0.41 ± 0.07	1.35 ± 0.86	0.001
Anaerococcus	1.08 ± 0.85	0.12 ± 0.06	0.001
Gardnerella	1.04 ± 0.87	0.03 ± 0.02	< 0.001

[1]Insemination outcome groups at d 30: not pregnant (NP30, n = 16) or pregnant (PS30, n = 12).
[2]Taxa abundance is represented as average relative abundance of taxa ± standard error of the mean. Phyla and genera that were different based on insemination outcome and had a relative abundance ≥ 1.00% are presented.
[3]A false discovery rate of 5% was assumed and a false discovery rate corrected *P*-value ≤ 0.05 was considered significant.

Table 3.4. Differential abundance analyses at the ranks phylum and genus within the uterine microbiome of virgin Holstein heifers were performed based on insemination outcome at 84 d.[1]

	NP84, %[2] (n = 16)	**PL84, %**[2] (n = 2)	**PS84, %**[2] (n = 10)	***P*-value**[3]
Phylum				
Bacteroidetes	16.84 ± 2.45[a]	21.06 ± 14.51[a,b]	27.27 ± 5.80[b]	0.017
Kiritimatiellaeota	0.40 ± 0.19[a]	2.53 ± 1.74[b]	0.28 ± 0.07[a,b]	0.001
Genus				
Ureaplasma	0.09 ± 0.02[a]	0.00 ± 0.00[a]	6.84 ± 6.37[b]	< 0.001
Alloprevotella	0.21 ± 0.03	2.91 ± 2.10	0.11 ± 0.03	< 0.001
Lactobacillus	3.10 ± 0.67	0.41 ± 0.26	1.09 ± 0.29	0.011
Methylobacterium	1.56 ± 0.64	0.06 ± 0.04	3.08 ± 1.42	0.047
Xanthobacteraceae (Unknown Genus)	2.87 ± 0.67[a]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	< 0.001
Kiritimatiellae WCHB1-41	0.24 ± 0.13	1.70 ± 1.15	0.16 ± 0.04	0.006
Romboutsia	0.56 ± 0.11	1.63 ± 0.86	0.24 ± 0.05	0.011
Afipia	1.95 ± 1.32[a]	0.12 ± 0.22[a,b]	0.18 ± 0.06[b]	0.001
Prevotellaceae UCG-004	0.35 ± 0.08	1.54 ± 1.07	0.52 ± 0.22	0.042
Clostridiales (Unknown Family)	0.02 ± 0.01[a]	1.40 ± 1.01 [b]	0.03 ± 0.02[a,b]	< 0.001
Ruminococcus	0.41 ± 0.07[a]	0.46 ± 0.19 [a,b]	1.58 ± 1.04[b]	0.001
Prevotella	0.21 ± 0.04	0.07 ± 0.05	1.18 ± 0.87	0.008
Anaerococcus	1.07 ± 0.85	0.00 ± 0.00	0.15 ± 0.07	0.002
Gardnerella	1.04 ± 0.87[a]	0.00 ± 0.00 [a,b]	0.03 ± 0.02[b]	< 0.001
Succinivibrionaceae UCG-002	0.05 ± 0.03[a]	0.00 ± 0.00 [a,b]	1.05 ± 0.87[b]	< 0.001

[1]Insemination outcome groups at d 84: not pregnant (NP84, n = 16), pregnant but lost before 84 d (PL84, n = 2) or successfully pregnant through 84 d (PS84, n = 10).
[2]Taxa abundance is represented as mean relative abundance of taxa ± standard error of the mean. Phyla and genera that were different based on insemination outcome and had a relative abundance ≥ 1.00% are presented .
[3]False discovery rate corrected *P*-values from across group differential abundance analysis. A false discovery rate of 5% was assumed and $P \leq 0.05$ were considered significant
[a,b]Superscripts indicate a difference for pairwise comparisons between groups. A Bonferroni corrected *P*-value ≤ 0.05 was considered significant

Table 3.5. Differential abundance analyses at the ranks phylum and genus within the uterine microbiome of virgin Holstein heifers were performed based on season of breeding.

	Spring, %[1] (n = 3)	**Summer, %**[1] (n = 12)	**Fall, %**[1] (n = 8)	**Winter, %**[1] (n = 5)	***P***[2]
Phylum					
Bacteroidetes	29.51 ± 10.57^{a,b}	12.14 ± 2.34^{a}	22.53 ± 7.18^{b}	15.50 ± 4.05^{a,b}	0.020
Tenericutes	15.40 ± 21.30^{a}	8.37 ± 4.36^{a}	1.11 ± 0.25^{b}	2.33 ± 1.20^{a,b}	0.004
Cyanobacteria	2.32 ± 2.13	0.67 ± 0.16	0.28 ± 0.88	0.46 ± 0.17	0.037
Chloroflexi	0.11 ± 0.07^{a}	0.82 ± 0.23^{a,b}	0.89 ± 0.28^{a,b}	2.40 ± 2.24^{b}	0.005
Kiritimatiellaeota	1.67 ± 1.20^{a}	0.21 ± 0.07^{b}	0.13 ± 0.37^{a,b}	0.21 ± 0.10^{a,b}	0.004
Genus					
Ureaplasma	14.77 ± 21.38^{a}	1.25 ± 0.90^{a,b}	0.20 ± 0.08^{b}	0.04 ± 0.03^{b}	< 0.001
Xanthobacteraceae (Unknown Genus)	0.00 ± 0.00^{a}	0.76 ± 0.60^{a,b}	7.70 ± 4.04^{b}	0.33 ± 0.20^{a,b}	0.004
Methylobacterium	0.08 ± 0.03	1.98 ± 0.95	3.82 ± 1.48	0.48 ± 0.14	0.027
Mycoplasma	0.08 ± 0.02^{a,b}	6.00 ± 4.45^{a}	0.52 ± 0.19^{b}	1.54 ± 1.22^{a,b}	0.003
Afipia	0.02 ± 0.01^{a}	0.50 ± 0.21^{a,b,c}	5.14 ± 2.66^{b}	0.23 ± 0.09^{a,c}	< 0.001
Ruminococcus	0.18 ± 0.09^{a,b}	0.42 ± 0.05^{a}	0.39 ± 0.11^{a,b}	2.88 ± 2.02^{b}	<0.001
Alloprevotella	1.90 ± 1.43	0.14 ± 0.03	0.20 ± 0.06	0.13 ± 0.04	0.001
Prevotella	0.06 ± 0.03^{a,b}	0.22 ± 0.05^{a}	0.30 ± 0.08^{a,b}	2.02 ± 1.75^{b}	< 0.001
Succinivibrionaceae UCG-002	0.01 ± 0.01^{a}	0.05 ± 0.03^{a}	0.13 ± 0.03^{a,b}	1.91 ± 1.75^{b}	< 0.001
Anaerococcus	0.01 ± 0.00^{a}	2.22 ± 1.13^{b}	0.15 ± 0.07^{a,b}	0.13 ± 0.06^{a,b}	< 0.001
Gardnerella	0.00 ± 0.00^{a}	2.18 ± 1.15^{b}	0.06 ± 0.02^{a,b}	0.05 ± 0.02^{a}	< 0.001
Kiritimatiellae WCHB1-41 (Unknown Family)	1.11 ± 0.81	0.12 ± 0.03	0.09 ± 0.25	0.12 ± 0.06	0.016

[1]Taxa abundance is represented as mean relative abundance of taxa ± SEM. Phyla and genera that were different based on season and had a relative abundance ≥ 1.00% in at least one group are presented.

[2]False discovery rate corrected *P*-values from across group differential abundance analysis. A false discovery rate of 5% was assumed and $P \leq 0.05$ were considered significant.

[a-c]Superscripts indicate a difference for pairwise comparisons between groups. A Bonferroni corrected *P*-value ≤ 0.05 was considered significant.

3.9 FIGURES

Figure 3.1 A) Venn diagram of the operational taxonomic units (OTU) among insemination outcome group at d 30: not pregnant (NP30, n = 16), or pregnant (PS30, n =12). A total of 8,158 OTU were identified across all samples, with 2,766 OTU shared between insemination outcome groups. **B)** Venn diagram of OTU among insemination outcome group at d 84: not pregnant (NP84, n = 16), pregnant but lost before d 84 (PL84, n =2), and successfully pregnant through d 84 (PS30, n =10). A total of 8,158 OTU were identified across all samples, with 1,038 OTU shared between insemination outcome groups. **C**) Relative abundance of phyla within the virgin Holstein heifer uterine microbiome -3 d relative to first insemination based on insemination outcome on d 30 or d 84. Firmicutes was the most abundant phyla in all heifers (NP30 = 38.51%, PS30 = 37.62%; NP84 = 38.51%, PL84 = 41.96%, PS84 = 36.50%), followed by Bacteroidetes (NP30 = 16.84 %, PS30 = 22.33%; NP84 = 16.84%, PL84 = 27.27%, PS84 = 21.06%) and Proteobacteria (NP30 = 24.46%, PS30 = 19.11%; NP84 = 24.46%, PL84 = 15.24%, PS84 = 20.11%).

Figure 3.2 A) Venn diagram of the operational taxonomic units (OTU) among heifers based on season: spring (n = 3), summer (n = 12), fall (n = 8), and winter (n = 5). A total of 8,158 OTU were identified across all samples, with 1,713 OTU shared between seasons. **B)** Relative abundance of phyla within the virgin Holstein heifer uterine microbiome -3 d relative to first insemination based on season. Firmicutes was the dominant phylum in all seasons (spring = 35.42%, summer = 43.01%, fall = 31.96%, winter = 37.79%), followed by Bacteroidetes (spring = 29.51%, summer = 16.01%, fall = 20.92%, winter = 15.50%), Proteobacteria (spring = 5.38%, summer = 20.45%, fall = 30.27%, winter = 24.88%), and Tenericutes (spring = 15.40%, summer = 6.19%, fall = 1.22%, winter = 2.33%).

Figure 3.3 Alpha diversity, or microbial diversity within each sample, of virgin Holstein heifer uterine microbiome -3 d relative to first insemination. Heifers were separated into groups based on insemination outcome at d 30 [not pregnant (NP, n = 16) and pregnant (PS, n = 12)] or d 84 [not pregnant (NP84, n = 16), pregnant but lost before d 84 (PL84, n = 2), and successfully pregnant through d 84 (PS84, n = 10)]. **A**) Bacterial richness was evaluated using the Chao1 index. **B**) Species diversity was measured using phylogenetic diversity. Difference in alpha diversity measures was analyzed using Kruskel-Wallace H test. There was not difference in alpha diversity measures based on insemination outcome at d 30, but there was a decrease in bacterial richness in PL84 heifers compared to NP84 and PS84 heifers.

Figure 3.4. Beta diversity, or microbial diversity between samples, of the virgin Holstein heifer uterine microbiome -3 d relative to first insemination. Heifers were separated into groups based on insemination outcome at d 30 [not pregnant (NP, n = 16) and pregnant (PS, n = 12)] or d 84 [not pregnant (NP84, n = 16), pregnant but lost before d 84 (PL84, n = 2), and successfully pregnant through d 84 (PS84, n = 10)]. Beta diversity was measured using **A**) unweighted and **B**) weighted Unifrac distances. Difference in beta diversity was analyzed using a permutational multivariate ANOVA based on insemination outcome at d 30 and d 84. There was a difference in unweighted Unifrac distance based on insemination outcome at both time points, but not weighted Unifrac distances, indicating taxa with lower abundance are responsible for differences in microbial community structure.

Figure 3.5 Alpha and beta diversity of the virgin Holstein heifer uterine microbiome -3 d relative to first insemination. Heifers were separated into groups based on season: spring (n = 3), summer (n = 12), fall (n = 8), and winter (n = 5). Alpha diversity, microbial diversity within samples, was measured using **A)** the Chao1 index and **B)** phylogenetic diversity. There was lower bacterial

richness and phylogenetic diversity in spring heifers compared to other seasons. Beta diversity, microbial diversity between samples, was measured using **C**) unweighted and **D**) weighted Unifrac distances. There was not a difference in unweighted Unifrac distances based on season, but there was a difference in weighted Unifrac distances.

Figure 3.6 Pearson correlations between taxa relative abundance in the uterine microbiome of virgin Holstein heifers and **A)** serum progesterone concentration -3 d before insemination or **B,C**) fetal growth rate. Proteobacteria were negatively correlated with serum progesterone concentration. Actinobacteria and Thermomicrobia were negatively correlated with fetal growth rate.

Figure 3. 1

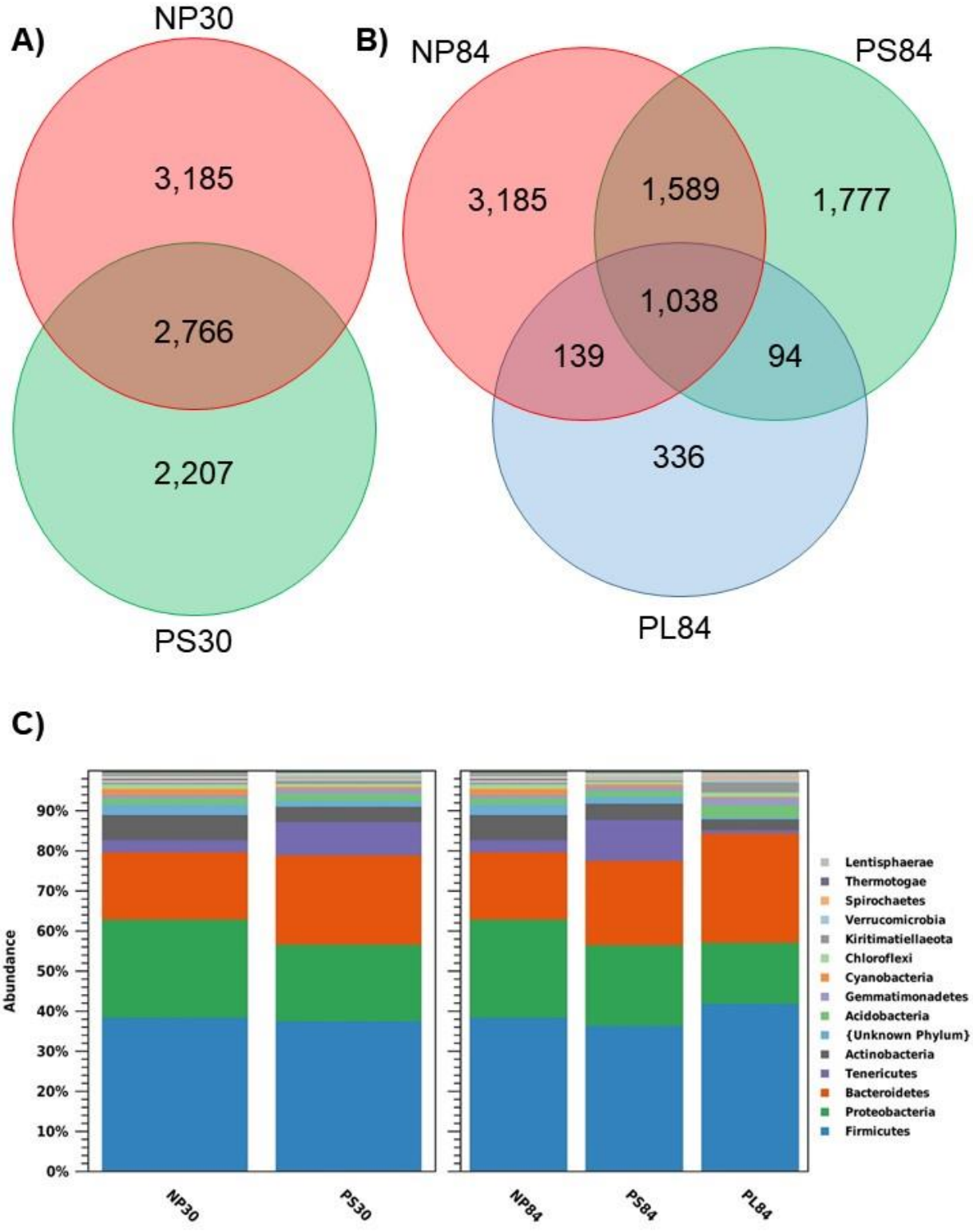

A)
NP30
3,185
2,766
2,207
PS30
B)
NP84
PS84
3,185
1,589
1,777
1,038
139
94
336
PL84
C)
Abundance
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
NP30
PS30
NP84
PS84
PL84
Lentisphaerae
Thermotogae
Spirochaetes
Verrucomicrobia
Kiritimatiellaeota
Chloroflexi
Cyanobacteria
Gemmatimonadetes
Acidobacteria
{Unknown Phylum}
Actinobacteria
Tenericutes
Bacteroidetes
Proteobacteria
Firmicutes

Figure 3. 2

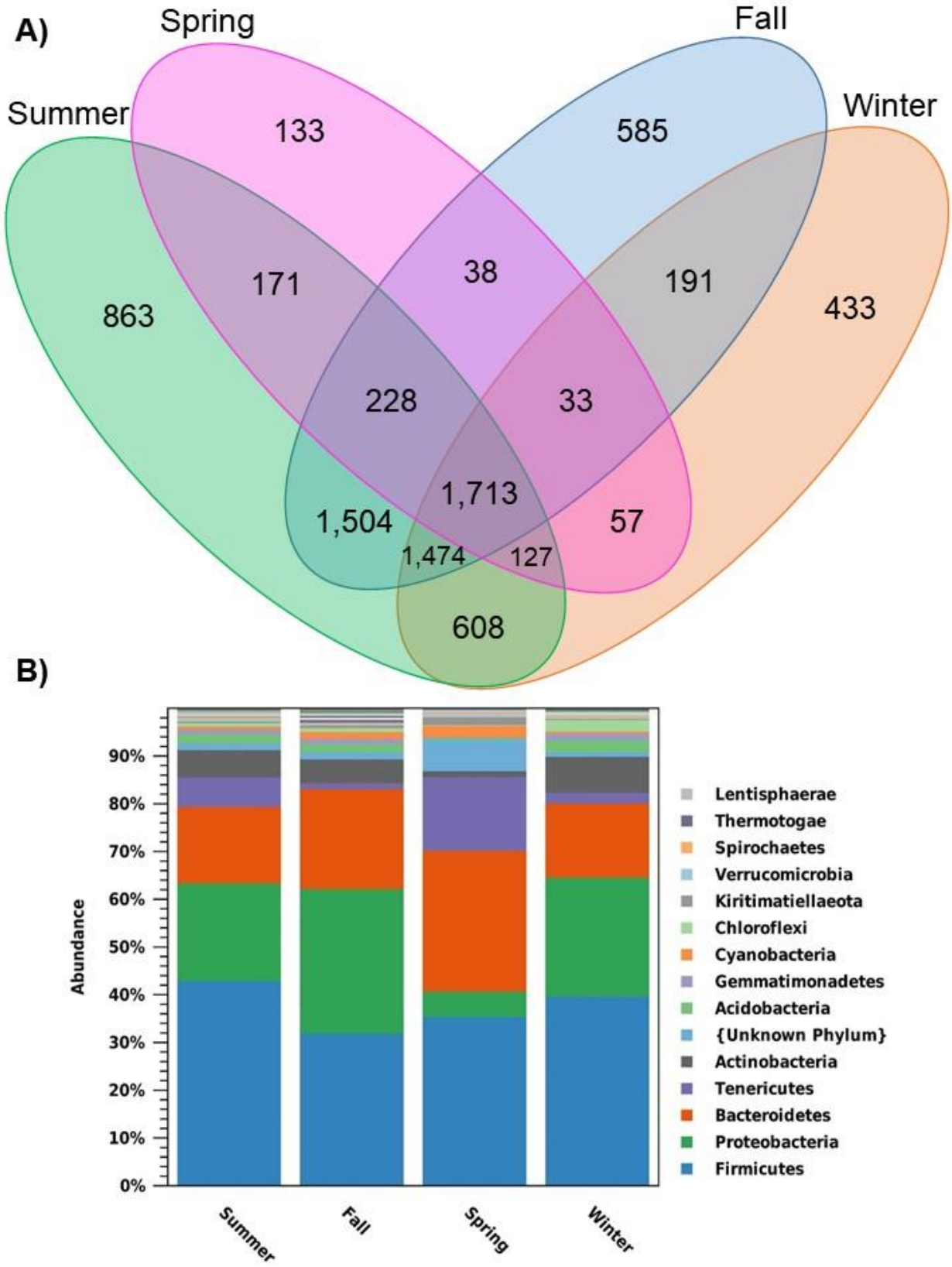

A)
Spring
Fall
Summer
Winter
133
585
863
171
38
191
433
228
33
1,713
1,504
57
1,474
127
608
B)
Abundance
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Summer
Fall
Spring
Winter
Lentisphaerae
Thermotogae
Spirochaetes
Verrucomicrobia
Kiritimatiellaeota
Chloroflexi
Cyanobacteria
Gemmatimonadetes
Acidobacteria
{Unknown Phylum}
Actinobacteria
Tenericutes
Bacteroidetes
Proteobacteria
Firmicutes

Figure 3. 3

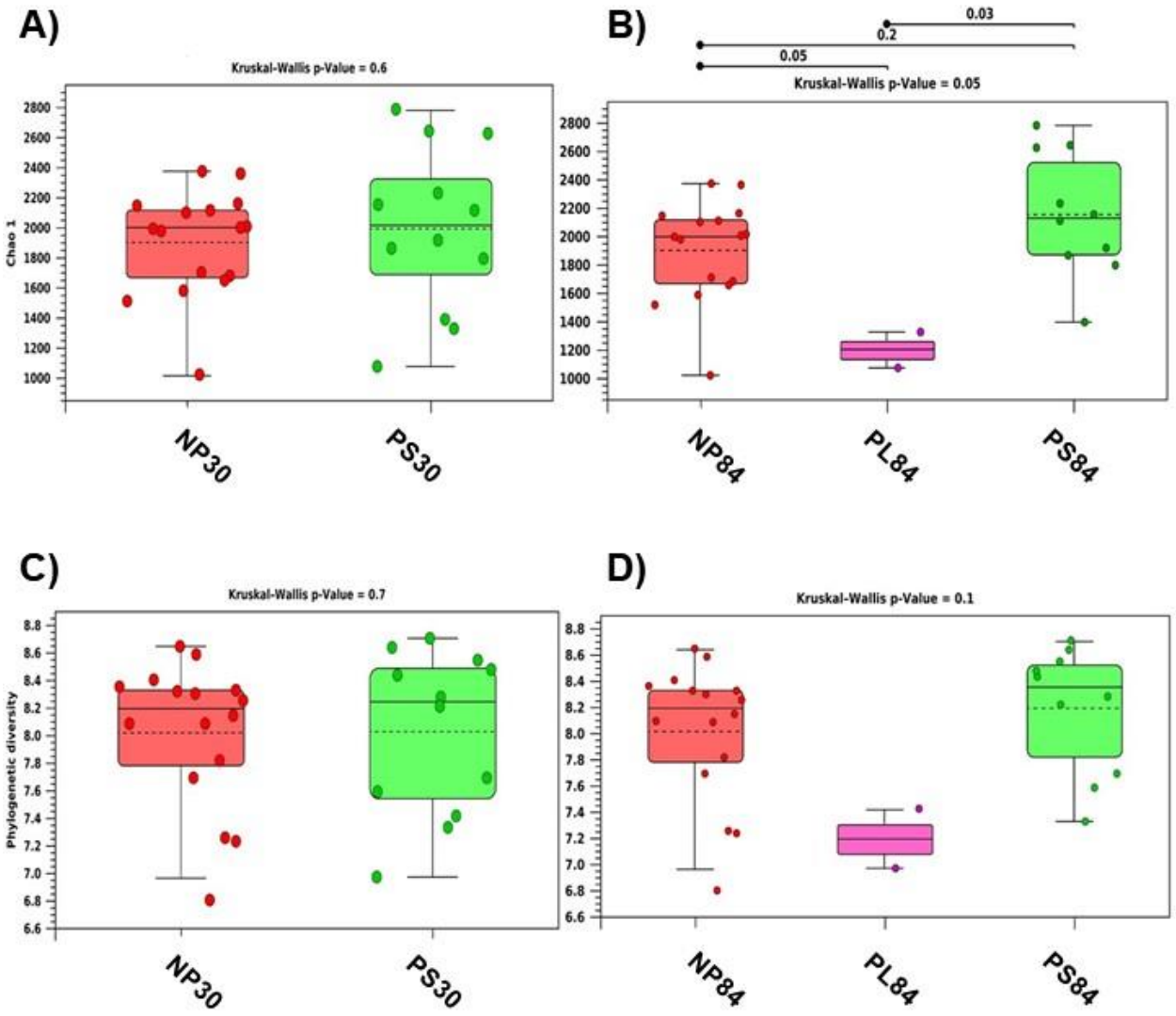

A)
Kruskal-Wallis p-Value = 0.6
Chao 1
NP30
PS30
B)
0.03
0.2
0.05
Kruskal-Wallis p-Value = 0.05
NP84
PL84
PS84
C)
Kruskal-Wallis p-Value = 0.7
Phylogenetic diversity
NP30
PS30
D)
Kruskal-Wallis p-Value = 0.1
NP84
PL84
PS84

Figure 3. 4

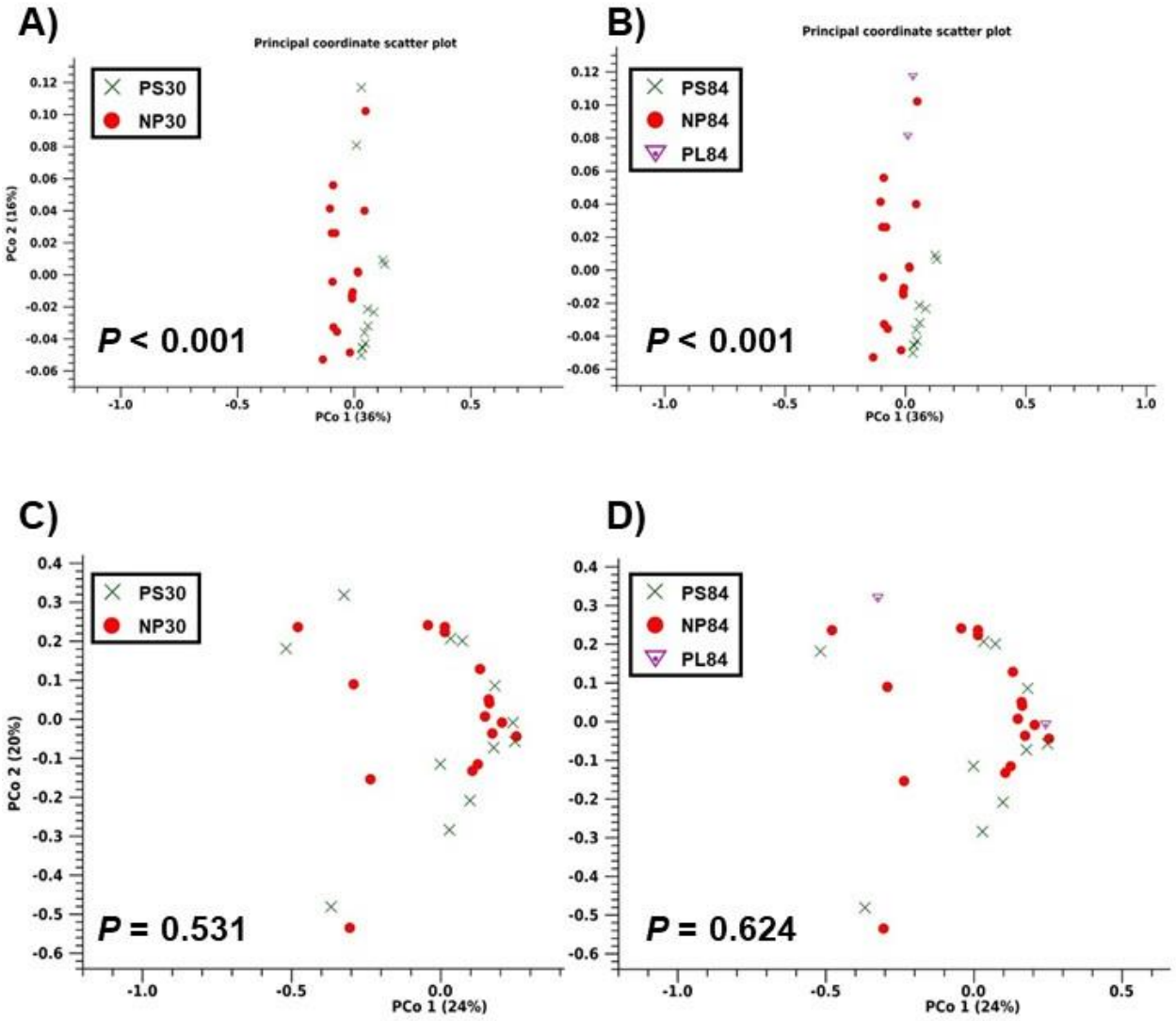

A)
Principal coordinate scatter plot
PS30
NP30
P < 0.001
PCo 2 (16%)
PCo 1 (36%)
B)
Principal coordinate scatter plot
PS84
NP84
PL84
P < 0.001
PCo 1 (36%)
C)
PS30
NP30
P = 0.531
PCo 2 (20%)
PCo 1 (24%)
D)
PS84
NP84
PL84
P = 0.624
PCo 1 (24%)

Figure 3. 5

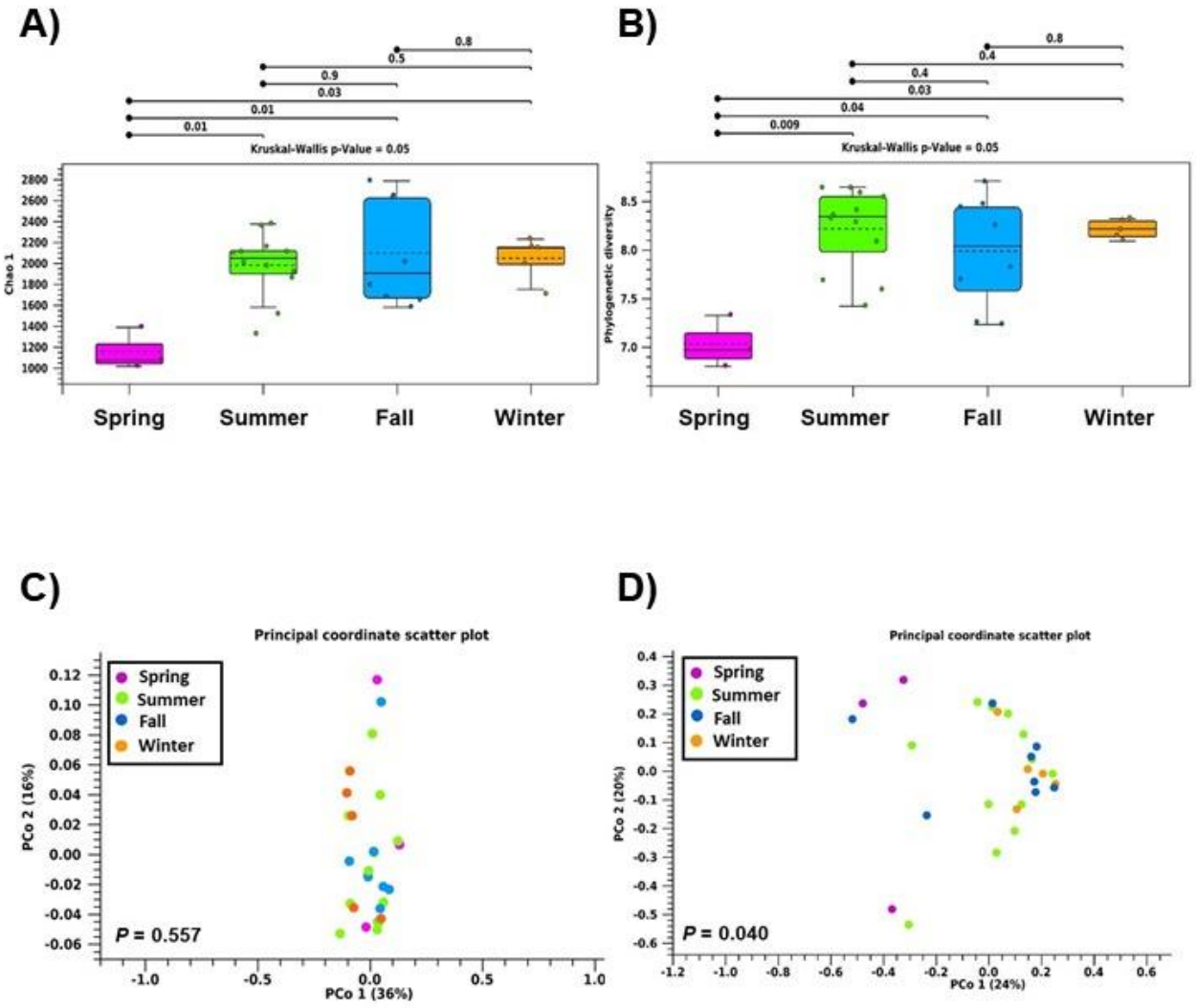
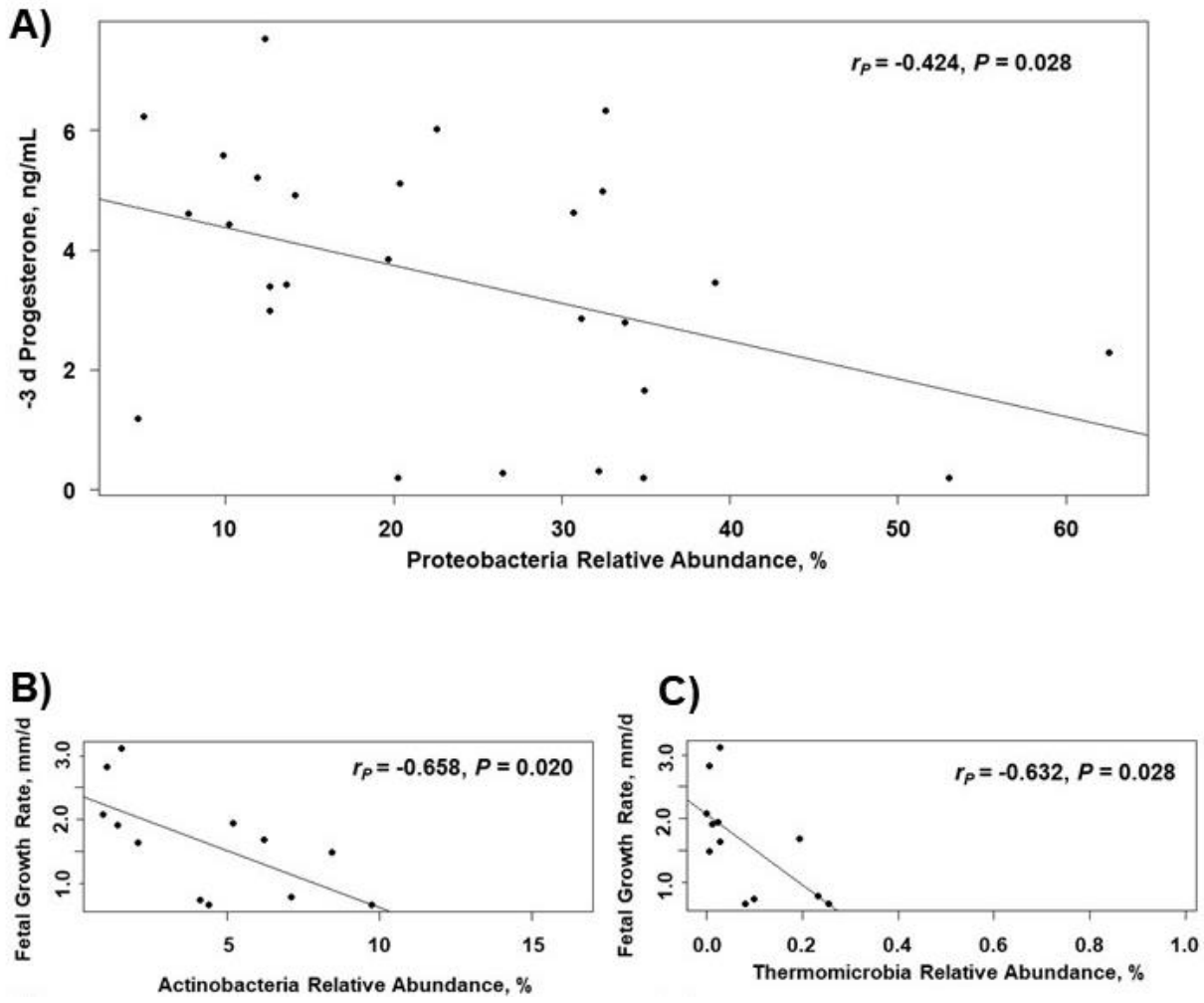

A)
0.8
0.5
0.9
0.03
0.01
0.01
Kruskal-Wallis p-Value = 0.05
Chao 1
Spring
Summer
Fall
Winter
B)
0.8
0.4
0.4
0.03
0.04
0.009
Kruskal-Wallis p-Value = 0.05
Phylogenetic diversity
Spring
Summer
Fall
Winter
C)
Principal coordinate scatter plot
Spring
Summer
Fall
Winter
PCo 2 (16%)
PCo 1 (36%)
P = 0.557
D)
Principal coordinate scatter plot
Spring
Summer
Fall
Winter
PCo 2 (20%)
PCo 1 (24%)
P = 0.040

Figure 3. 6


A)
r_P = -0.424, P = 0.028
-3 d Progesterone, ng/mL
0
2
4
6
10
20
30
40
50
60
Proteobacteria Relative Abundance, %
B)
r_P = -0.658, P = 0.020
Fetal Growth Rate, mm/d
1.0
2.0
3.0
5
10
15
Actinobacteria Relative Abundance, %
C)
r_P = -0.632, P = 0.028
Fetal Growth Rate, mm/d
1.0
2.0
3.0
0.0
0.2
0.4
0.6
0.8
1.0
Thermomicrobia Relative Abundance, %

3.10 SUPPLEMENTARY INFORMATION

Supplementary Tables S3.1 – S3.3: Relative abundance of genera in the uterine microbiome of virgin Holstein heifers based on insemination outcome at d 30 (**S3.1**), insemination outcome at d 84 (**S3.2**), and season (**S3.3**).

CHAPTER 4: Maternal oral, fecal, colostrum, and reproductive microbiomes predictive of pre-weaning calf fecal microbiome inoculation and development

4.1 ABSTRACT

The calf gut microbiome can be inoculated from multiple maternal sources, including the dam's vagina, colostrum, feces, and oral cavity, and may also be inoculated *in utero*. The objectives of this study were 1) to characterize the various maternal and calf fecal microbiomes and predicted metagenomes during peri-partum and post-partum periods and 2) examine the influence of the maternal microbiome on calf gut development during the pre-weaning phase. Multiparous Holstein cows (n = 12) were placed in individual, freshly bedded box stalls 14 d before expected calving. Sterile swabs of the posterior vagina were collected approximately 24 h before calving. Calves (n = 12; bulls = 8, heifers = 4) were immediately isolated after parturition to prevent environmental contamination. Sterile swabs were used to collect dam fecal, dam oral, and calf meconium samples. Representative colostrum samples were collected aseptically within 1 h of calving and 4 L of colostrum was fed to calves using a clean bottle that was assigned to the calf for the duration of the study. Cotyledon sections of placenta were collected within 6 h after calving. Calves were individually housed for 60 d until weaning. Sterile swabs were used to collect calf fecal samples at 24 h, 7 d, 42 d, and 60 d of age. A subset of calf-dam pairs (n = 6; bulls = 3, heifers = 3) were selected and DNA was extracted from all samples. Amplicons covering V4-V5 16S rDNA regions were generated using extracted DNA and sequenced using 300 bp paired end sequencing via Illumina MiSeq. Sequences were aligned into operational taxonomic units using the 97% Greengenes reference database. Spearman correlations were performed between maternal and calf fecal microbiomes. Negative binomial regression models were created for genera in calf fecal samples at each time point using genera in maternal

microbiomes. Metagenomes were predicted and collapsed into gene pathways using PiCRUST (Phylogenetic Investigation of Communities by Reconstruction of Unobservable States). Differences in predicted metagenomes were analyzed using an analysis of variance within STAMP (Statistical Analysis of Metagenomic Profiles). We determined that Bacteroidetes dominated the calf fecal microbiome at all time points (relative abundance ≥ 42.55%) except for 24 h post-calving, where Proteobacteria were the dominant phylum (relative abundance = 85.10%). Colostrum and placenta had low diversity within samples and clustered independently from fecal samples. Each maternal microbiome was a significant predictor for calf fecal microbiome during at least 2 time points. Genes for infectious disease and neurodegenerative disease were greater in colostrum and 24 h calf fecal samples compared to other samples. Results indicated that calf fecal microbiome inoculation and development stems from various maternal sources. Maternal microbiomes could be used to predict calf microbiome inoculation and development, but further research including environmental and potential genetic influences is needed.

Keywords: microbiome, inoculation, dairy calf.

4.2 INTRODUCTION

Enteric infections and incidence of diarrhea are the main causes of neonatal calf death, even with the use of preventive measures like antibiotics (Uetake, 2012). This brings into question what other factors play a role in early calf health. One factor that is not thoroughly understood is the early calf microbiome and its development. Newborn gut colonization during and after parturition influences intestinal development and immune system function (Malmuthuge et al., 2012; Maynard et al., 2015). Understanding sources of colonization and their magnitude of influence on gut development could lead to future preventative measures.

Major sources of calf gut inoculation include the dam's vagina, feces, and the calf's diet. Vaginal bacteria seem to transfer the majority of bacteria to the early calf gut, as the vagina shared the most bacteria with calf feces from 30 min to 48 h after birth when compared to dam feces or colostrum (Klien-Jöbstl et al., 2019). Rumen microbiota differed based on mode of birth (vaginal vs. cesarean section), which demonstrates the vaginal canal as a major influence on the entire gastrointestinal tract (Cunningham et al., 2018). Cow feces and colostrum doinfluence to the calf gut, as both shared abundant bacteria with calf feces during the first 24 h post-partum (Cunningham et al., 2018). There is also evidence that bacteria from these sources influence the microbiome up to 21 d of life (Yeoman et al., 2018). However, many bacteria in the calf gut are not found in the examined maternal microbiomes. Characterizing other unstudied sources could identify the origin of these bacteria and further explain calf gut colonization.

Another potential source of inoculation is the upper reproductive tract of the dam. While previously considered sterile, recent evidence in multiple species has identified microbiomes distinct to locations within the upper reproductive tract (Moore et al., 2017; Rowe et al., 2020). Bacteria have even been identified in multiple locations of the pregnant tract of dairy cattle and

could serve as another potential source of calf gut inoculation (Moore et al., 2017). It would be difficult and potentially dangerous to collect samples from the post-partum uterus, but the placenta could be representative of the upper reproductive microbiome. However, potential relationships between the placental and calf microbiomes have yet to be described. Also, due to the known influence of other maternal microbiomes, the placenta's potential influence cannot be studied independently. Therefore, the objectives of this study were 1) to characterize the various maternal and calf fecal microbiomes and predicted metagenomes during peri-partum and post-partum periods and 2) examine the influence of the maternal microbiome on calf gut development during the pre-weaning phase. We hypothesized that dam reproductive, fecal, and colostrum microbiomes would all play significant roles in calf gut colonization.

4.3 MATERIALS AND METHODS

4.3.1 *Animal Observation and Sample Collection*

Animal procedures were approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (protocol #17-187-DASC). Multiparous, pregnant Holstein cows (n = 12) were selected 12 to 14 d prior to expected calving date and housed in individual box stalls. Box stalls were bedded with sawdust and re-bedded after each calving to avoid contamination across dams. Cows were fed a close-up dry cow total mixed ration twice daily at 0900 h and 1900 h and were provided *ad libitum* access to water. The Moocall calving alert system (Moocall Ltd, Dublin, Ireland) was placed on the dam's tail 7 d prior to expected calving to monitor calving. Sterile, flocked swabs (Puritan, Guilford, ME, United States) were used to sample the dam's posterior vagina within 24 h prior to parturition and snap frozen in cryotubes using liquid nitrogen.

At parturition, calves (n = 13; bulls = 9, heifers = 4) were immediately separated from dams and transferred to a clean 111.28 × 55.40 × 46.13 cm plastic container containing fresh wood shavings to prevent environmental contact. The container was cleaned and rebedded between each calving. Calves were immediately weighed to measure birth weight. Sterile, flocked swabs (Puritan, Guilford, ME, United States) were used to collect meconium from newborn calves and oral samples from the left and right buccal wall of the dam immediately after parturition and snap frozen in cryotubes using liquid nitrogen. Dam fecal samples were removed from the rectum using a clean palpation sleeve and sterile flocked swab were used to collect samples before being snap frozen in cryotubes using liquid nitrogen.

Colostrum was collected using a stainless-steel portable bucket milking machine. Colostrum was required to have a Brix score ≥ 22 g/dL using a Brix refractometer (VEE GEE Scientific, Vernon Hills, IL), which correlates to ≥ 50 g/L of immunoglobulin G (**IgG**) in colostrum. If colostrum did not achieve a Brix score ≥ 22 g/dL, the dam-calf pair were removed from the study. One dam and one bull calf were removed due to failure to meet colostrum requirements. Calves were assigned individual bottles and nipples at colostrum feeding to be used for the remainder of the study. Calves were bottle fed 4 L of their dam's colostrum within 1 h post-birth. Representative colostrum samples were aseptically collected before milking and frozen at -20 °C. In order to mitigate use of antibiotics in the study, calves were bottle fed 2 L of colostrum at 12 h post-calving.

Sections of placenta were collected within 6 h post-birth after passage through the vagina and before coming in contact with the ground using a sterile scalpel. Cotyledon tissue was snap frozen in cryotubes using liquid nitrogen.

Calves were moved to individual, sawdust-bedded hutches after their initial colostrum feeding and remained there through the end of the study. Sterile, flocked swabs (Puritan, Guilford, ME, United States) were used to collect calf fecal samples at 24 h post birth. Blood was collected from each calf 24 h post birth via jugular venipuncture using Monoject blood tubes with no additive (Covidien, Dublin, Ireland). Blood was stored at 4 °C for 12 h and then centrifuged at 2200 x g for 20 min at 4 °C to isolate serum.

Calves were fed 4 L of 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 beginning approximately 24 h post-birth. Calves were fed using individually-assigned bottles and nipples to avoid cross-contamination. Calves were allowed *ad libitum* access to water at 1 d of age. At 28 d of age, calves were given *ad libitum* access to a 22% CP starter grain (Intensity 22% Textured Calf Starter Medicated, Cargill Animal Nutrition, Minneapolis, MN). Step down weaning began at 42 d of age, with calves fed 3 L of milk replacer twice daily from 42 – 49 d and 2 L of milk replacer twice daily from 50 – 56 d. Calves were completely weaned at 57 d and removed from the study at 60 d. Water and starter refusals were measured at each feeding. Calves were weighed weekly approximately 1 h prior to evening feeding. Sterile, flocked swabs (Puritan, Guilford, ME, United States) were used to collect calf fecal samples at 7 d, 42 d, and 60 d.

Colostrum and calf serum IgG concentrations were measured using a commercial Bovine IgG ELISA (Bethyl Laboratories, Inc., Montgomery, TX) according to manufacturer protocol in order to confirm successful passive transfer. Between each step, plates were washed five times. A 96-well plate was coated with a mix of 10 mL ELISA Coating Buffer (0.05 M Carbonate-Bicarbonate, pH 9.6) and 10 μL of Affinity purified Sheep anti-Bovine IgG Coating Antibody

(A10-130A, Bethyl Laboratories), and incubated at room temperature for 1 h. Plate was then blocked with 200 µL of ELISA Blocking solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) followed by a 30 min incubation at room temperature. Next, serial dilutions of samples in PBS (dilution factors ranged from initial 1:2000 to final 1:128,000) were loaded onto the plate in duplicate followed by a 1 h incubation at room temperature. Each well was then loaded with 100 µL of HRP Conjugated Sheep anti-Bovine IgG detection antibody (Dilution 1:200,000; A10-130P, Bethyl Laboratories) followed by a 1h incubation period. After incubation, 100 µL of Enzyme Substrate, TMB (Cat. No. E115, Bethyl Laboratories) were added to each well. Plates were then incubated in the dark for 5 min to minimize oversaturation. After final incubation, an additional 100 µL of ELISA stop solution (0.18 M H_2SO_4) was added to each well. An ELISA Plate reader (BioTek Instruments, Winooski, VT) was used for absorbance measurements (at 450 nm) and results from reading were input into MyAssays analysis software (MyAssays Ltd, Brighton, UK). A four-parameter logistic curve was generated and samples with absorbance measures with an intra assay CV of < 10% and inter assay CV of < 15% were used to determine IgG concentration.

4.3.2 *DNA Extraction and Sequencing*

Bacterial DNA was extracted from all oral, fecal, and vaginal swab samples using the QIAamp BiOstic Bacteremia DNA kit (Qiagen, Germantown, MD, United States). Bacterial DNA was extracted from placenta and colostrum samples using the Qiagen Mini Stool Kit (Qiagen, Germantown, MD, United States). Colostrum was initially centrifuged at 12,000 × *g* for 30 min at 4°C in order to pellet bacteria before DNA extraction. Before DNA precipitation, each sample was treated with 20 µg RNAse A at room temperature for 3 minutes to remove any potential RNA contamination. Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay kit

(Invitrogen, Carlsbad, CA, United States) were used to measure DNA quality and quantity before sequencing.

Samples were submitted to the Virginia Bioinformatics Institute Genomics Research Laboratory (Blacksburg, VA, United States) for library preparation and 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). Amplicon libraries were sequenced using 300 bp paired end sequencing via Illumina MiSeq (Illumina, San Diego, Ca, United States).

4.3.3 *Bioinformatics Analysis*

4.3.3.1 *Taxonomic Profiling*

Taxonomic profiling was performed using CLC Genomics Workbench Microbial Genomics Module version 12.0 (Qiagen, Germantown, MD). Amplicon sequences had adapters removed and were filtered to remove reads with a Phred score < 0.05. Filtered reads were aligned to the 97% Greengenes database version 13.8 to be separated into operational taxonomic units (**OTU**). These OTU were aligned using Multiple Sequence Comparison by Log-Expectation (**MUSCLE**, version 3.8.31; Edgar, 2004) with a maximum of 16 iterations and a minimum combined abundance of 10. A phylogenetic tree was constructed using aligned OTU with a Neighbor Joining method, General Time Reversible nucleotide substitution model, and Whelan and Goldman (**WAG**) protein substitution model (Saitou and Nei, 1987; Yang, 1994; Whelan and Goldman, 2001).

4.3.3.2 *Alpha and Beta Diversity*

Alpha diversity, the diversity within a sample, was measured using phylogenetic diversity (*PD*) based on the constructed phylogenetic tree.

$$PD = \sum_{i=1}^{n} b_i \, I \, (p_i > 0)$$

where *n* was the number of branches within the phylogenetic tree, b_i was the length of branch *I*, p_i was proportion of taxa descending from branch *i*, and the $I(p_i > 0)$ assumed the value of 1 if any taxa descending from branch *i* was present in the sample or 0 otherwise.

Beta diversity, diversity between samples, was measured using weighted Unifrac distances ($d^{(W)}$) based on the constructed phylogenetic tree.

$$d^{(W)} = \frac{\sum_{i=1}^{n} b_i \left| p_i^A - p_i^B \right|}{\sum_{i=1}^{n} b_i \, (p_i^A + \, p_i^B)}$$

where *n* was the number of branched in the phylogenetic tree, b_i was the length of branch *i*, and p_i^A and p_i^B were the proportion of taxa descending from branch *i* in samples *A* and *B*. A permutational multivariate analysis of variance (PERMANOVA) was used to measure difference in Beta diversity based on the main effects of sample type and calf sex (Anderson, 2001). A *P*-value < 0.05 was considered significant. A Bonferroni *P*-value < 0.05 was considered significant when multiple pair-wise comparisons were made between various sample types.

4.3.3.3 *Microbiome Associations*

Spearman ranked correlations were performed between maternal microbiomes and early calf fecal microbiomes based on genera relative abundance using cor.test function in the package stats in R version 3.6.1 (R Core Team, 2019). A *P*-value < 0.05 was considered significant.

A negative binomial regression model was created using genera count data to evaluate the ability of dam's placental, colostrum, vaginal, fecal, and oral microbiomes to predict calf fecal microbiomes. The following model was created in R version 3.6.1 (R Core eam, 2019) and the glm.nb function within the MASS package version 7.3-51.5 (Venables and Ripley, 2002):

$$\ln \mu = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5$$

where μ is calf fecal bacteria count at a given time point, β_0 is the intercept, $x_1 - x_5$ are the dam placental, colostrum, vaginal, oral, and fecal bacteria count, respectively, and $\beta_1 - \beta_5$ are the expected change in ln μ if x_i changes by 1. Predictors were considered significant if $P \leq 0.05$.

4.3.4 *Functional Analysis*

Functional capacity of microbiomes was predicted using the **PICRUSt** (Phylogenetic Investigation of Communities by Reconstruction of Unobservable States) package version 1.1.4 in python version 3.8.1 (Langille et al., 2013). Operational taxonomic units were normalized by their predicted 16S rRNA copy number in order to account for variation in 16S rRNA gene operons between organisms. Functional capacity was then estimated and predicted functions were collapsed into 2 hierarchical levels within Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathways. A principle component analysis plot was created to visualize clustering of predicted metagenomes. Differences in relative abundance of sequences within KEGG pathways based on type of sample were analyzed using an analysis of variance in Statistical Analysis of Metagenomic Profiles (**STAMP**) version 2.1.3 (Parks and Beiko, 2010; Parks et al., 2014). A Bonferroni corrected *P*-value ≤ 0.05 was considered significantly different.

4.4 RESULTS

4.4.1 *Descriptive Statistics*

Dams gave birth to calves (n = 12; bulls = 8, heifers = 4) with a birth weight of 46.12 ± 0.94 (**Table 4.1**). There were no signs of dystocia and calvings did not require assistance. Serum IgG concentrations indicated successful passive transfer of immunity in all calves (**Table 4.1**). Calves had no signs of scouring or illness during the experiment.

4.4.2 *Bioinformatics Analyses*

Across all samples, a total of 18,852 OTU were identified using 11,777,504 reads (**Table 4.2**). Alpha diversity of microbiomes indicated placenta and colostrum have the least phylogenetic diversity and the cow oral microbiome had the most phylogenetic diversity compared to other samples (**Figure 4.1**). Beta diversity indicated placenta and colostrum samples independently clustered from other samples (**Figure 4.2**). There was a difference in beta diversity based on sample type ($P < 0.001$), but further pairwise comparisons did not indicate a difference between specific sample types ($P \geq 0.097$; **Table S4.1**). There was not a difference in beta diversity based on calf sex ($P = 0.842$).

The predominant phylum in colostrum, placenta, vagina, cow oral, and calf 24 h fecal samples was Proteobacteria (96.15%, 47.70%, 57.84%, 69.33%, and 85.10%, respectively; **Figure 4.3**, **Table S4.2**). The predominant phylum in cow fecal, meconium, calf 7 d, 42 d and 60d fecal was Bacteroidetes (48.81%, 42.55%, 43.36%, 49.35%, and 45.58%, respectively; **Figure 4.3**). In meconium, 42 d calf fecal, and 60 d calf fecal, *Prevotella* had the greatest abundance (11.56%, 30.23%, and 27.83%, respectively; **Figure 4.4**; **Table S4.3**). No genus was dominant in other sample types.

4.4.3 *Microbiome Associations*

Spearman ranked correlations were performed between maternal microbiomes and calf fecal microbiomes using genera relative abundance. Colostrum had a low correlation with calf fecal microbiomes (r_s = 0.05 – 0.12), while oral microbiome had a moderate correlation with meconium (r_s = 0.527; **Table 4.3**). Correlations between calf fecal microbiomes and dam and vaginal microbiomes increased with age (**Table 4.3**).

Negative binomial regression models were created to estimate predictive ability of maternal microbiomes on calf fecal microbiomes (**Table 4.4**). Each maternal microbiome was a significant predictor for at least 2 time points. None of the maternal microbiomes were significant predictors for all calf fecal microbiomes.

4.4.4 *Functional Analysis*

Functional potential of the microbiomes was estimated using PICRUSt and functions were collapsed into 2 hierarchical levels of KEGG pathways (**Tables S4.4-S4.5**). Of the predicted metagenomes at the first and broadest hierarchical level, the most abundant metagenomes in all sample types were Metabolism (43.36% - 49.48%) followed by Genetic Information Processing (16.20% - 24.52%). At the second hierarchical level, the three most abundant functions associated with OTU in all sample types were Membrane Transport, Amino Acid Metabolism, and Carbohydrate Metabolism.

Of the 41 KEGG Pathways identified within the second hierarchical level, 33 were significantly different based on sample type (**Table S4.5**). Predicted metagenomes for Infectious Diseases and Neurodegenerative Diseases were greater in colostrum and 24 h fecal samples than 7 d, 42 d, or 60 d fecal samples ($P < 0.001$), while predicted metagenomes related to the Immune System were greater in 42 d and 60 d fecal samples than colostrum or 24 h fecal samples ($P <$

0.001, **Figure 4.5**). In the principle component analysis, colostrum and 24 h fecal samples clustered independently from other sample types (**Figure 4.6**).

4.5 DISCUSSION

The objectives of this study were to characterize the various maternal and calf fecal microbiomes during peri-partum and post-partum periods and examine the influence of the maternal microbiome on calf gut development during the pre-weaning phase. Using 16S sequencing, we identified unique microbiomes within the dam's placenta, vagina, colostrum, feces, and oral cavity and calf's feces. Dam oral microbiome had a moderate positive correlation with early calf fecal microbiomes. All maternal microbiomes were a significant predictor for the calf microbiome during at least 2 time points during pre-weaning. No maternal microbiome was a significant predictor at every time point.

Calf microbiome development can have long-term effects on animal health (Oikonomou et al., 2013). Inoculation of this microbiome can stem from a multitude of sources, like the dam before and during birth as well as the diet and environment. Previous research has investigated influence of the dam on early rumen or intestinal inoculation (birth to 7 d) or exclusively diet on rumen microbiome development in dairy calves (Alipour et al., 2018; Rey et al., 2014; Yeoman, et al., 2018). This was the first study aimed at associating how the maternal microbiomes, including placental, vaginal, colostrum, oral, and fecal, influenced the calf gut inoculation and microbiome development during the pre-weaning phase. Measures were taken to prevent contamination from the environment and between calf-dam pairs.

Time of colostrum sampling relative to birth may influence microbiome results. Our results reflect findings in some previous literature related to characterizing the colostrum microbiomes. Colostrum samples were dominated by Proteobacteria (relative abundance =

96.15%), which is similar to some previous research examining the dairy cow microbiome (relative abundance = 84.9%; Klien-Jöbstl et al., 2019). However, other research examining colostrum microbiome and its relation to mastitis identified colostrum dominated by Firmicutes (relative abundance = 40.8 – 46.1%; Lima et al., 2017). Both previous studies and the current study utilized similar methods of DNA extraction, 16S library preparation, and sequencing platform. The only major difference is time of sample collection; Klien-Jobstl et al. (2019) collected colostrum within 2 h of calving, while Lima et al. (2017) sampled within 24 h. Colostrum composition changes dramatically within 24 h after calving, including decreased concentrations of fat, protein, and immunoglobulins (Puppel et al., 2019). This shift in colostrum composition could influence the microbiome, therefore changing bacteria that inoculate the calf gut. Time of calf colostrum feeding influences rate of absorption and could also influence which bacteria can successfully inoculate the calf gut (Staley and Bush, 1985). Further research is needed examining time of colostrum collection, calf feeding, and their effects on the microbiome.

Identification of a placental microbiome provides evidence of potential inoculation of the calf gastrointestinal tract *in utero*. While limited, previous evidence has identified bacteria in amniotic fluid, placenta, and endometrium of dairy cattle (Moore et al., 2017) as likely sources of inoculation. In this study, cotyledonary regions of the placenta were dominated by Firmicutes (40%), Bacteroidetes (35%), and Proteobacteria (15%). Our cotyledonary placenta samples were also dominated by these three phyla, but with Proteobacteria being the most abundant and Firmicutes being the least. One cause of this conflicting evidence could be due to our method of sample collection and subsequent analysis. Even though measures were taken to choose placenta sections with little vaginal contamination, there was still the likelihood of vaginal bacteria in

placental samples may have contained vaginal bacteria. We removed OTU from the placental microbiome that also appeared in vaginal samples to account for this, but some of these removed OTU may have actually been native to the placenta. Evidence in humans identified a microbiome in the placenta and amniotic fluid that could inoculate the fetal intestines *in utero* (Aargaard et al., 2014; Collado et al., 2016). There is conflicting evidence on the existence of a distinct placental microbiome, but fluorescent immunohistochemistry has identified live bacteria in the cow's placenta (Karstrup et al., 2017; Leiby et al., 2018; de Goffau et al., 2019). *In utero* inoculation could occur in dairy cows, but genera relative abundance in the placenta was not predictive of those in meconium. Inoculation could occur from the microbiome of other locations in the cow, like the oral cavity or respiratory tract.

One potential component that shapes microbiomes we were unable to account for is heritability. A core rumen microbiome has been identified in beef and dairy cattle with an estimated narrow sense heritability ≥ 0.15 (Sasson et al., 2017; Li et al., 2019). This heritable subset of rumen bacteria has also been associated with feed efficiency and methane emissions (Wallace et al., 2019). This could mean portions of the fecal microbiome are inherited and could influence cow performance or calf disease resistance. If so, these microbiomes could be used alongside genomic testing as an additional phenotype for genetic prediction. However, a much larger study examining various maternal sources of calf fecal bacteria are needed to estimate their heritability.

Instead of direct passage of from parent to progeny, microbiome heritability may be due to genetic influence on tissue morphology. Which taxa dominates a particular location is influenced by the available proteins, metabolites, and molecular substrates, as certain bacteria are more efficient at surviving in a particular environment than others (Russel and Baldwin, 1978;

Erbilgin et al., 2017; Tuncil et al., 2017). In an animal's body, this environment influences and is influenced by tissue morphology, including type of cells, abundance of each type, and level of activity within these cells (Senoo and Hata, 1994). In humans, genomic markers have been associated with tissue morphology, including skeletal muscle, pancreas, and reproductive tissues (Ash et al., 2018). This genetic influence on morphology would then influence the tissue environment and subsequently the microbiome. We observed that samples clustered based on location within the dam's body, with placenta, vagina, oral cavity, udder, and large intestine all having distinct morphology. It is possible there is a genetic influence on this morphology and the microbiomes of each sample type, but further research is needed to examine the genetic influence on morphology in cattle as well as its association with the various microbiomes.

Immunoglobulin G is considered the main factor of passive immunity in colostrum, but there may be other components that aide in developing newborn calf immunity. Colostrum and colostrum feeding management are one of the most important factors in regulating newborn calf health (Weaver et al., 2000; MgGuirk and Collins, 2004). Successful passive transfer of immunoglobulins within 24 h after birth helps prevent bacterial infection and diarrhea in newborns (Glass et al., 1983). Cytokines and antimicrobial peptides have also been identified within colostrum and influence calf immune system, while insulin-like growth factor I from colostrum influences calf gut development (Blum and Hammon, 2000; Stelwagon et al., 2009). These smaller factors could play a role in microbial inoculation and gut microbiome development as well. Metagenomes predicted by PICRUSt indicated a greater number of bacteria with genes related to infectious diseases in colostrum and 24 h fecal samples compared to other samples. Despite the apparent presence of pathogens in the colostrum, we did not observe signs of diarrhea or other illness during this study. Infection by these bacteria may have

been prevented by various cytokines and antimicrobial peptides within colostrum. Future studies should examine all colostrum components and their influence on the colostrum and calf gut microbiome.

Each sample type from the dam seems to predict the calf fecal microbiome during at least 2 time points and no one maternal microbiome seems to be the sole influencer of calf fecal development. This supports our hypothesis that each maternal microbiome plays some role in calf gut inoculation and development. However, our sample size was limited to only 6 calf-dam pairs. We attempted to limit potential environmental contamination during and following parturition, but analyzing the microbiome from environmental sources like water or bedding would allow separation of source contributions to the calf microbiome. Future studies with increased sample size and accounting for further sources of variation would support mathematical modelling to predict calf microbiome development.

4.6 CONCLUSION

The current study supports our hypothesis that maternal microbiomes, including fecal, oral, colostrum, and reproductive, play a role in calf fecal microbiome inoculation and development. Presence of a placental microbiome and its relationship with early calf fecal microbiomes suggests potential *in utero* colonization of the calf gut. Studies further validating relationships between these microbiomes as well as other maternal or calf microbiomes are necessary to use these microbiomes as a tool to monitor calf health.

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4.8 TABLES

Table 4.1: Descriptive statistics for Holstein calves and colostrum during experiment. Colostrum was collected within 1 h of parturition. Calf serum was collected 24 h after colostrum feeding. Fecal scores were on a scale of 0-3.

	Mean	SEM
Birth weight, kg	46	0.94
ADG, kg/d	0.70	0.03
Water intake, kg/d	0.60	0.06
Feed intake, kg/d	0.61	0.02
Fecal score, 0-3[1]	0.46	0.05
Colostrum brix, g/dL	26	0.88
Colostrum volume, L	6.47	0.86
Colostrum IgG, mg/dL	13,502	1,976
Calf serum IgG, mg/dL	2,997	251

[1.] Fecal scores ranged from 0 (normal, solid feces) to 3 (water stool that sifts through bedding) according to the Univeristy of Wisconsin Madision School of Veterinary Medicine's Calf Health Scoring Chart.

Table 4.2: Results from 300 bp PE sequencing of V4 region of 16S rDNA amplicons on the Illumina MiSeq platform. Results are separated based on type of sample.

Tissue	Total Reads	Reads in OTU	Number of OTU
Placenta	385,350 ± 26,534	61,688 ± 15,016	187.00 ± 56.44
Colostrum	362,749 ± 49,633	32,776 ± 6,923	20.50 ± 7.42
Vagina	314,095 ± 57,108	184,739 ± 74,332	1,436.25 ± 114.11
Oral	431,700 ± 58,540	313,742 ± 50,289	2,202.67 ± 466.09
Dam Fecal	204,011 ± 29,443	110,857 ± 16,556	1,498.83 ± 176.39
Meconium	244,533 ± 16,662	107,453 ± 14,227	1,223.33 ± 146.94
24 h Fecal	543,118 ± 51,403	490,960 ± 44,588	339.33 ± 29.01
7 d Fecal	372,971 ± 44,982	338,514 ± 41,614	406.00 ± 35.87
42 d Fecal	208,827 ± 29,414	176,874 ± 27,221	797.33 ± 27.06
60 d Fecal	262,002 ± 40,738	216,843 ± 33,375	1,063.83 ± 95.34

Table 4.3: Spearman rank correlation r_s between genus relative abundance of the maternal microbiomes at calving and its calf's fecal microbiome from calving until 60 d of age. Microbiomes were from multiparous Holstein cow-calf pairs (n = 6). All correlations had a *P*-value < 0.001.

	Meconium	**24 h**	**7 d**	**42 d**	**60 d**
Colostrum	0.128	0.097	0.073	0.052	0.050
Placenta	0.267	0.221	0.204	0.193	0.210
Vagina	0.142	0.312	0.309	0.329	0.404
Oral	0.527	0.310	0.309	0.319	0.347
Fecal	0.337	0.329	0.335	0.420	0.477

Table 4.4: Coefficient estimates for negative binomial regression models between maternal microbiomes and calf fecal microbiomes from multiparous Holstein cow-calf pairs (n =6). Models were created using genera count data. Maternal predictors were considered significant when $P \leq 0.05$.

	Meconium		**24 h Fecal**		**7 d Fecal**		**42 d Fecal**		**60 d Fecal**	
	Estimate	***P***	**Estimate**	***P***	**Estimate**	***P***	**Estimate**	***P***	**Estimate**	***P***
Placenta	4.96×10^{-4}	0.390	1.87×10^{-2}	<0.001	-3.02×10^{-3}	<0.001	-1.75×10^{-3}	0.27	1.72×10^{-4}	0.722
Colostrum	2.72×10^{-3}	<0.001	-4.26×10^{-3}	<0.001	8.58×10^{-2}	0.001	-1.81×10^{-2}	0.180	-2.30×10^{-3}	0.003
Vagina	1.23×10^{-5}	0.130	-2.29×10^{-6}	0.770	-1.79×10^{-5}	0.025	-2.93×10^{-5}	0.180	-1.70×10^{-5}	0.014
Oral	1.73×10^{-4}	<0.001	-7.70×10^{-6}	0.100	3.31×10^{-5}	<0.001	5.57×10^{-4}	<0.001	2.78×10^{-5}	<0.001
Fecal	1.59×10^{-4}	<0.001	-5.90×10^{-5}	<0.001	-2.34×10^{-6}	0.870	4.78×10^{-5}	0.201	1.19×10^{-5}	0.312

4.9 FIGURES

Figure 4.1 Alpha diversity, or microbial diversity within a sample, as measured by phylogenetic diversity for each maternal (n = 6) and calf (n = 6) microbiome sample type from multiparous Holstein cow-calf pairs. Dam's oral cavity seems to have the greatest diversity within samples, while placenta and colostrum seem to have the least diversity within samples.

Figure 4.2 Principle component scatter plot using beta diversity, or microbial diversity between samples, as measured by weighted Unifrac distances for each maternal (n = 6) and calf (n = 6) microbiome sample type from multiparous Holstein cow-calf pairs. Placenta and colostrum clustered independently from other sample types, potentially due to low phylogenetic diversity within these samples. Meconium and dam fecal samples clustered closely. Clustering of fecal samples from 24 h to 60 d indicated calf fecal samples became more similar to dam fecal samples over time.

Figure 4.3 Relative abundance of phyla each multiparous Holstein maternal (placenta, colostrum, vagina, oral, fecal; n = 6) and calf (meconium, 24 h, 7d, 42 d, and 60 d fecal; n = 6) sample type. Proteobacteria were the dominant phylum within placenta, vagina, colostrum, oral, and 24 h calf fecal samples, while Bacteroides were the dominant phylum in dam fecal, meconium, 7 d, 42 d, and 60 d calf fecal samples.

Figure 4.4 Relative abundance of genera within each multiparous Holstein maternal (placenta, colostrum, vagina, oral, fecal; n = 6) and calf (meconium, 24 h, 7d, 42 d, and 60 d fecal; n = 6)

sample type. Sample types greatly varied from one another at the genus level. Calf 42 d and 60 d fecal samples were the only samples similar to each other.

Figure 4.5. Average relative abundance of normalized sequences within KEGG pathways (2nd hierarchical level) from predicted metagenomes related to disease or infection. Calf fecal microbiome samples were collected at birth (meconium), 24 h (H 24), 7 d (D 7), 42 d (D 42), and 60 d (D 60). Each color represents a sample type and analysis of variance based on sample type was performed. **A**) Genes related to infectious disease were of greater relative abundance in colostrum and 24 h fecal samples than other sample types. **B**) Genes related to the immune system were of greater relative abundance in 7 d, 42 d, and 60 d fecal samples than other sample types. **C**) Genes related to metabolic disease were of lower relative abundance in colostrum than in all calf fecal samples except for meconium. **D**) Genes related to neurodegenerative diseases were of lower relative abundance in 7 d, 42 d, and 60 d calf fecal samples than other sample types.

Figure 4.6. Principle component scatterplot of predicted metagenomes from Holstien dam and calf microbiomes using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobservable States) at the 2nd hierarchical level when metagenomes were collapsed into KEGG pathways. Calf fecal microbiome samples were collected at birth (meconium), 24 h (H 24), 7 d (D 7), 42 d (D 42), and 60 d (D 60). Most sample types clustered together, except for colostrum and 24 h fecal, which clustered independently.

Figure 4.1

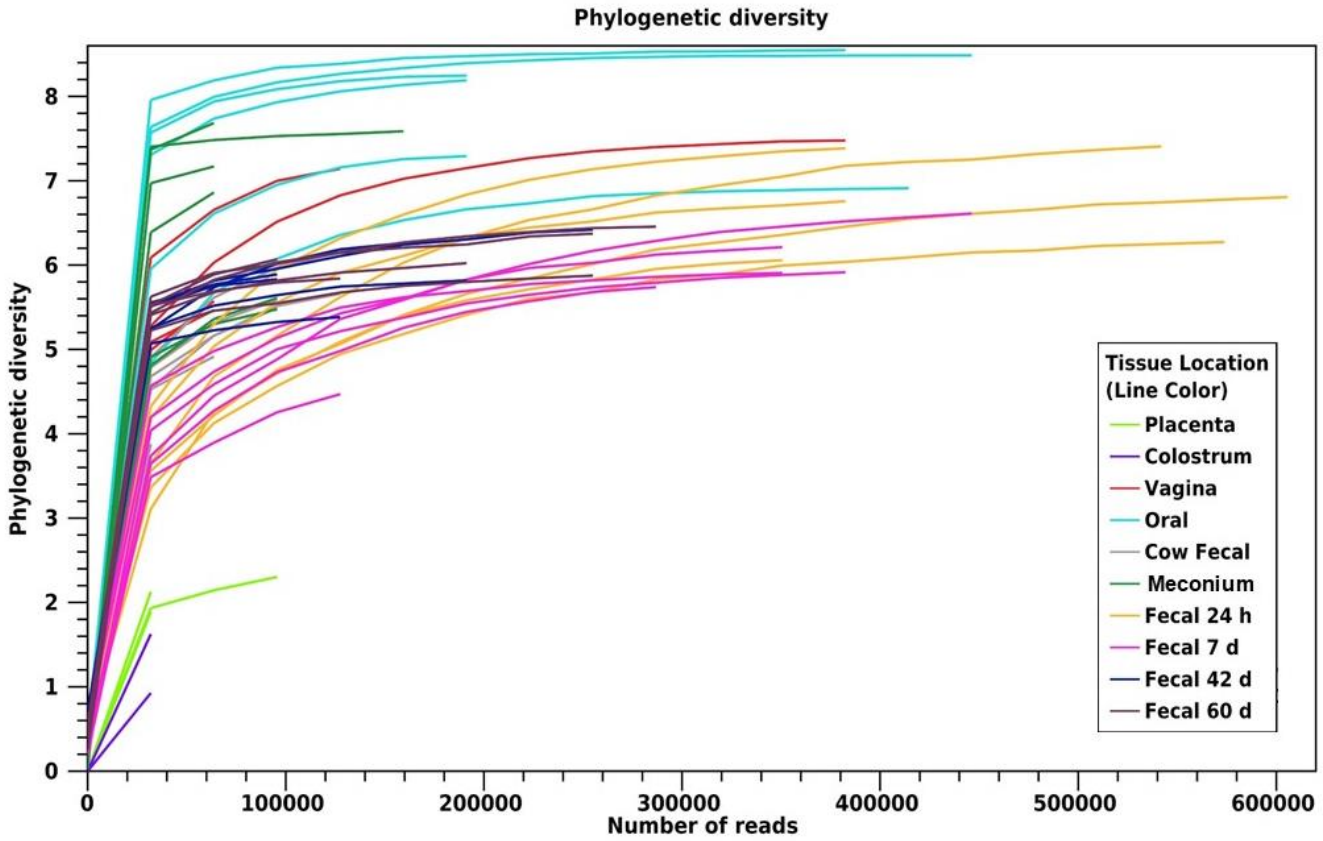

Phylogenetic diversity
Phylogenetic diversity
Number of reads
0
100000
200000
300000
400000
500000
600000
1
2
3
4
5
6
7
8
Tissue Location
(Line Color)
Placenta
Colostrum
Vagina
Oral
Cow Fecal
Meconium
Fecal 24 h
Fecal 7 d
Fecal 42 d
Fecal 60 d

Figure 4.2

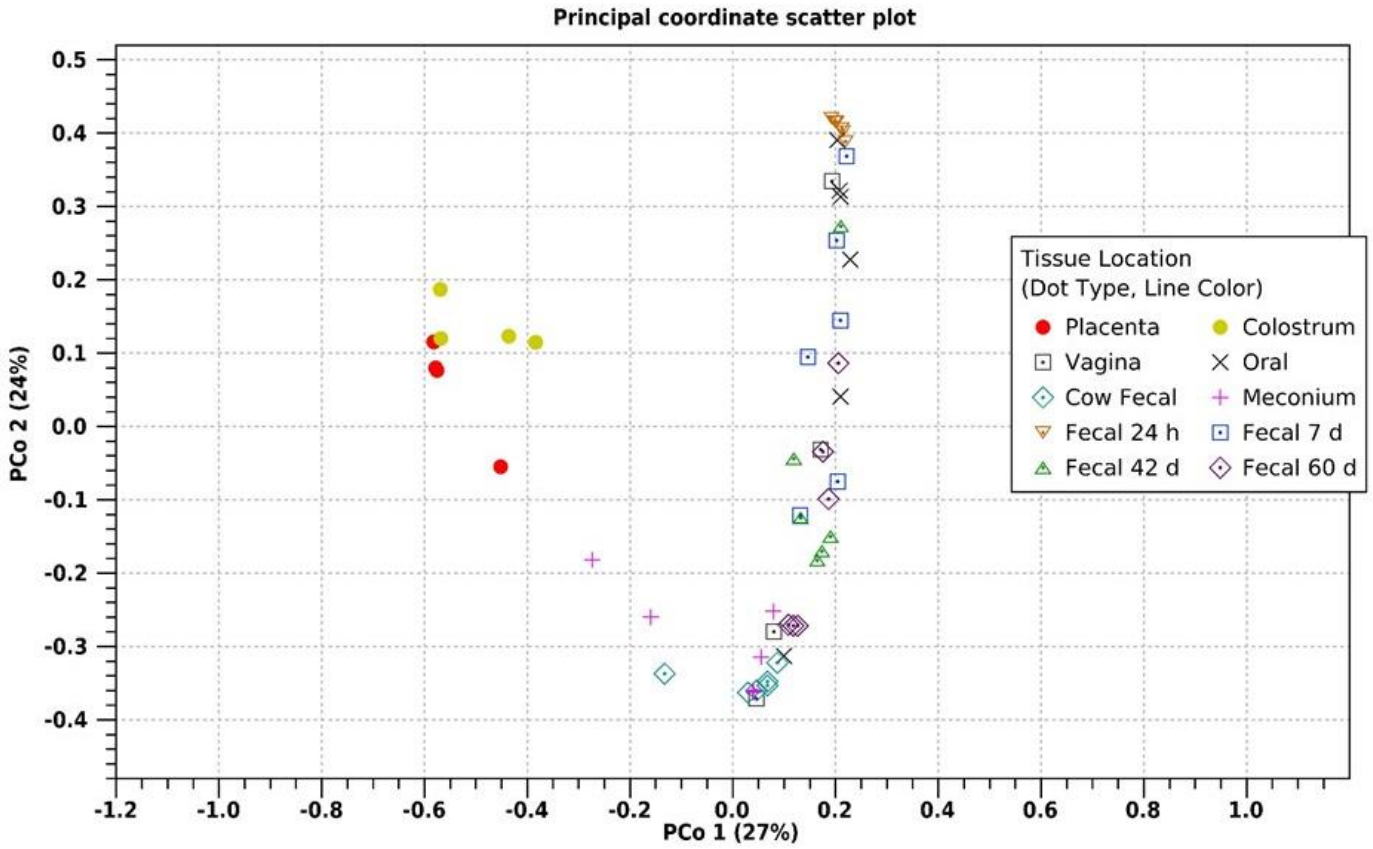

Principal coordinate scatter plot
PCo 2 (24%)
PCo 1 (27%)
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
Tissue Location
(Dot Type, Line Color)
Placenta
Colostrum
Vagina
Oral
Cow Fecal
Meconium
Fecal 24 h
Fecal 7 d
Fecal 42 d
Fecal 60 d

Figure 4.3

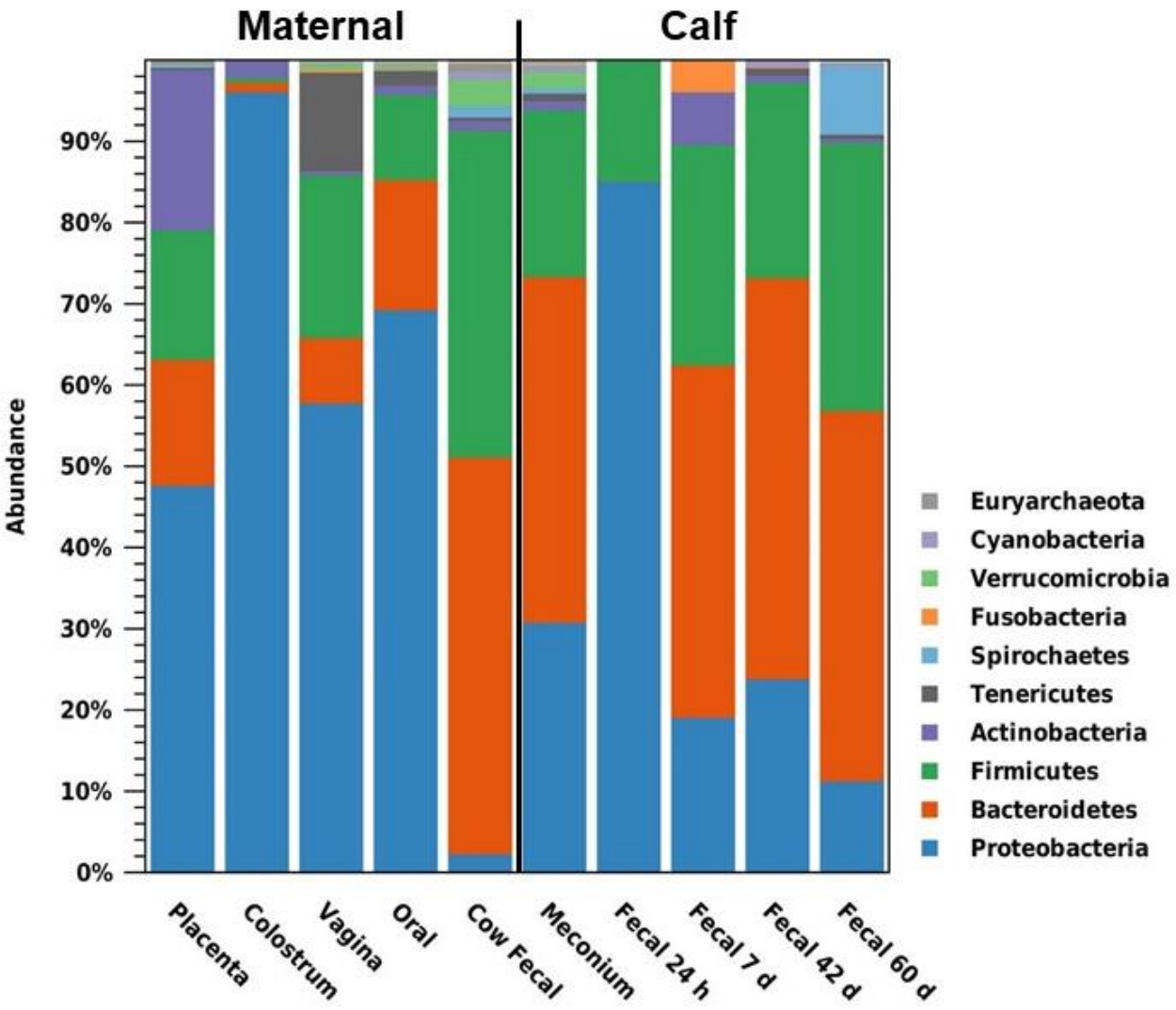

Maternal
Calf
Abundance
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Placenta
Colostrum
Vagina
Oral
Cow Fecal
Meconium
Fecal 24 h
Fecal 7 d
Fecal 42 d
Fecal 60 d
Euryarchaeota
Cyanobacteria
Verrucomicrobia
Fusobacteria
Spirochaetes
Tenericutes
Actinobacteria
Firmicutes
Bacteroidetes
Proteobacteria

Figure 4.4

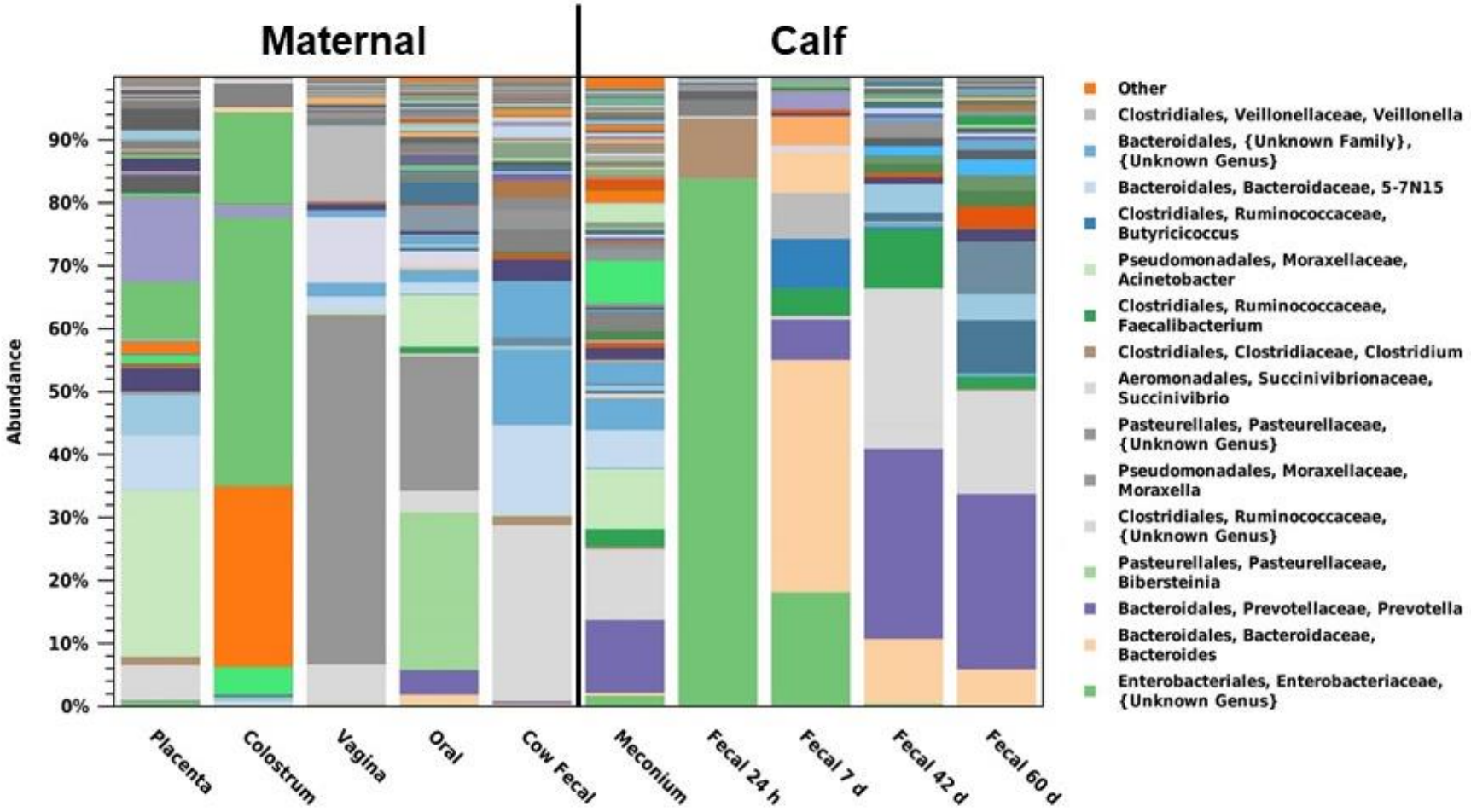


Figure 4.5

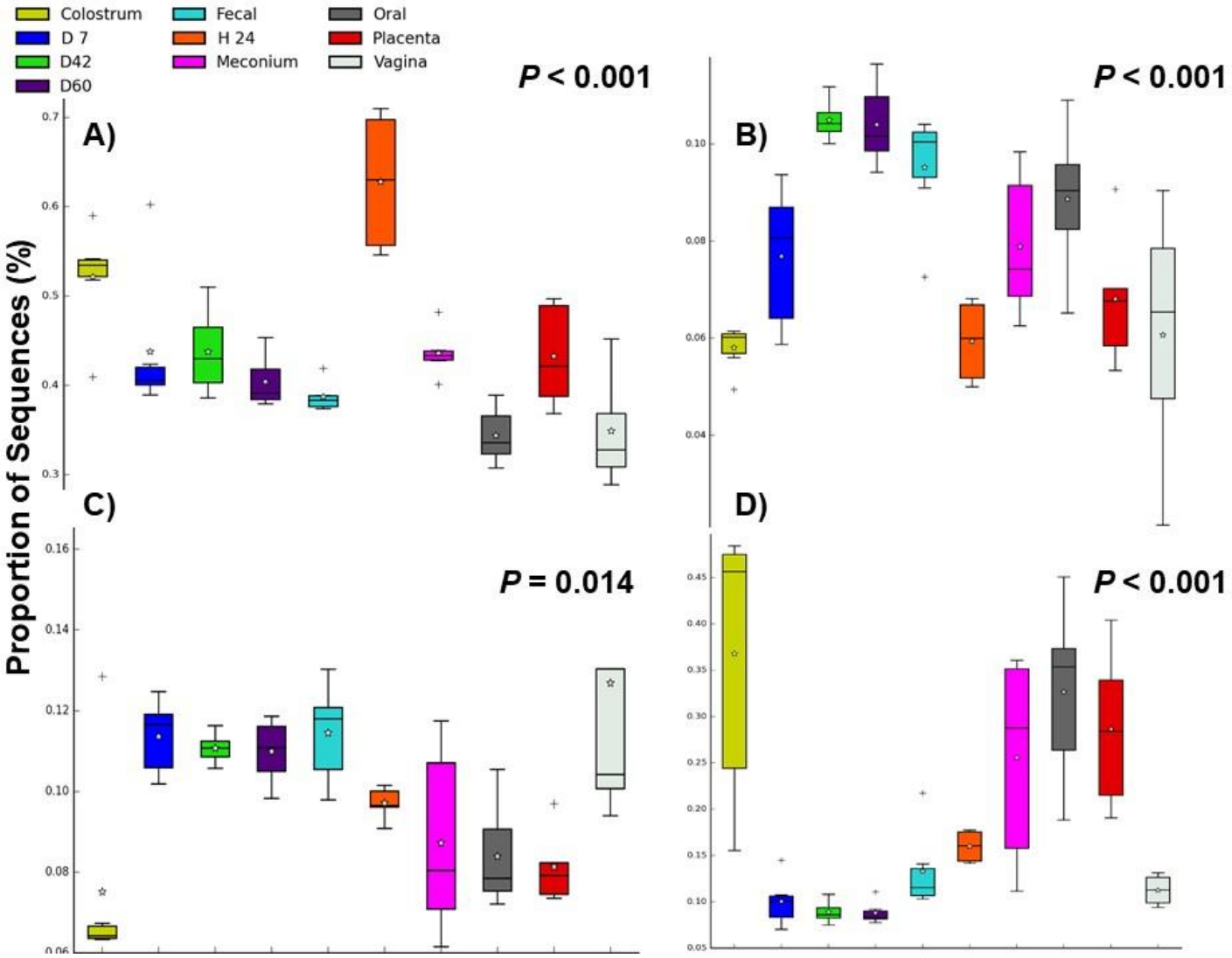
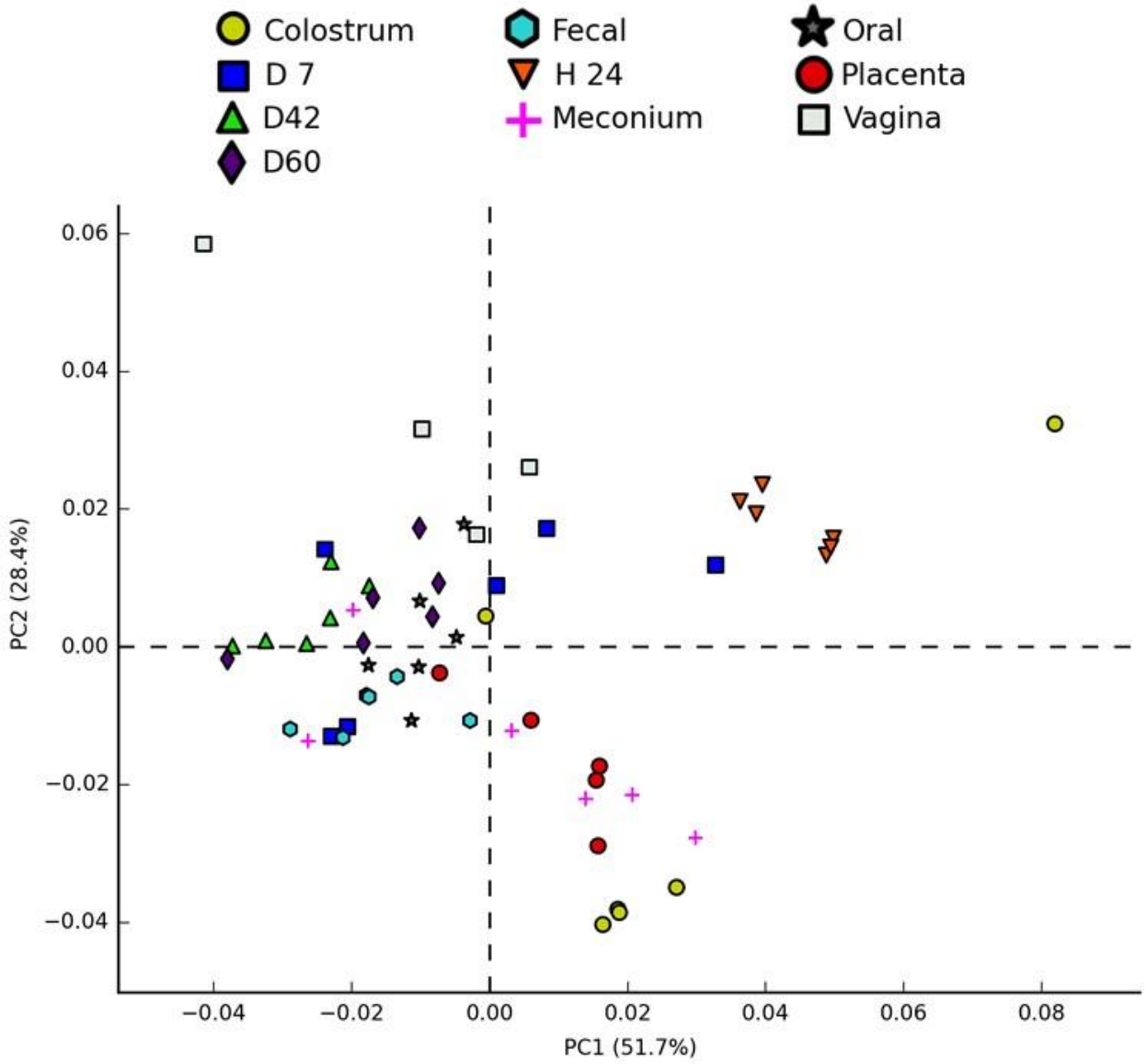

Colostrum
D 7
D42
D60
Fecal
H 24
Meconium
Oral
Placenta
Vagina
Proportion of Sequences (%)
A)
P < 0.001
B)
P < 0.001
C)
P = 0.014
D)
P < 0.001

Figure 4.6


Colostrum
D 7
D42
D60
Fecal
H 24
Meconium
Oral
Placenta
Vagina
0.06
0.04
0.02
0.00
−0.02
−0.04
PC2 (28.4%)
−0.04
−0.02
0.00
0.02
0.04
0.06
0.08
PC1 (51.7%)

4.10 SUPPLEMENTAL INFORMATION

Table S4.1 Permutational Analysis of Variance (PERMANOVA) pairwise comparison results based on sample type using Weighted Unifrac distances. A Bonferroni corrected *P*-value ≤ 0.05 was considered significant.

Sample Type 1	Sample Type 2	Pseudo-f statistic	Bonferonni *P* - value
Placenta	Colostrum	2.071	0.999
Placenta	Vagina	6.093	0.714
Placenta	Oral	10.224	0.097
Placenta	Cow Fecal	11.906	0.097
Placenta	Meconium	6.408	0.097
Placenta	24 h Fecal	16.495	0.097
Placenta	7 d Fecal	9.981	0.097
Placenta	42 d Fecal	9.601	0.097
Placenta	60 d Fecal	10.572	0.097
Colostrum	Vagina	4.702	0.214
Colostrum	Oral	7.883	0.097
Colostrum	Cow Fecal	9.284	0.097
Colostrum	Meconium	6.140	0.097
Colostrum	24 h Fecal	10.747	0.097
Colostrum	7 d Fecal	7.659	0.097
Colostrum	42 d Fecal	7.418	0.097
Colostrum	60 d Fecal	8.084	0.097
Vagina	Oral	3.194	0.999
Vagina	Cow Fecal	4.611	0.214
Vagina	Meconium	2.445	0.999
Vagina	24 h Fecal	12.008	0.214
Vagina	7 d Fecal	3.625	0.999
Vagina	42 d Fecal	2.424	0.999
Vagina	60 d Fecal	3.016	0.643
Oral	Cow Fecal	14.984	0.097
Oral	Meconium	6.932	0.195
Oral	24 h Fecal	6.953	0.097
Oral	7 d Fecal	3.105	0.999
Oral	42 d Fecal	4.535	0.999
Oral	60 d Fecal	6.109	0.584
Cow Fecal	Meconium	2.085	0.999
Cow Fecal	24 h Fecal	77.318	0.097
Cow Fecal	7 d Fecal	14.099	0.097
Cow Fecal	42 d Fecal	9.707	0.097
Cow Fecal	60 d Fecal	8.474	0.097
Meconium	24 h Fecal	21.121	0.097
Meconium	7 d Fecal	6.941	0.097
Meconium	42 d Fecal	4.252	0.097
Meconium	60 d Fecal	3.623	0.195
24 h Fecal	7 d Fecal	9.944	0.097

24 h Fecal	42 d Fecal	17.294	0.097
24 h Fecal	60 d Fecal	26.499	0.097
7 d Fecal	42 d Fecal	3.221	0.974
7 d Fecal	60 d Fecal	4.998	0.195
42 d Fecal	60 d Fecal	0.857	0.999

Table S4.2 Relative abundance of phyla within microbiome of each sample type from multiparous Holstein cows (n = 6) and their calves (n =6; bulls = 3, heifers =3).

Phylum	Placenta	Colostrum	Vagina	Oral	Cow Fecal	Meconium	24 h Fecal	7 d Fecal	42 d Fecal	60 d Fecal
Crenarchaeota	0.000	0.000	0.000	0.000	0.000	0.005	0.000	0.000	0.000	1.560E-4
Euryarchaeota	0.414	0.000	0.114	0.059	1.025	0.232	0.000	0.000	0.024	0.232
{Unknown Phylum} Bacteria-1	0.000	0.000	0.001	1.100E-4	3.360E-4	0.000	0.000	0.000	0.000	0.000
{Unknown Phylum} Bacteria-2	0.000	0.000	0.001	4.380E-4	0.009	0.002	0.000	0.000	0.000	0.000
[Thermi]	0.000	0.000	0.016	0.021	0.002	0.006	0.000	0.000	0.000	0.000
Acidobacteria	0.000	0.000	0.006	0.008	0.005	0.061	0.000	1.000E-4	2.870E-4	1.560E-4
Actinobacteria	19.729	2.049	0.476	1.150	1.280	1.149	0.003	6.500	0.914	0.487
Armatimonadetes	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000	0.000
Bacteroidetes	15.510	1.177	8.057	16.013	48.812	42.554	0.043	43.362	49.352	45.575
BRC1	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.000	0.000	0.000
Chlamydiae	0.000	0.000	0.000	3.290E-4	0.000	0.000	0.000	0.000	0.000	0.000
Chlorobi	0.000	0.000	4.940E-4	0.001	0.000	0.000	0.000	0.000	0.000	0.000
Chloroflexi	0.000	0.000	0.017	0.020	0.005	0.094	7.050E-5	2.010E-4	0.001	0.001
Cyanobacteria	0.050	0.000	0.119	0.213	0.969	0.585	7.050E-5	2.010E-4	0.753	0.257
Deferribacteres	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000
Elusimicrobia	0.000	0.000	0.000	0.001	0.030	0.002	0.000	1.000E-4	0.000	0.253
FBP	0.000	0.000	3.290E-4	0.001	0.000	0.001	0.000	0.000	0.000	0.000
Fibrobacteres	0.000	0.000	0.003	0.010	0.048	0.128	0.000	0.000	0.000	0.000
Firmicutes	15.961	0.599	20.043	10.504	40.153	20.502	14.847	27.153	24.050	32.975
Fusobacteria	0.013	0.000	0.317	0.191	0.001	0.003	0.001	3.830	0.127	0.007
Gemmatimonadetes	0.000	0.000	0.003	0.002	0.000	0.022	0.000	0.000	0.000	0.000
GN02	0.000	0.000	0.001	0.012	0.000	0.000	0.000	0.000	0.000	0.000
GN04	0.000	0.000	0.000	1.100E-4	0.000	0.000	0.000	0.000	0.000	0.000
LD1	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000
Lentisphaerae	0.000	0.000	0.063	0.078	0.145	0.127	2.470E-4	1.510E-4	0.000	0.003
Nitrospirae	0.000	0.000	0.000	1.100E-4	0.001	0.014	7.050E-5	0.000	0.000	0.000
NKB19	0.000	0.000	0.000	3.290E-4	3.360E-4	0.001	0.000	0.000	0.000	0.000
OD1	0.000	0.000	0.002	0.003	0.000	0.012	0.000	0.000	0.000	0.000
OP11	0.000	0.000	0.001	0.002	0.000	0.000	0.000	0.000	0.000	0.000
Planctomycetes	0.006	0.000	0.036	0.032	0.067	0.081	1.060E-4	0.000	0.000	0.000
Proteobacteria	47.702	96.153	57.843	69.330	2.373	30.859	85.104	19.144	23.902	11.327
Spirochaetes	0.233	0.011	0.050	0.179	1.355	0.627	4.580E-4	0.001	0.002	8.320
Synergistetes	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.001
Tenericutes	0.276	0.000	12.147	1.777	0.436	0.921	0.001	3.010E-4	0.875	0.562
TM6	0.000	0.000	0.000	1.640E-4	0.000	0.000	0.000	0.000	0.000	0.000

TM7	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.000	0.000	0.000
Verrucomicrobia	0.107	0.011	0.681	0.388	3.279	2.006	3.530E-4	0.009	0.000	1.560E-4
WPS-2	0.000	0.000	0.002	0.001	0.000	0.001	0.000	0.000	0.000	0.000
WWE1	0.000	0.000	0.000	1.640E-4	0.001	0.000	0.000	0.000	0.000	0.000

Table S4.3. Relative abundance of genera within microbiome of each sample type from multiparous Holstein cows (n = 6) and their calves (n =6; bulls = 3, heifers =3).

Genus	Placenta	Colostrum	Vagina	Oral	Cow Fecal	Meconium	24 h Fecal	7 d Fecal	42 d Fecal	60 d Fecal
Candidatus Nitrososphaera	0	0	0	0	0	0.004	0	0	0	0.000
{Unknown Genus} Methanobacteriaceae	0	0	0	5.48E-05	0	0	0	0	9.55E-05	0.000
Methanobacterium	0	0	0	0	0	0	0	0	9.55E-05	0
Methanobrevibacter	0.407	0	0.039	0.023	0.026	0.017	0	0	0.021	0.219
Methanosphaera	0	0	0.000	0.000	0	0.001	0	0	0.002	0.011
Methanocella	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Methanocorpusculaceae	0.006	0	0.071	0.028	0.972	0.196	0	0	0	0
Methanocorpusculum	0	0	0	0	0.000	0	0	0	0	0
vadinCA11	0	0	0.003	0.006	0.024	0.017	0	0	0	0
{Unknown Phylum} Bacteria-1	0	0	0.000	0.000	0.000	0	0	0	0	0
{Unknown Phylum} Bacteria-2	0	0	0.001	0.000	0.009	0.001	0	0	0	0
Deinococcus	0	0	0	0.000	0.000	0	0	0	0	0
B-42	0	0	0.015	0.019	0.000	0.006	0	0	0	0
Truepera	0	0	0	0.000	0.000	0	0	0	0	0
Thermus	0	0	0	0.000	0	0	0	0	0	0
{Unknown Family} RB41	0	0	0	0	0	0.018	0	0	0	0
{Unknown Genus} Ellin6075	0	0	0.000	0.000	0	0.000	0	0	0	0
{Unknown Family} iii1-15	0	0	0	0	0.000	0.035	0	5.02E-05	0.000	0
{Unknown Genus} mb2424	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} RB40	0	0	0.002	0.001	0.000	0	0	0	0	0
{Unknown Family} DS-18	0	0	0	0	0	0	0	5.02E-05	0	0
{Unknown Family} Solibacterales	0	0	0	0.000	0.000	0	0	0	0	0.000
{Unknown Genus} [Bryobacteraceae]	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Solibacteraceae	0	0	0	0.000	0	0	0	0	0	0
Candidatus Solibacter	0	0	0	0.000	0	0.003	0	0	0	0
{Unknown Family} Sva0725	0	0	0.003	0.005	0.003	0.002	0	0	0	0
{Unknown Family} Acidimicrobiales-1	0	0	0	0.000	0	0	0	0	0	0

{Unknown Family} Acidimicrobiales-2	0.006	0	0.008	0.007	0.001	0.002	0	0	0	0
{Unknown Genus} C111	0	0	0.003	0.003	0	0	0	0	0	0
{Unknown Genus} Microthrixaceae	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Order} Actinobacteria	0	0	0.000	0	0	0	0	0	0	0
{Unknown Family} Actinomycetales-1	0	0	0	0.000	0.000	0	0	0	0	0
{Unknown Family} Actinomycetales-2	0.081	0.021	0.002	0.006	0.006	0.003	0	0	0	0
{Unknown Genus} Actinomycetaceae	0.025	0	0.010	0.002	0.001	0.001	0	0	0	0
Actinobaculum	0	0	0	0	0.000	0	0	0	0	0
Actinomyces	0.062	0	0.000	0.001	0	0	0	0.000	0	0
Arcanobacterium	0	0	0.010	0	0	0	0	0	0	0
N09	0	0	0	0.000	0.000	0.000	0	0	0	0
Trueperella	0	0	0.214	0.000	0	0	0	0.001	0	0
{Unknown Genus} Actinopolysporaceae	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Actinosynnemataceae	0	0	0	0	0.000	0	0	0	0	0
Salana	0	0	0	0.000	0	0.000	0	0	0	0
Georgenia	0.006	0	0	0.000	0.004	0.000	0	0	0	0
Brevibacterium	0.012	0	0	0.015	0.043	0.039	0	0	0	0
{Unknown Genus} Cellulomonadaceae	0.050	0	0.000	0.003	0.018	0.001	0	0	0	0
Actinotalea	0	0	0	5.48E-05	0.000	0.000	0	0	0	0
Cellulomonas	0.006	0	0.000	0.001	0.001	0.002	0	0	0	0
Demequina	0	0	0.002	0.004	0	0	0	0	0	0
Corynebacterium	13.353	1.902	0.103	0.080	0.678	0.103	7.05E-05	0	0	0.000
{Unknown Genus} Dermabacteraceae	0	0	0	0.000	0	0	0	0	0	0
Brachybacterium	0.062	0	0.000	0.016	0.117	0.011	0	0	0	0
Dermacoccus	0	0	0	0.000	0.000	0	0	0	0	0
Piscicoccus	0	0	0	5.48E-05	0	0	0	0	0	0
{Unknown Genus} Dietziaceae	0	0	0	0	0.000	0	0	0	0	0
Dietzia	1.253	0	0.000	0.011	0.138	0.016	0	0	0	0
{Unknown Genus} Geodermatophilaceae	0	0	0	0.000	0	0	0	0	0	0

Glycomyces	0	0	0.000	0	0	0	0	0	0	0
Gordonia	0	0	0	0.000	0	0.012	0	0	0	0
{Unknown Genus} Intrasporangiaceae	0.031	0	0.006	0.026	0.012	0.012	0	0	0	0
Arsenicicoccus	0	0	0	0.000	0	0	0	0	0	0
Janibacter	0	0	0.006	0.009	0.005	0.018	0	0	0	0.000
Knoellia	0.043	0	0.000	0.000	0	0.000	0	0	0	0
Phycicoccus	0	0	0	0.000	0	0	0	0	0	0
Serinicoccus	0	0	0	0.000	0	0	0	0	0	0
Terracoccus	0	0	0	5.48E-05	0	0	0	0	0	0
{Unknown Genus} Jonesiaceae	0.006	0	0	0.001	0.000	0.001	0	0	0	0
Jonesia	0	0	0	0.000	0	0	0	0	0	0
Kineococcus	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Microbacteriaceae	0.144	0.010	0.000	0.005	0.001	0.001	0	0	0	0
Agrococcus	0	0	0	0.000	0.010	0.003	0	0	0	0
Agromyces	0.012	0	0	0.001	0.002	0.001	0	0	0	0
Clavibacter	0	0	0.000	0.000	0	0.000	0	0	0	0
Cryobacterium	0	0	0.000	0.000	0.000	0.000	0	0	0	0
Cryocola	0	0	0	0.000	0.000	0.000	0	0	0	0
Curtobacterium	0.006	0	0	0.000	0	0	0	0	0	0
Frigoribacterium	0.006	0	0	0.000	0.000	0.000	0	0	0	0
Frondihabitans	0	0	0	5.48E-05	0	0.000	0	0	0	0
Leucobacter	0.025	0	0.003	0.012	0.003	0.026	0	0	0	0
Microbacterium	0.025	0	0.001	0.014	0.005	0.021	0	0	0	0.000
Mycetocola	0	0	0	0.000	0.003	0.029	0	0	0	0
Pseudoclavibacter	0.018	0.010	0.001	0.003	0.001	0.001	0	0	0	0
Rathayibacter	0	0	0	5.48E-05	0	0	0	0	0	0
Salinibacterium	0.006	0	0.000	0.001	0.000	0.004	0	0	0	0
Subtercola	0	0	0.000	0.000	0.000	0	0	0	0	0
{Unknown Genus} Micrococcaceae-1	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Micrococcaceae-2	0.445	0.010	0.008	0.222	0.026	0.040	0	0	0	0
Arthrobacter	1.987	0.052	0.025	0.074	0.038	0.096	7.05E-05	0	0	0
Citricoccus	0	0	0	5.48E-05	0.001	0.012	0	0	0	0
Kocuria	0.269	0.010	0.000	0.012	0.014	0.002	7.05E-05	0	0	0
Microbispora	0.050	0	0.000	0.001	0.013	0.002	0	0	0	0
Micrococcus	0.050	0	0.000	0.001	0.001	0	0	0	0	0
Nesterenkonia	0.018	0	0	0.003	0.011	0.000	0	0	0	0

Renibacterium	0	0	0	0	0.000	0	0	0	0	0
Rothia	0.043	0	0.000	0.068	0	0.003	0	0	0	0
Sinomonas	0	0	0	0.000	0.001	0	0	0	0	0
{Unknown Genus} Micromonosporaceae	0	0	0	0.000	0	0	0	0	0	0
Pilimelia	0	0	0.000	0	0	0	0	0	0	0
Mycobacterium	0.006	0	0	0.001	0.000	0.002	0	0	0	0
Nocardia	0	0	0	0.000	0	0	0	0	0	0
Rhodococcus	0.075	0	0.001	0.003	0.000	0.003	0	0	0	0
{Unknown Genus} Nocardioidaceae-1	0	0	0	0	0.000	0	0	0	0	0
{Unknown Genus} Nocardioidaceae-2	1.416	0	0.002	0.017	0.018	0.034	0	0	0	0
Aeromicrobium	0.006	0	0.001	0.004	0.003	0.003	0	0	0	0
Nocardioides	0.056	0	0.002	0.003	0.005	0.001	0	0	0	0
Pimelobacter	0	0	0	0.000	0.000	0	0	0	0	0
Propionicimonas	0	0	0	0.000	0.002	0	0	0	0	0
{Unknown Genus} Nocardiopsaceae	0	0	0	5.48E-05	0	0	0	0	0	0
Nocardiopsis	0	0	0	0.000	0	0	0	0	0	0
Prauseria	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Genus} Promicromonosporaceae	0	0	0.000	0.000	0.000	0	0	0	0	0
Cellulosimicrobium	0	0	0	0.000	0.000	0	0	0	0	0
Promicromonospora	0	0	0	0.000	0	0	0	0	0	0
Xylanimicrobium	0	0	0	0.001	0.000	0.000	0	0	0	0
{Unknown Genus} Propionibacteriaceae-1	0	0	0	0.000	0.000	0	0	0	0	0
{Unknown Genus} Propionibacteriaceae-2	0	0	0.000	0.001	0.001	0.001	0	0	0	0
Luteococcus	0	0	0	0.000	0	0	0	0	0	0
Propionibacterium	0.006	0	0.000	0.002	0.000	0.027	0.000	0	0	0.001
Tessaracoccus	0	0	0	0.000	0.000	0.000	0	0	0	0
{Unknown Genus} Pseudonocardiaceae	0	0	0	0.000	0	0.000	0	0	0	0
Actinomycetospora	0	0	0	0.000	0	0	0	0	0	0
Pseudonocardia	0	0	0	5.48E-05	0.000	0.000	0	0	0	0
Saccharomonospora	0	0	0	0	0	0.001	0	0	0	0
Saccharopolyspora	0	0	0.000	0.000	0.000	0	0	0	0	0
Thermocrispum	0	0	0	5.48E-05	0	0	0	0	0	0

{Unknown Genus} Rarobacteraceae	0	0	0	0.000	0	0.000	0	0	0	0
Rarobacter	0	0.021	0	0.000	0.000	0	0	0	0	0
Ruania	0	0	0	0.000	0	0	0	0	0	0
Sanguibacter	0.031	0	0.000	0.012	0.011	0.006	0	0	0	0
{Unknown Genus} Sporichthyaceae	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Streptomycetaceae	0	0	0.000	0.000	0.000	0.000	0	0	0	0
Streptomyces	0	0	0.000	0.000	0	0.003	0	0	0	0
{Unknown Genus} Thermomonosporaceae	0	0	0	0.000	0	0	0	0	0	0
Williamsia	0	0	0	0.000	0	0	0	0	0	0
Yaniella	0.012	0	0	0.001	0.001	0.001	0	0	0	0
{Unknown Genus} Bifidobacteriaceae	0	0	0.000	0.002	0.000	0.013	7.05E-05	0.000	0.012	0.133
Aeriscardovia	0	0	0	0.000	0	0	0	0	0	0
Bifidobacterium	0.006	0.010	0.028	0.406	0.027	0.369	0.002	6.489	0.230	0.026
Gardnerella	0	0	0	0	0	0.000	0	0	0	0
{Unknown Genus} Coriobacteriaceae	0	0	0.011	0.014	0.014	0.113	0	0	0.078	0.311
Adlercreutzia	0	0	0.004	0.001	0.007	0.005	0	0	0.000	0
Atopobium	0	0	0.001	0.000	0.001	0	0	0.000	0	0
Collinsella	0	0	0.000	0.035	0.000	0.072	7.05E-05	0.008	0.591	0.012
Eggerthella	0	0	0	0.000	0	0	0	0	0	0
Slackia	0	0	0	0.000	0.003	0	0	0	0.001	0.000
{Unknown Genus} Nitriliruptoraceae	0	0	0	0.000	0	0	0	0	0	0
{Unknown Order} OPB41	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} AK1AB1_02E	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Gaiellaceae	0	0	0	0	0	0.000	0	0	0	0
{Unknown Family} Solirubrobacterales	0	0	0.002	0.002	0.001	0	0	0	0	0
Patulibacter	0	0	0	0.001	0	0.000	0	0	0	0
Solirubrobacter	0	0	0.000	0	0	0	0	0	0	0
{Unknown Family} SJA-22	0	0	0	0	0	0.003	0	0	0	0
{Unknown Genus} [Balneolaceae]	0	0	0.000	0.000	0	0	0	0	0	0
Balneola	0	0	0	0.000	0	0	0	0	0	0

KSA1	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Genus} Rhodothermaceae	0	0	0.000	0.000	0	0	0	0	0	0
Rubricoccus	0	0	0	0.000	0.000	0.009	0	0	0	0
{Unknown Family} [Saprospirales]-1	0	0	0	0	0	0.000	0	0	0	0
{Unknown Family} [Saprospirales]-2	0	0	0	0.002	0	0.012	0	0	0	0
{Unknown Genus} Chitinophagaceae	0	0	0.002	0.028	0.001	0.092	0	0	0.000	0
Chitinophaga	0	0	0	5.48E-05	0	0	0	0	0	0
Flavihumibacter	0	0	0	0.000	0	0	0	0	0	0
Flavisolibacter	0	0	0	0.000	0	0.001	0	0	0.000	0
Lacibacter	0	0	0	0	0	0.014	0	0	0	0
{Unknown Genus} Saprospiraceae	0	0	0.098	0.104	0.001	0.034	0	0	0	0
Saprospira	0	0	0	0.001	0	0	0	0	0	0
{Unknown Order} At12OctB3	0	0	0	0	0.000	0	0	0	0	0
{Unknown Family} Bacteroidales-1	0	0	0	0	0.000	0	0	0	0	0
{Unknown Family} Bacteroidales-2	0.031	0.010	2.137	1.949	11.988	5.035	0.006	0.003	0.675	0.471
{Unknown Genus} [Barnesiellaceae]	0	0	0.021	0.028	0.143	0.041	0	0	0.018	0.009
Butyricimonas	0	0	0.013	0.000	0.004	0.000	0	0.000	0.003	0.109
Odoribacter	0	0	0.006	0.027	0.033	0.047	0	0.000	0.439	0.295
{Unknown Genus} [Paraprevotellaceae]	0	0	0	0.002	0.000	0	0	0	0.001	0.000
[Prevotella]	6.438	0.641	0.063	0.695	0.573	0.834	0.001	0.001	4.570	4.146
CF231	0.018	0	0.246	0.308	2.756	0.608	0.000	0.000	0.041	0.060
Paraprevotella	0	0	0.008	0.008	0.040	0.012	7.05E-05	0	0	0.039
YRC22	0	0	0.021	0.024	0.184	0.053	0	0	0	0
{Unknown Genus} Bacteroidaceae	0.012	0	0.815	0.971	3.468	2.910	0.000	0.000	0	0
5-7N15	8.732	0.514	2.458	1.783	14.188	6.030	0.002	0.000	0.000	0.000
Bacteroides	0.006	0.010	0.173	1.570	0.402	0.463	0.015	36.936	10.314	5.947
BF311	0	0	0.000	0.002	0	0.024	0	0	0	0
{Unknown Genus} BS11	0	0	0.183	0.133	2.112	0.654	0.000	0.000	0	0
{Unknown Genus} Marinilabiaceae	0	0	0.000	0.000	0	0	0	0	0	0

Cytophaga-1	0	0	0	0	0	0	0	0	0	0.000
{Unknown Genus} ML635J-40	0	0	0	0.000	0.000	0	0	0	0	0
{Unknown Genus} p-2534-18B5	0	0	0.011	0.084	0.116	0.495	0.000	0	0	0
{Unknown Genus} Porphyromonadaceae	0	0	0.000	0.026	0.008	0.019	0	0	0	0
Dysgonomonas	0	0	0	0.000	0	0	0	0	0	0
Paludibacter	0.006	0	0.090	0.154	0.634	0.178	7.05E-05	0	0	0
Parabacteroides	0	0	0.005	0.099	0.019	0.285	0.001	0.005	1.507	2.483
Porphyromonas	0	0	0.031	0.027	0.000	0	0	0	0	0
{Unknown Genus} Prevotellaceae	0	0	0	0.033	0.000	0.035	0.000	0.000	0.053	1.389
Prevotella	0.125	0	0.072	3.894	0.369	11.561	0.011	6.411	30.225	27.831
{Unknown Genus} RF16	0.006	0	0.272	0.599	1.905	0.881	0.000	0	0.000	0.009
{Unknown Genus} Rikenellaceae	0	0	1.046	1.252	9.005	3.464	0.000	0.000	0.001	0.029
Alistipes	0	0	0.011	0.002	0.025	0.025	0	0	0	0
{Unknown Genus} S24-7	0.018	0	0.127	0.212	0.377	1.475	0.000	0.000	1.479	2.448
{Unknown Genus} SB-1	0	0	0.001	0.001	0.000	0	0	0	0	0
{Unknown Genus} Cyclobacteriaceae	0	0	0.001	0.018	0.008	0.072	0	0	0	0.000
Algoriphagus	0	0	0	0.000	0.000	0	0	0	0	0
{Unknown Genus} Cytophagaceae	0	0	0.002	0.004	0.001	0.004	0	0	0	0
Adhaeribacter	0	0	0	0	0	0.000	0	0	0.000	0
Cytophaga-2	0	0	0	0.001	0.000	0.010	0	0	0	0
Dyadobacter	0	0	0	0.003	0.000	0.059	0	0	0	0
Flectobacillus	0	0	0	0.000	0	0	0	0	0	0
Hymenobacter	0	0	0	0.000	0	0	0	0	0	0
Leadbetterella	0	0	0.001	0.005	0.000	0.004	0	0	0	0
Pontibacter	0	0	0	0.000	0.000	0.005	0	0	0	0
Spirosoma	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Flammeovirgaceae	0	0	0.001	0.001	0	0	0	0	0	0
Flexibacter	0	0	0	0	0.000	0	0	0	0	0
{Unknown Family} Flavobacteriales-1	0	0	0	0.002	0	0.010	0	0	0	0
{Unknown Family} Flavobacteriales-2	0	0	0.007	0.007	0.014	0.027	0	0	0	0

{Unknown Genus} [Weeksellaceae]	0	0	0.003	0.794	0.019	0.237	0.000	0	0	0
Chryseobacterium	0.106	0	0.000	0.025	0.046	0.682	0	0	0	0
Cloacibacterium	0	0	0	0.016	0	0.002	0	0	0	0
Ornithobacterium	0	0	0	0.004	0	0	0	0	0	0
Riemerella	0	0	0	0.003	0	0	0	0	0	0
Wautersiella	0	0	0.006	0.041	0.002	0.188	0	0	0	0
Weeksella	0	0	0.000	0.003	0.000	0.002	0	0	0	0
{Unknown Genus} Cryomorphaceae	0	0	0.002	0.011	0	0.000	0	0	0	0
Brumimicrobium	0	0	0.001	0.005	0.000	0.002	0	0	0	0
Crocinitomix	0	0	0	0.000	0.000	0	0	0	0	0
Cryomorpha	0	0	0.000	0.009	0.000	0.000	0	0	0	0
Fluviicola	0	0	0.002	0.016	0.005	0.114	0	0	0	0
{Unknown Genus} Flavobacteriaceae-1	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Flavobacteriaceae-2	0	0	0.045	0.152	0.033	0.633	0	0	0	0
Aequorivita	0	0	0.014	0.016	0.003	0.008	0	0	0	0
Aquimarina	0	0	0.001	0.000	0	0	0	0	0	0
Arenibacter	0	0	0	0.000	0.000	0	0	0	0	0
Capnocytophaga	0	0	0.000	0.001	0	0	0	0	0	0
Flavivirga	0	0	0.007	0.004	0	0	0	0	0	0
Flavobacterium	0	0	0.004	0.297	0.244	2.844	0.000	0	0	0
Gaetbulibacter	0	0	0	0.004	0.003	0	0	0	0	0
Gelidibacter	0	0	0.001	0.024	0.001	0.020	0	0	0	0
Gillisia	0	0	0	0	0	0.000	0	0	0	0
Mariniflexile	0	0	0.000	0.000	0.000	0	0	0	0	0
Myroides	0	0	0.000	0.070	0.000	0.114	0	0	0	0
Polaribacter	0	0	0.001	0.001	0	0	0	0	0	0
Tenacibaculum	0	0	0.000	0.000	0	0	0	0	0	0
Vitellibacter	0	0	0	0	0	0.000	0	0	0	0
Winogradskyella	0	0	0.003	0.001	0	0	0	0	0	0
Yeosuana	0	0	0	5.48E-05	0	0	0	0	0	0
Zhouia	0	0	0.004	0.001	0	0	0	0	0	0
{Unknown Family} Sphingobacteriales	0	0	0.000	0.001	0	0.008	0	0	0	0
{Unknown Genus} Sphingobacteriaceae-1	0	0	0	0.000	0	0	0	0	0	0

{Unknown Genus} Sphingobacteriaceae-2	0	0	0.001	0.047	0.030	0.230	0	0	0.017	0.300
Mucilaginibacter	0	0	0	5.48E-05	0	0	0	0	0	0
Olivibacter	0	0	0	0.000	0	0	0	0	0	0
Parapedobacter	0	0	0	0.000	0	0	0	0	0	0
Pedobacter	0	0	0.000	0.024	0.009	0.395	0	0	0	0.001
Sphingobacterium	0.006	0	0.009	0.342	0.014	1.552	0	0	0	0
{Unknown Order} VC2_1_Bac22	0	0	0	0.001	0	0.007	0	0	0	0
{Unknown Order} PRR-11	0	0	0	0.000	0	0.001	0	0	0	0
{Unknown Genus} Parachlamydiaceae	0	0	0	0.000	0	0	0	0	0	0
{Unknown Order} OPB56	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Genus} Anaerolinaceae	0	0	0	0	0	0.008	0	0	0	0
Anaerolinea	0	0	0	0	0	0.011	0	0	9.55E-05	0
SHD-231	0	0	0.000	0.000	0.000	0	0	0	0	0
T78	0	0	0	0.000	0.000	0.000	0	0	0	0
Ardenscatena	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Caldilineaceae	0	0	0.001	0.000	0.000	0	0	0	0	0
Caldilinea	0	0	0	0	0	0	0	0	0.000	0
{Unknown Family} DRC31	0	0	0.001	0.001	0	0	0	0	0	0
{Unknown Family} envOPS12	0	0	0.000	0.000	0.001	0.061	0	0.000	0.000	0.000
{Unknown Family} GCA004	0	0	0.000	0	0	0	0	0	0	0
{Unknown Genus} A4b	0	0	0.001	0.001	0	0.002	7.05E-05	0	0	0
{Unknown Genus} SHA-31	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Family} [Roseiflexales]	0	0	0	0.000	0	0	0	0	0	0
Chloronema	0	0	0	0.000	0	0	0	0	0	0
{Unknown Order} P2-11E	0	0	0	0	0	0.008	0	0	0	0
{Unknown Order} SAR202	0	0	0	0	0	0	0	0.000	0	0
{Unknown Family} AKYG1722	0	0	0.002	0.001	0	0	0	0	0	0
{Unknown Family} JG30-KF-CM45	0	0	0.006	0.010	0.002	0.002	0	0	0	0
{Unknown Order} TK17	0	0	0	0.000	0	0	0	0	0	0
{Unknown Family} YS2	0.031	0	0.118	0.166	0.967	0.447	0	0	0.753	0.254
{Unknown Family} Stramenopiles	0	0	0	0.000	0	0	0	0	0	0

{Unknown Family} Streptophyta	0.018	0	0	0.041	0.000	0.124	7.05E-05	0.000	0	0.001
{Unknown Order} ML635J-21	0	0	0.000	0.004	0.000	0.002	0	0	0	0
{Unknown Genus} Gomphosphaeriaceae	0	0	0	0	0	0	0	0	0	0.000
Arthronema	0	0	0	0	0	0.009	0	0	0	0
Mucispirillum	0	0	0	0	0.002	0	0	0	0	0
{Unknown Genus} Elusimicrobiaceae	0	0	0	0.001	0.030	0.002	0	0	0	0.008
Elusimicrobium	0	0	0	0	0	0	0	0.000	0	0.244
{Unknown Class} FBP	0	0	0.000	0.000	0	0.000	0	0	0	0
{Unknown Family} 258ds10	0	0	0.001	0.001	0.000	0.014	0	0	0	0
{Unknown Genus} Fibrobacteraceae	0	0	0	0.000	0	0	0	0	0	0
Fibrobacter	0	0	0.001	0.008	0.047	0.113	0	0	0	0
{Unknown Family} Bacillales-1	0	0	0	5.48E-05	0.000	0	0	0	0	0
{Unknown Family} Bacillales-2	0.006	0	0.000	0.001	0.002	0.011	0.000	0	0	0
Exiguobacterium	0	0	0.000	0.000	0.001	0	0	0	0	0
{Unknown Genus} Bacillaceae	0	0	0.000	0.000	0.002	0	0	0	0	0
Bacillus	0.031	0	0.006	0.017	0.013	0.005	0	0	0	0
Marinococcus	0	0	0	0.000	0	0	0	0	0	0
Sinobaca	0	0	0	0.000	0.003	0	0	0	0	0
{Unknown Genus} Paenibacillaceae	0	0	0	0.000	0	0	0	0	0	0
Brevibacillus	0	0	0	0.000	0	0	0	0	0	0
Paenibacillus	0	0	0.000	0.000	0	0.002	0	0	0	0
{Unknown Genus} Planococcaceae-1	0	0	0	0	0.000	0	0	0	0	0
{Unknown Genus} Planococcaceae-2	0.006	0	0.001	0.003	0.025	0.001	3.53E-05	0	0	0
Kurthia	0	0	0.000	0.000	0.000	0	0	0	0	0
Lysinibacillus	0	0	0	0.000	0.151	0.027	0	0	0	0.001
Planococcus	0.043	0	0	0.002	0.175	0.006	0	0	0	0
Planomicrobium	0.156	0	0.003	0.027	1.881	0.097	0.000	0	0	0
Rummeliibacillus	0	0	0.000	0.002	0	0.000	0	0	0	0
Solibacillus	0.037	0	0.013	0.004	0.003	0.002	0	0	0	0
Sporosarcina	0	0	0.000	0.000	0.002	0	0	0	0	0
Viridibacillus	0	0	0	0.000	0	0	0	0	0	0

Jeotgalicoccus	0.031	0	0.001	0.013	0.165	0.011	0	0	0	0
Macrococcus	0	0	0.003	0.001	0.000	0	0	0	0	0
Salinicoccus	0	0	0.000	0.001	0.006	0.000	0	0	0	0
Staphylococcus	0.031	0	0.032	0.036	0.083	0.054	0.000	0	0	0.002
Shimazuella	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Gemellaceae	0	0	0	0	0	0.009	0	0	0	0
Gemella	0	0	0	0.000	0	0.000	0	0	0	0
{Unknown Family} Lactobacillales-1	0	0	0	0	0	0	0.001	0	0	0
{Unknown Family} Lactobacillales-2	0	0	0.001	0.009	0.002	0.002	0.049	0.000	9.55E-05	0
{Unknown Genus} Aerococcaceae	0.012	0	0.049	0.010	0.031	0.025	0	0	0	0
Aerococcus	0	0	0.019	0.004	0.000	0	0	0	0	0.001
Alkalibacterium	0	0	0	0.000	0.003	0	0	0	0	0
Alloiococcus	0	0	0	0.000	0	0	0	0	0	0
Facklamia	0.031	0	0.002	0.019	0.052	0.014	0	0	0	0
{Unknown Genus} Carnobacteriaceae	0.006	0	0.000	0.001	0	0.000	7.05E-05	0	0	0
Carnobacterium	0.106	0	0.000	0.012	0.024	0.013	0.000	0	0	0
Desemzia	0	0	0.000	0.001	0.021	0.001	0	0	0	0
Granulicatella	0	0	0	0.000	0.003	0.000	0.001	0	0	0
Isobaculum	0	0	0	0.000	0	0	0.000	0	0	0
Trichococcus	0	0	0.000	0.001	0.001	0.001	0	0	0	0
{Unknown Genus} Enterococcaceae	0	0	0	0	0	0	0.001	5.02E-05	0	0
Enterococcus	0.006	0	0.003	0.009	0.001	0.065	1.338	0.006	9.55E-05	0
Melissococcus	0	0	0	0	0	0	3.53E-05	0	0	0
Vagococcus	0	0	0.000	0.000	0.000	0	0.000	0	0	0
Lactobacillus	0.156	0.010	0.005	0.076	0.002	0.267	0.026	4.621	0.005	0.013
Pediococcus	0.068	0	0	0.000	0	0.000	0	0	0	0
{Unknown Genus} Leuconostocaceae	0.420	0	0.000	0.004	0	0	0	0	0	0
Leuconostoc	0.031	0.042	0	0.000	0	0	0	0	0	0
Oenococcus	0.012	0	0	0	0	0	0	0	0	0
Weissella	0.043	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Streptococcaceae	0.012	0.010	0	0.000	0	0	3.53E-05	0	0	0
Lactococcus	0	0.073	0.000	0.005	0	0.112	0.169	0.002	0	0

Streptococcus	0.050	0.010	10.374	2.286	0.011	0.357	0.333	1.078	0.003	0.004
Turicibacter	0.006	0	0.036	0.010	0.030	0.016	0.002	0.000	0	0.023
{Unknown Order} Clostridia-1	0	0	0	0	0	0	0.000	0	0	0
{Unknown Order} Clostridia-2	0	0	0.002	0.000	0.004	0.001	0.049	0	0	0.003
{Unknown Family} Clostridiales-1	0	0	0.001	0.005	0.015	0.011	0.006	5.02E-05	0.000	0.012
{Unknown Family} Clostridiales-2	3.667	0.231	0.976	0.501	3.409	1.952	0.004	0.426	1.065	1.894
Fusibacter	0	0	0	0.000	0	0.001	7.05E-05	0	0	0
{Unknown Genus} [Mogibacteriaceae]	2.683	0.063	0.101	0.038	0.201	0.076	0	0	0.018	0.060
Anaerovorax	0	0	0	0.000	0.000	0	0	0	0	0
Mogibacterium	0.012	0	0.044	0.018	0.015	0.011	0	0	0.001	0.002
{Unknown Genus} [Tissierellaceae]	0	0	0.000	0.004	0.003	0.004	0	0	0	0
Anaerococcus	0	0	0.001	0.000	0	0.027	0	0	0.000	0
Finegoldia	0.012	0	0	0.000	0	0.000	0	0.000	0	0
Gallicola	0	0	0	0.000	0	0	0	0	0	0
GW-34	0	0	0	0.000	0	0	0	0	0	0
Helcococcus	0	0	0.106	0.000	0	0	0	0	0	0
Parvimonas	0	0	0.055	0.000	0	0	0	0	0	0
Peptoniphilus	0	0	0.012	0.000	0	0.000	0	0	0	0
ph2	0	0	0	0.000	0	0	0	0	0	0
Sedimentibacter	0	0	0	0.000	0.000	0	0	0	0	0
Sporanaerobacter	0	0	0.000	0.000	0	0	0	0	0	0
Tissierella_Soehngenia	0	0	0	0.000	0.001	0	0	0	0	0
Caldicoprobacter	0	0	0.000	0	0.000	0	0	0	0	0
{Unknown Genus} Christensenellaceae	0	0	0.016	0.009	0.059	0.008	0	0	0	0
Christensenella	0	0	0	0	0	0	0	0	0	0.002
{Unknown Genus} Clostridiaceae-1	0	0	0	0	0	0	0.000	0	0	0
{Unknown Genus} Clostridiaceae-2	0.357	0	0.087	0.044	0.183	0.084	2.591	0.018	0.003	0.173
02d06	0	0	0	0.000	0.000	0.000	0	0	0	0.000
Candidatus Arthromitus	0.050	0.042	0	0	0	0.011	0	0	0	0
Clostridium-1	1.303	0.010	0.217	0.145	1.426	0.420	9.543	0.116	0.015	0.292
Proteiniclasticum	0	0	0.000	0.002	0.006	0.008	0	0	0	0
Sarcina	0	0	0.000	0.000	0.000	0	0.036	0	0	0.000
SMB53	0.025	0	0.102	0.039	0.127	0.058	0.590	5.02E-05	0.000	0.020

{Unknown Genus} Dehalobacteriaceae	0	0	0.001	0.000	0.006	0.000	0	0	0	0
Dehalobacterium	0	0	0.000	0.000	0.001	0.001	0	0	0	0
{Unknown Genus} EtOH8	0	0	0	0	0	0	0	0	0.000	0
Acetobacterium	0	0	0	0.000	0	0	0	0	0	0
Anaerofustis	0	0	0.001	0.000	0.004	0	0	0	0	0
Pseudoramibacter_Eubacterium	0	0	0.000	0.000	0.000	0.001	0	0	0.005	0.005
{Unknown Genus} Gracilibacteraceae	0	0	0	5.48E-05	0.001	0.001	0	0	0	0
{Unknown Genus} Lachnospiraceae-1	0	0	0	0.000	0	0	0	0	9.55E-05	0.000
{Unknown Genus} Lachnospiraceae-2	0.413	0	0.212	0.230	0.691	0.717	0.000	0.520	0.631	3.487
[Ruminococcus]	0.006	0	0	0.020	0.000	0.077	0.000	0.075	0.063	0.078
Anaerostipes	0	0	0.005	0.007	0.015	0.024	0	0	0	0.041
Blautia	0.012	0	0.007	0.152	0.009	0.324	0.001	0.275	1.248	1.494
Butyrivibrio	0.106	0	0.158	0.057	0.036	0.086	0	0	0.022	0.003
Clostridium-2	0	0	0	0	0	0	0	0	0	0.000
Coprococcus	0.006	0	0.016	0.018	0.040	0.066	0	0.000	0.043	0.171
Dorea	0.018	0	0.101	0.055	0.191	0.113	0	0.000	0.114	0.266
Epulopiscium	0.006	0	0.006	0.003	0.010	0.003	0.000	0	0	0
Lachnobacterium	0	0	0	0.001	0.000	0.007	0	0	0.040	0.128
Lachnospira	0	0	0	0	0.001	0	0	0	0.000	0.000
Moryella	0	0	0	0.000	0.001	0	0	0	0	0
Oribacterium	0	0	0	0.001	0	0	0	0	0	0
Pseudobutyrivibrio	0	0	0	0.000	0	0	0	0	0	0
Roseburia	0	0	0.002	0.016	0.005	0.025	0	5.02E-05	0.028	1.070
Shuttleworthia	0	0	0	0.000	0.000	0	0	0	0.001	0.000
{Unknown Genus} Peptococcaceae	0	0	0.011	0.008	0.051	0.030	0.000	0	0.014	0.031
Desulfotomaculum	0	0	0.000	0	0	0	0	0	0	0
Niigata-25	0	0	0	5.48E-05	0	0	0	0	0	0
Peptococcus	0	0	0.000	0	0	0	0	0	0	0
rc4-4	0.137	0	0.010	0.004	0.035	0.005	0	0	0	0.003
{Unknown Genus} Peptostreptococcaceae	0.056	0	0.081	0.026	0.120	0.041	0.078	0.000	0	0.043
[Clostridium]	0	0	0	0.001	0	0	0	0.008	0	0
Clostridium-3	0	0	0.000	5.48E-05	0.000	0	0.000	0	0	0
Filifactor	0	0	0	0.000	0	0	0	0	0	0
Peptostreptococcus	0	0	0.001	0.001	0.000	0.000	0	0	0	0

{Unknown Genus} Ruminococcaceae-1	0	0	0.000	0.000	0.017	0.004	0	0.000	0.002	0.000
{Unknown Genus} Ruminococcaceae-2	5.592	0.105	6.176	3.450	27.987	9.187	0.004	0.518	3.520	8.083
Butyricicoccus	0	0	0.002	0.101	0.001	0.115	0.004	7.868	0.358	0.004
Clostridium-4	0	0	0.000	0	0	0	0	0	9.55E-05	0
Ethanoligenens	0	0	0.006	0.008	0.054	0.004	0	0	0	0
Faecalibacterium	0	0	0.004	0.951	0.002	2.796	0.002	4.302	9.345	1.913
Oscillospira	0.112	0	0.239	0.169	1.060	0.475	0.000	0	0.553	0.422
Ruminococcus	0.037	0	0.497	0.151	0.686	0.250	7.05E-05	0.003	0.333	0.579
{Unknown Genus} Veillonellaceae-1	0	0	0	0	0	0	0	0	0	0.000
{Unknown Genus} Veillonellaceae-2	0	0	0.006	0.081	0.028	0.207	0.000	0.001	0.638	1.605
Acidaminococcus	0	0	0	0.001	0	0.002	0	0	0.095	0.000
Anaerovibrio	0	0	0.000	0.099	0.001	0.188	0.000	0.000	0.482	0.441
BSV43	0	0	0.000	0.000	0	0.006	0	0	0	0
Dialister	0	0	0	0.000	0	0	0	0.004	0.003	0.001
Megamonas	0	0	0.002	0.307	0	0.615	0.001	0.006	2.630	0.017
Megasphaera	0	0	0	0.003	0	0.059	0	0	0.066	0.044
Mitsuokella	0	0	0	0.000	0	0.004	0	0	0.007	0.003
Pelosinus	0	0	0	0.000	0	0	0	0	0	0
Phascolarctobacterium	0.018	0	0.135	0.130	0.601	0.376	0.000	0.000	0.523	0.516
Selenomonas	0	0	0	0.000	0.001	0	0	0	0	0
Succiniclasticum	0	0	0	0.001	0.001	0.010	0	0	0.007	0.000
Veillonella	0	0	0.001	0.345	0.002	0.017	0.001	7.287	0.001	0.001
{Unknown Genus} Erysipelotrichaceae	0.006	0	0.032	0.138	0.118	0.139	0	0.005	0.484	0.744
[Eubacterium]	0	0	0.013	0.017	0.125	0.068	0	0.000	0.276	0.348
Allobaculum	0.006	0	0	0	0	0.126	7.05E-05	0	0	0
Bulleidia	0	0	0	0	0	0.002	0	0	0.001	0.001
Clostridium-5	0	0	0	0.000	0	0.000	0	0	0	0.031
Coprobacillus	0	0	0.001	0.002	0.025	0.002	7.05E-05	0	0	0.365
Erysipelothrix	0	0	0.000	0.002	0.009	0.002	0	0	0	0
gut	0	0	0.001	0.004	0.018	0.003	0	0	0	0.000
Holdemania	0	0	0	0	0	0	0	0	0.000	0.009
L7A_E11	0	0	0.001	0.001	0.000	0	0	0	0	0
p-75-a5	0	0	0.001	0.002	0.005	0.003	0	0	0.000	0.024
RFN20	0	0	0.001	0.003	0.007	0.011	0	0	0.004	0.008
Sharpea	0	0	0.001	0.522	0.000	0.496	0.000	0.003	1.377	8.464

{Unknown Order} OPB54	0	0	0.001	0.000	0.003	0.002	0	0	0	0
{Unknown Genus} Fusobacteriaceae-1	0	0	0	0	0	0	0	0.000	0	0
{Unknown Genus} Fusobacteriaceae-2	0	0	0.000	0.000	0.000	0.000	0.000	0.866	0.092	0.000
Fusobacterium	0.012	0	0.314	0.121	0.000	0.001	0.000	2.963	0.034	0.006
{Unknown Genus} Leptotrichiaceae	0	0	0.001	0.002	0	0	0	0	0	0
Leptotrichia	0	0	0	0.066	0	0	0	0	0	0
{Unknown Order} Gemm-1	0	0	0.001	0.000	0	0.006	0	0	0	0
{Unknown Order} Gemm-2	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Order} Gemm-3	0	0	0.000	0.000	0	0.008	0	0	0	0
{Unknown Order} Gemm-5	0	0	0	0.000	0	0	0	0	0	0
{Unknown Family} Gemmatimonadales	0	0	0.000	0.000	0	0.001	0	0	0	0
Gemmatimonas	0	0	0	5.48E-05	0	0	0	0	0	0
{Unknown Family} N1423WL	0	0	0	0	0	0.006	0	0	0	0
{Unknown Class} GN02	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Order} 3BR-5F	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Order} BD1-5	0	0	0	0.011	0	0	0	0	0	0
{Unknown Class} GN04	0	0	0	0.000	0	0	0	0	0	0
{Unknown Class} LD1	0	0	0	0	0.000	0	0	0	0	0
{Unknown Genus} Victivallaceae	0	0	0.045	0.059	0.103	0.093	0.000	0.000	0	0.002
Victivallis	0	0	0.017	0.017	0.039	0.027	0	0	0	0.000
{Unknown Genus} R4-45B	0	0	0.000	0.001	0.001	0.006	0	0	0	0
Leptospirillum	0	0	0	0.000	0	0	0	0	0	0
4-29	0	0	0	0	0	0.013	0	0	0	0
Nitrospira	0	0	0	0	0.000	0	7.05E-05	0	0	0
{Unknown Order} TSBW08	0	0	0	0.000	0.000	0.000	0	0	0	0
{Unknown Class} OD1	0	0	0	0.000	0	0	0	0	0	0
{Unknown Order} ABY1	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Order} SM2F11	0	0	0	0.000	0	0	0	0	0	0
{Unknown Order} ZB2	0	0	0.001	0.002	0	0.011	0	0	0	0
{Unknown Class} OP11	0	0	0.000	0.001	0	0	0	0	0	0
{Unknown Family} d153	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Family} agg27	0	0	0	0.000	0	0	0	0	0	0
{Unknown Family} MSBL9	0	0	0	0	0	0.004	0	0	0	0
{Unknown Family} Phycisphaerales	0	0	0	0.000	0	0	0	0	0	0

{Unknown Genus} Phycisphaeraceae	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Family} WD2101	0	0	0	0.000	0	0.019	0	0	0	0
{Unknown Genus} Gemmataceae	0	0	0	0.000	0	0	0	0	0	0
Gemmata	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Pirellulaceae	0.006	0	0.031	0.021	0.066	0.054	0.000	0	0	0
A17	0	0	0	0.000	0	0	0	0	0	0
Rhodopirellula	0	0	0	5.48E-05	0	0	0	0	0	0
Planctomyces	0	0	0.004	0.008	0.000	0.002	0	0	0	0
{Unknown Order} Alphaproteobacteria	0	0	0.033	0.027	0.309	0.102	0	0	0	0
{Unknown Family} BD7-3	0.006	0.010	0.000	0.007	0.001	0.023	0	0	0	0
{Unknown Family} Caulobacterales	0	0	0	0	0	0	0	0	0	0.000
{Unknown Genus} Caulobacteraceae	0	0	0.000	0.010	0.009	0.106	0	0	0	0
Asticcacaulis	0	0	0	0.000	0	0.000	0	0	0	0
Brevundimonas	0	0	0.000	0.005	0.001	0.049	0	0	0	0
Caulobacter	0	0	0	0.000	0	0.004	0	0	0	0
Mycoplana	0	0	0.000	0.004	0.006	0.050	0	0	0	0
Nitrobacteria	0	0	0	0.001	0.000	0.012	0	0	0	0
Phenylobacterium	0	0	0	0.000	0.000	0.009	0	0	0	0
{Unknown Family} Kiloniellales	0	0	0	0.000	0	0	0	0	0	0
Thalassospira	0	0	0.001	0.000	0	0	0	0	0	0
{Unknown Family} RF32	0.012	0	0.031	0.037	0.070	0.065	0	0	0.051	0.116
{Unknown Family} Rhizobiales-1	0	0	0	0.000	0	0	0	0	0	0
{Unknown Family} Rhizobiales-2	0.006	0	0.004	0.004	0.002	0.003	0	0	0	0
{Unknown Genus} Aurantimonadaceae	0.006	0	0.000	0	0.000	0	0	0	0	0
{Unknown Genus} Beijerinckiaceae	0	0	0	0.001	0.000	0.004	0	0	0	0
Chelatococcus	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Bradyrhizobiaceae-1	0	0	0	0.000	0	0	0	0	0	0

{Unknown Genus} Bradyrhizobiaceae-2	0.006	0.021	0	0.000	0	0	0	0	0	0
Balneimonas	0	0	0	0.000	0	0.000	0	0	9.55E-05	0
Bosea	0	0	0	0.000	0	0.005	0	0	0	0.000
Ochrobactrum	0.194	0.472	0.000	0.006	0.001	0.036	0	0	0	0.000
Paenochrobactrum	0	0	0.000	0.000	0	0.004	0	0	0	7.8E-05
Pseudochrobactrum	0	0	0	0.000	0.000	0.004	0	0	0	0
{Unknown Genus} Hyphomicrobiaceae	0	0	0	0.000	0	0	0	0	0	0
Devosia	0.006	0	0.005	0.023	0.016	0.084	0	0	0	0
Hyphomicrobium	0	0	0.000	0.000	0.000	0	0	0	0	0
Parvibaculum	0	0	0	0	0	0.001	0	0	0	0
Rhodoplanes	0	0	0.000	0.000	0.000	0.003	0	0	0	0
{Unknown Genus} Methylobacteriaceae	0	0	0	0	0	0.001	0	0	0	0
Methylobacterium	0	0	0	0.001	0.000	0.000	0	0	0	0
{Unknown Genus} Methylocystaceae	0	0	0	0.000	0	0	0	0	0	0
Pleomorphomonas	0	0	0	0	0	0.000	0	0	0	0
{Unknown Genus} Phyllobacteriaceae	0	0	0.000	0.004	0.003	0.007	0	0	0	0
Aminobacter	0	0	0	0.001	0.002	0.007	0	0	0	0
Aquamicrobium	0	0	0.000	0.009	0.004	0.023	0	0	0	0.000
Chelativorans	0	0	0	0.000	0	0	0	0	0	0
Mesorhizobium	0	0	0	0.000	0.000	0	0	0	0	0
Nitratireductor	0	0	0.000	0.000	0.000	0.000	0	0	0	0
Phyllobacterium	0	0	0	0	0	0.000	0	0	0	0
{Unknown Genus} Rhizobiaceae	0	0	0	0.002	0.002	0.008	0	0	0	0
Agrobacterium	0	0	0.001	0.016	0.005	0.042	0	0	0	0
Kaistia	0	0	0	0.000	0.000	0.001	0	0	0	0
Rhizobium	0	0	0	0.000	0.000	0	0	0	0	0
Shinella	0	0	0	5.48E-05	0	0	0	0	0	0
Ancylobacter	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Hyphomonadaceae	0	0	0	0	0.000	0.000	0	0	0	0
Hyphomonas	0.062	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Rhodobacteraceae-1	0	0	0	5.48E-05	0	0	0	0	0	0

{Unknown Genus} Rhodobacteraceae-2	0.112	0.021	0.012	0.039	0.011	0.281	0	0	0	0
Amaricoccus	0.006	0	0.001	0.001	0	0.000	0	0	0	0
Anaerospora	0	0	0.000	0.001	0.000	0.014	0	0	0	0
Oceaniovalibus	0	0	0.000	0.000	0.000	0	0	0	0	0
Octadecabacter	0	0	0.000	0.000	0	0.001	0	0	0	0
Paracoccus	0	0	0.002	0.019	0.031	0.061	0	0	0	0
Rhodobacter	0.006	0	0.000	0.006	0.003	0.047	0	0	0	0
Rubellimicrobium	0	0	0	0.000	0	0	0	0	0	0
Tropicibacter	0	0	0	0	0	0.000	0	0	0	0
{Unknown Family} Rhodospirillales	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Acetobacteraceae	0.006	0	0.000	0.001	0.002	0.001	0	0	0	0
Acetobacter	0.012	0	0	0.000	0	0.000	0	0	0	0
Acidisoma	0	0	0	0.000	0	0	0	0	0	0
Gluconobacter	0	0	0	0.005	0	0	0	0	0	0
Granulibacter	0	0	0	0.000	0	0	0	0	0	0
Roseococcus	0	0	0	0	0	0.001	0	0	0	0
{Unknown Genus} Rhodospirillaceae	0	0	0	0.000	0	0	0	0	0	0
Azospirillum	0	0	0	0	0	0	0	0.000	0	0
Novispirillum	0	0	0	0.000	0	0	0	0	0	0
Oceanibaculum	0	0	0.000	0.000	0	0	0	0	0	0
Skermanella	0	0	0	0.000	0	0	0	0	0	0
{Unknown Family} Rickettsiales	0.313	0	0.002	0.001	0.008	0.002	0	0	0	0
{Unknown Genus} mitochondria	0	0	0	0.001	0	0	0	0	0	0
Hedyosmum	0	0	0	0.000	0	0.000	0	0	0	7.8E-05
Podophyllum	0	0	0	0.000	0	0.004	0	0	0	0
Pythium	0	0	0	0.000	0	0.000	0	0	0	0
Raphanus	0	0	0	0.000	0	0	0	0	0	0
Zea	0	0	0	0.000	0	0	0	0	0	0
{Unknown Family} Sphingomonadales	0	0	0.000	0.005	0.000	0.019	0	0	0	0
{Unknown Genus} Erythrobacteraceae	0	0	0.005	0.009	0.006	0.030	0	0	0	0
Erythrobacter	0	0	0	5.48E-05	0	0	0	0	0	0
Lutibacterium	0	0	0	0.000	0.000	0.004	0	0	0	0

{Unknown Genus} Sphingomonadaceae	0	0	0.001	0.008	0.001	0.062	0	0	0	0
Blastomonas	0	0	0	0	0	0.000	0	0	0	0
Kaistobacter	0	0	0	0.000	0	0.000	0	0	0	0
Novosphingobium	0	0	0.000	0.016	0.006	0.227	0	0	0	0
Sphingobium	0.012	0	0	0.010	0.002	0.066	0	0	0	0
Sphingomonas	0	0	0.000	0.004	0.006	0.008	0	0	0	0
Sphingopyxis	0	0	0.002	0.053	0.007	0.217	0	0	0	0
{Unknown Order} Betaproteobacteria-1	0	0	0	0	0	0.000	0	0	0	0
{Unknown Order} Betaproteobacteria-2	0	0	0	0.000	0	0.004	0.000	0.000	9.55E-05	0.000
{Unknown Genus} UD5	0	0	0	0	0	0.002	0	0	0	0
{Unknown Family} ASSO-13	0	0	0	0.000	0	0	0	0	0	0
{Unknown Family} Burkholderiales	0.006	0	0.000	5.48E-05	0	0.015	0.000	0	0	0
{Unknown Genus} Alcaligenaceae-1	0	0	0	0	0	0	0	0	0	7.8E-05
{Unknown Genus} Alcaligenaceae-2	0.006	0	0.003	0.036	0.008	0.291	0	0	0.001	0.015
Achromobacter	0	0	0	0.002	0	0.017	0	0	0	0
Alcaligenes	0	0	0.000	0.001	0	0.019	0	0	0	0
Brackiella	0	0	0.000	0	0	0	0	0	0	0
Kerstersia	0	0	0	5.48E-05	0	0.000	0	0	0	0
Oligella	0	0	0	0.001	0	0.000	0	0	0	0
Pelistega	0	0	0	0.000	0	0.002	0	0	9.55E-05	0.002
Pigmentiphaga	0	0	0	0.000	0	0.005	0	0	0	0
Rhodospirillum	0	0	0	0.000	0	0.007	0	0	0	0
Sutterella	0.018	0	0.029	0.084	0.068	0.218	0.000	0.313	1.323	2.534
Tetrathiobacter	0	0	0	0.000	0	0.000	0	0	0	0
{Unknown Genus} Burkholderiaceae	0	0	0.001	0.000	0.000	0	0	0	0	0
Burkholderia	0.131	0.010	0	0.003	0.000	0.002	0	0	0	0
Lautropia	0	0	0	0.036	0	0	0	0	0	0
{Unknown Genus} Comamonadaceae	0.068	0	0.003	0.052	0.012	0.454	7.05E-05	0.000	0	0
Acidovorax	0	0	0	0.000	0	0.008	0	0	0	0
Alicycliphilus	0	0	0	0	0	0.000	0	0	0	0
Aquabacterium	0	0	0	0	0	0.000	0	0	0	0
Comamonas	0.188	0.010	0.007	0.089	0.011	0.700	0	0.004	0.001	0

Delftia	0	0	0	0.000	0.000	0.002	0	0	0	0
Diaphorobacter	0	0	0	0.000	0	0	0	0	0	0
Giesbergeria	0.006	0	0	0.000	0.000	0.007	0	0	9.55E-05	0
Hydrogenophaga	0	0	0.000	0.008	0.001	0.013	0	0.000	0.000	0
Hylemonella	0	0	0.000	0.001	0.000	0.003	0	0	0	0
Lampropedia	0	0	0.000	0.001	0.000	0.029	0	0	0	0
Leptothrix	0	0	0	5.48E-05	0	0	0	0	0	0
Limnohabitans	0	0	0	0.002	0.000	0.004	0	0	0	0
Methylibium	0	0	0	0.000	0	0.001	0	0	0	0
Polaromonas	0	0	0	0.001	0	0.029	0	0	0	0
Ramlibacter	0	0	0	0.000	0.000	0.016	3.53E-05	0	0	0
Rhodoferax	0	0	0	0.000	0.000	0.011	0	0	0	0
Roseateles	0	0	0	0.000	0	0	0	0	0	0
Rubrivivax	0	0	0	0	0	0.000	0	0	0	7.8E-05
Simplicispira	0	0	0	0.000	0.000	0.003	0	0	0	0
Tepidimonas	0	0	0	0	0	0.000	0	0	0	0
Variovorax	0	0	0	0.001	0	0.004	0	0	0	0
Xylophilus	0	0	0.000	0.001	0	0.008	0	0	0	0
{Unknown Genus} Oxalobacteraceae	0	0	0.002	0.022	0.009	0.184	7.05E-05	0	0	0
Cupriavidus	0	0	0	0	0	0.000	0	0	0	0
Janthinobacterium	0	0	0.001	0.011	0.003	0.254	0	0	0	0
Massilia	0	0	0.000	0	0	0	0	0	0	0
Oxalobacter	0	0	0	0	0	0.001	0	0	0	0
Ralstonia	0.075	0	0	0	0	0	0	0	0	0
{Unknown Family} Ellin6067	0	0	0	0	0.000	0.004	0	0	0	0
Gallionella	0.018	0	0	0	0	0	0	5.02E-05	0.000	7.8E-05
{Unknown Family} Methylophilales	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Methylophilaceae	0.025	0.010	0.000	0.013	0.003	0.055	0	0	0	0
Methylobacillus	0	0	0	0.000	0	0	0	0	0	0
Methylotenera	0	0.010	0	0.000	0.000	0.002	0	0	0	0
{Unknown Family} MKC10	0	0	0	0.000	0	0.003	0	0	0	0
{Unknown Genus} Neisseriaceae-1	0	0	0	5.48E-05	0	0	0	0	0	0
{Unknown Genus} Neisseriaceae-2	0	0	0	0.641	0	0.012	3.53E-05	0.000	0	0
Alysiella	0	0	0.000	3.572	0.000	0.000	0.000	0.000	0	0.000
Bergeriella	0	0	0	0.133	0	0	0	0	0	0

Conchiformibius	0	0	0	0.070	0	0	0	0	0	0
Eikenella	0	0	0	0.013	0	0.003	0	0	0	0
Kingella	0	0	0.000	0.412	0.005	0.010	0	0.000	0	0
Neisseria	0	0	0.000	1.112	0	0.001	3.53E-05	0.000	0	0
Vitreoscilla	0	0	0	0.000	0	0.001	0	0	0	0
{Unknown Family} Nitrosomonadales	0	0	0	0.000	0.001	0	0	0	0	0
{Unknown Genus} Nitrosomonadaceae	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Procabacteriaceae	0	0	0	0.000	0.003	0.006	0	0	0	0
{Unknown Genus} Rhodocyclaceae	0.031	0	0.001	0.024	0.005	0.446	0	5.02E-05	0	7.8E-05
Azospira	0	0	0	0	0	0	0	0	0	7.8E-05
Dechloromonas	0.006	0	0	0.000	0	0.003	0	5.02E-05	0	0
KD1-23	0	0	0.000	0.000	0	0.009	0	0	0	0
Propionivibrio	0.037	0	0	0	0	0	0	0	0.000	0
Thauera	0	0	0.004	0.003	0.000	0.001	0	0	0	0
{Unknown Family} SC-I-84	0	0	0	0	0	0.000	0	0	0	0
{Unknown Order} Deltaproteobacteria	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Bacteriovoracaceae	0	0	0	0.002	0.000	0.013	0	0	0	0
Bacteriovorax	0	0	0	0.001	0	0.001	0	0	0	0
Peredibacter	0	0	0	0.000	0	0.002	0	0	0	0
Bdellovibrio	0	0	0.001	0.002	0.000	0.000	0	0	0	0
{Unknown Genus} Desulfarculaceae	0	0	0	0.000	0	0	0	0	0	0
Desulfobulbus	0	0	0	0.000	0.000	0	0	0	0	0
Desulfomicrobium	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Desulfovibrionaceae	0.006	0	0.094	0.076	0.534	0.393	0.000	0	0.008	0.038
Bilophila	0	0	0	0.000	0.000	0.000	0	0	0.000	0
Desulfovibrio	0	0	0	0.001	0	0	0	0	0.019	0.106
{Unknown Genus} Desulfuromonadaceae	0	0	0	5.48E-05	0	0	0	0	0	0
{Unknown Genus} Geobacteraceae	0	0	0	0	0	0.000	0	0	0	0
Geobacter	0	0	0	0	0	0.006	0	0	0	0

{Unknown Genus} Pelobacteraceae	0	0	0	0.000	0	0	0	0	0	0
{Unknown Family} GMD14H09	0.025	0	0.000	0.001	0.000	0.020	0	0	0	0
{Unknown Family} GW-28	0	0	0	0	0.000	0	0	0	0	0
{Unknown Family} MBNT15	0	0	0	0	0	0.000	0	0	0	0
{Unknown Family} MIZ46	0	0	0	0.000	0	0	0	0	0	0
{Unknown Family} Myxococcales	0	0	0.002	0.002	0	0.001	0	0	0	0
{Unknown Genus} Cystobacterineae	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Haliangiaceae	0	0	0	0	0.000	0	0	0	0	0
{Unknown Genus} Myxococcaceae	0	0	0	0.000	0	0	0	0	0	0
Anaeromyxobacter	0	0	0.000	0	0	0	0	0	0	0
Nannocystis	0	0	0	0.000	0	0	0	0	0	0
Plesiocystis	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} OM27	0	0	0.002	0.003	0.000	0	0	0	0	0
{Unknown Genus} Polyangiaceae	0	0	0	0.000	0	0	0	0	0	0
{Unknown Family} PB19	0	0	0.000	0	0	0	0	0	0	0
{Unknown Family} Spirobacillales	0	0	0.000	0.001	0.000	0.001	0	0	0	0
{Unknown Genus} Syntrophaceae	0	0	0	0.000	0	0	0	0	0	0
Syntrophus	0	0	0	0.000	0.000	0	0	0	0	0
{Unknown Genus} Syntrophobacteraceae	0	0	0	0.000	0.000	0	0	0	0	0
Arcobacter	0	0	0.000	0.001	0.000	0.006	0	0	0	0
Campylobacter	0	0	0.002	0.002	0.006	0.006	0	0.000	0	0
Sulfurospirillum	0	0	0	0.000	0	0	0	0	0	0
{Unknown Order} Gammaproteobacteria-1	0	0	0.000	5.48E-05	0	0	3.53E-05	0	0	0
{Unknown Order} Gammaproteobacteria-2	0	0	0	0.000	0	0.001	0	0	0	0
{Unknown Family} 34P16	0	0	0	0.000	0	0.000	0	0	0	0
{Unknown Genus} [Marinicellaceae]	0	0	0	0.000	0	0	0	0	0	0
Marinicella	0.006	0	0.003	0.003	0	0	0	0	0	0

{Unknown Genus} Aeromonadaceae	0.112	0.819	0.000	0.005	0.001	0.056	0.033	5.02E-05	0	0
Aeromonas	0	0	0	0	0	0	3.53E-05	0	0	0
Zobellella	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Succinivibrionaceae	0.012	0	0.001	0.009	0.005	0.001	0	0	0.033	0.010
Ruminobacter	0.144	0	0.012	0.020	0.046	0.003	0	0	0	0.054
Succinivibrio	0.006	0	0.006	0.348	0.002	2.002	0.002	0.003	21.93	8.343
{Unknown Family} Alteromonadales	0	0	0	0.002	0.000	0.007	0	0	0	0
{Unknown Genus} 211ds20	0	0	0.009	0.009	0.001	0.009	0	0	0	0
{Unknown Genus} [Chromatiaceae]-1	0	0	0	0	0	0.000	0	0	0	0
{Unknown Genus} [Chromatiaceae]-2	0	0	0.000	0.014	0.006	0.571	0	0	0	0
Rheinheimera	0.018	0	0.000	0.006	0.001	0.238	0	0	0	0
{Unknown Genus} Alteromonadaceae	0.006	0	0.009	0.021	0.003	0.024	0	0	0	0
BD2-13	0.018	0	0.000	0.003	0.000	0.023	0	0	0	0
Candidatus Endobugula	0	0	0.000	0.001	0	0.009	0	0	0	0
Cellvibrio	0.043	0.010	0.012	0.045	0.019	0.394	0	0	0	0
Gilvimarinus	0	0	0.000	0.000	0	0	0	0	0	0
Marinobacter	0	0	0.001	0.003	0	0.006	0	0	0	0
Microbulbifer	0	0	0	0.000	0	0	0	0	0	0
ND137	0	0	0.001	0.000	0	0	0	0	0	0
nsmpVI18	0	0	0	0.000	0	0	0	0	0	0
Simiduia	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} HTCC2188	0	0	0	0.000	0	0	0	0	0	0
HTCC	0	0	0.000	0.001	0.001	0.006	0	0	0	0
{Unknown Genus} Idiomarinaceae	0	0	0	0.002	0	0.000	0	0	0	0
Pseudidiomarina	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} J115	0.006	0	0.000	0.002	0	0.001	0	0	0	0
{Unknown Genus} OM60	0	0	0.000	0.000	0	0.000	0	0	0	0
Haliea	0	0	0.000	0.000	0	0	0	0	0	0
Psychromonas	0	0	0	0.001	0	0	0	0	0	0
Shewanella	0.012	0	0.001	0.013	0.002	0.134	0	0	0	0
{Unknown Genus} Cardiobacteriaceae	0	0	0	0.000	0	0	0	0	0	0
Suttonella	0	0	0	0.002	0	0	0	0	0	0

Chromatium	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Enterobacteriaceae-1	0	0	0.006	0.002	0.001	0.000	0.036	0.006	0.000	0.000
{Unknown Genus} Enterobacteriaceae-2	0.971	0.210	0.361	0.427	0.142	1.811	83.943	18.242	0.521	0.083
Aquamonas	0	0	0	0	0	0	0.000	0	9.55E-05	0
Citrobacter	0.006	0	0.000	0.001	0.000	0.004	0.501	0.011	9.55E-05	0
Cronobacter	0	0	0.004	0.010	0.000	0.003	0.175	0.020	0.001	0.000
Enterobacter	0	0	0	0.000	0	0.000	0.024	0.002	0	0.000
Erwinia	0.037	0	0.002	0.008	0.000	0.024	0.026	0.000	9.55E-05	7.8E-05
Escherichia	0	0	0	0.000	0.000	0.000	0.057	0.010	9.55E-05	0
Ewingella	1.278	3.604	0	0.000	0	0.000	0	0	0	0.000
Gluconacetobacter	0	0.010	0	0	0	0	0	0	0	0
Klebsiella	0.313	0.136	0	0	0	0	0.002	0.001	0	0
Leminorella	0	0	0.000	0	0	0	0	0	0	0
Morganella	0	0	0	0	0	0	0.000	0.000	0	0
Pantoea	0	0	0.001	0.000	0	0.000	0.006	0.001	9.55E-05	7.8E-05
Photorhabdus	0	0	0.000	0	0	0	0	0	0	0
Pragia	0	0	0	0	0	0	3.53E-05	0	0	0
Proteus	0	0	0	0	0	0.000	0.000	0.000	0	0
Providencia	0	0	0	0	0	0.000	0	0	0	0
Rahnella	0	0.031	0	0	0	0	0	0	0	0
Raoultella	0	0	0	0	0	0.000	0.000	0	0	0
Salmonella	0	0	0	0	0	0	0.000	0	0	0
Serratia	0.012	0	0.001	0.001	0.000	0.008	0.233	0.004	0.000	0.000
Shigella	0	0	0.000	0.000	0	0.001	0.026	0.007	9.55E-05	7.8E-05
Sodalis	0	0	0	0.000	0	0.000	7.05E-05	0	0	0
Trabulsiella	0	0	0	0.000	0	0	0.014	5.02E-05	0	0
Xenorhabdus	0	0	0	0.000	0	0.000	0.000	0	0	0
Yersinia	0.633	14.555	0	0.025	0.002	0.061	7.05E-05	0	0	0.000
{Unknown Family} Legionellales	0	0	0.000	0.000	0	0	0	0	0	0
Rickettsiella	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Legionellaceae	0	0	0.001	0.002	0.000	0	0	0	0	0
Legionella	0	0	0	0.000	0	0.000	0	0	0	0
Methylocaldum	0	0	0	0	0	0.000	0	0	0	0
Alcanivorax	0	0	0	0	0	0.000	0	0	0	0
{Unknown Genus} Halomonadaceae	0	0	0.001	0.005	0.000	0.040	0	0	0	0

Candidatus Portiera	0	0	0.000	0.008	0.001	0.003	0	0	0	0
Halomonas	0.112	0	0	0.003	0.000	0.002	0	0	0	0
{Unknown Genus} Oceanospirillaceae	0	0	0	5.48E-05	0	0.008	0	0	0	0
Marinobacterium	0	0	0	0.000	0	0	0	0	0	0
Marinomonas	0	0	0	0.000	0	0	0	0	0	0
Oleibacter	0	0	0.000	0.003	0	0.006	0	0	0	0
{Unknown Genus} Saccharospirillaceae	0	0	0	0.000	0	0	0	0	0	0
Reinekea	0	0	0.000	0	0	0	0	0	0	0
{Unknown Genus} SUP05	0	0	0	0	0	0.000	0	0	0	0
{Unknown Genus} Pasteurellaceae-1	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Genus} Pasteurellaceae-2	0	0	55.314	0.826	0.002	0.000	0.002	0.001	0.002	0.002
Actinobacillus	0	0	0.001	0.509	0.000	0.005	3.53E-05	0.002	0	0
Aggregatibacter	0	0	0.963	0.749	0.000	0.005	0.000	0.000	0	0
Avibacterium	0	0	0	0.011	0	0	0	0	0	0
Bibersteinia	0	0	0.002	25.030	0.003	0.031	0.003	0.002	0.001	0.002
Gallibacterium	0	0	0.145	0.047	0.001	0.000	0.000	0.502	0.000	0.003
Haemophilus	0.043	0	0.000	0.035	0	0.039	0	0.000	0	0
Mannheimia	0	0	0.086	4.041	0	0.004	0.001	0.000	9.55E-05	0.000
Nicoletella	0	0	0	5.48E-05	0	0	0	0	0	0
Pasteurella	0.006	0	0	0.001	0	0	0	0	0	0
{Unknown Family} Pseudomonadales	0	0	0	0	0	0.000	0	0	0	0
{Unknown Genus} Moraxellaceae-1	0	0	0	0.000	0.000	0.000	0	0	0	0
{Unknown Genus} Moraxellaceae-2	0.539	0	0.011	0.336	0.423	0.911	0	0.000	0	0
Acinetobacter	26.474	0.126	0.435	8.339	0.278	9.589	0.003	0.000	0.000	0.000
Alkanindiges	0	0	0	0.000	0	0.000	0	0	0	0
Enhydrobacter	0.006	0	0.013	0.095	0	0.031	0	0	0	0.001
Moraxella	0	0	0.048	20.578	0.005	0.021	0.003	0.001	0.001	0.001
Perlucidibaca	0.006	0	0	0.001	0.000	0.000	0	0	0	0
Psychrobacter	0	0	0.002	0.034	0.014	0.049	0	0	0	0
{Unknown Genus} Pseudomonadaceae-1	0	0	0	0	0	0.000	0	0	0	0
{Unknown Genus} Pseudomonadaceae-2	1.561	28.670	0.016	0.155	0.026	1.907	0.000	0	0	0.001

Azomonas	0	0	0	0.000	0	0	0	0	0	0
Pseudomonas	1.253	4.498	0.031	0.437	0.047	6.760	0.000	0	0.000	0.001
Serpens	0.056	0	0.001	0.003	0.000	0.011	3.53E-05	0	0	0
{Unknown Genus} Piscirickettsiaceae	0.006	0	0.001	0.001	0	0.000	0	0	0	0
Methylophaga	0	0	0.000	0.002	0.000	0.007	0	0	0	0
{Unknown Genus} Pseudoalteromonadaceae	0	0	0	0.000	0	0	0	0	0	0
Pseudoalteromonas	0	0	0.001	0.004	0	0	0	0	0	0
Vibrio	3.335	0.147	0	0.000	0	0.001	0	0	0	0
{Unknown Genus} Sinobacteraceae	0	0	0.002	0.006	0.001	0.010	7.05E-05	0	0	0
Hydrocarboniphaga	0	0	0	0.000	0	0	0	0	0	0
Steroidobacter	0	0	0	0.000	0	0	7.05E-05	0	0	0
{Unknown Genus} Xanthomonadaceae-1	0	0	0.000	0	0	0	0	0	0	0
{Unknown Genus} Xanthomonadaceae-2	0.131	0.042	0.007	0.048	0.009	0.132	0	0	0	0
Arenimonas	0	0	0	0.000	0.000	0	0	0	0	0
Dokdonella	0	0	0.000	0.003	0.002	0.012	0	0	0	0
Dyella	0	0	0	0.000	0	0.000	0	0	0	0
Fulvimonas	0	0	0	0.000	0.000	0	0	0	0	0
Ignatzschineria	0	0	0.000	0.000	0	0.000	0	0	0	0
Luteibacter	0.006	0	0	0.004	0.001	0.014	0	0	0	0
Luteimonas	0.062	0	0.011	0.057	0.071	0.236	0	0	0	0
Lysobacter	0	0	0	0.002	0.002	0.005	0	0	0	0
Pseudofulvimonas	0	0	0	0.000	0.000	0.003	0	0	0	0
Pseudoxanthomonas	0.006	0	0	0.000	0.000	0.003	0	0	0	0
Rhodanobacter	0	0	0	0.000	0.001	0.000	0	0	0	0
Stenotrophomonas	9.015	42.722	0.005	0.084	0.011	0.469	0	0	0	0
Thermomonas	0.012	0	0.000	0.008	0.004	0.023	0	0	0	0
{Unknown Family} PHOS-HD29	0	0	0	0.000	0	0	0	0	0	0
{Unknown Family} M2PT2-76	0	0	0.015	0.068	0.049	0.323	7.05E-05	0	0	0
Sphaerochaeta	0	0	0.000	0.002	0.019	0.007	0	0	0	0
Treponema	0.231	0.010	0.033	0.109	1.285	0.296	0.000	0.000	0.002	8.319
Synergistes	0	0	0	0	0	0	0	0	0	0.001
vadinCA02	0	0	0	0	0.001	0	0	0	0	0
{Unknown Genus} Acholeplasmataceae	0	0	0	0	0.000	0	0	0	0	0

Acholeplasma	0.006	0	0.000	0.007	0.000	0.009	0	0	0	0
{Unknown Genus} Anaeroplasmataceae	0	0	0.004	0.003	0.014	0.002	0	0	0	0
Anaeroplasma	0	0	0.001	0.003	0.017	0.002	0	0	0	0
{Unknown Genus} Mycoplasmataceae	0	0	0	0.000	0	0	0	0	0	0
Mycoplasma	0	0	0.000	1.635	0	0	0.000	0.000	0	0
Ureaplasma	0	0	11.962	0.000	0.000	0.339	0.000	0.000	0.000	0
{Unknown Family} RF39	0.012	0	0.153	0.108	0.288	0.497	0.000	5.02E-05	0.874	0.562
{Unknown Family} ML615J-28	0.257	0	0.024	0.017	0.113	0.069	0	0	0	0
{Unknown Order} SJA-4	0	0	0	0.000	0	0	0	0	0	0
{Unknown Class} TM7	0	0	0	0.000	0	0	0	0	0	0
{Unknown Order} TM7-1	0	0	0	0.000	0	0.000	0	0	0	0
{Unknown Family} [Pedosphaerales]	0	0	0	0	0	0.017	0	0	0	0
{Unknown Genus} Ellin515	0	0	0	0	0	0.017	0	0	0	0
{Unknown Genus} [Chthoniobacteraceae]	0	0	0	0.000	0.000	0.000	0	0	0	0
Candidatus Xiphinematobacter	0	0	0.000	0.000	0.000	0.000	0	0	0	0
DA101	0	0	0	0.000	0	0	0	0	0	0
heteroC45_4W	0	0	0	0.000	0.000	0	0	0	0	0
{Unknown Order} Opitutae	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Genus} [Cerasicoccaceae]	0	0	0.007	0.005	0.013	0.017	0	0	0	0
{Unknown Family} HA64	0	0	0	0.000	0.001	0.000	0	0	0	0
{Unknown Genus} Opitutaceae	0	0	0	0.000	0	0	0	0	0	0
Opitutus	0	0	0.000	0.003	0.000	0.003	0	0	0	0
{Unknown Genus} Puniceicoccaceae	0	0	0.001	0.000	0	0.000	0	0	0	0
{Unknown Order} Verruco-5	0	0	0.000	0.000	0	0	0	0	0	0
{Unknown Family} LD1-PB3	0	0	0	0.000	0.000	0	0	0	0	0
{Unknown Family} WCHB1-41	0	0	0	0	0	0.000	0	0	0	0
{Unknown Genus} RFP12	0.068	0	0.081	0.113	0.220	0.377	0	0	0	0
{Unknown Genus} WCHB1-25	0	0	0	0.000	0	0	0	0	0	0
{Unknown Genus} Verrucomicrobiaceae	0	0	0.000	0.005	0.000	0.029	0	0	0	0
Akkermansia	0.037	0	0.586	0.241	3.036	1.498	0.000	0.009	0	0.000

Luteolibacter	0	0.010	0	0.009	0.002	0.036	0	0	0	0
Prosthecobacter	0	0	0	0.001	0.000	0.002	0	0	0	0
Rubritalea	0	0	0.000	0.000	0	0	0	0	0	0
Verrucomicrobium	0	0	0.000	0.002	0.000	0.001	0	0	0	0
{Unknown Class} WPS-2	0	0	0.001	0.000	0	0.001	0	0	0	0
{Unknown Genus} [Cloacamonaceae]	0	0	0	0.000	0	0	0	0	0	0
Candidatus Cloacamonas	0	0	0	0	0.000	0	0	0	0	0
Candidatus Nitrososphaera	0	0	0	0	0	0.004	0	0	0	0.000

Table S4.4 Relative abundance of Kyoto Encyclopedia of Genes and Genomes from Predicted metagenomes from Holstein dam (n = 6) and calf (n = 6) microbiomes. Pathways are from 1st and broadest hierarchical level

Pathway	Placenta	Colostrum	Vagina	Oral	Cow Fecal	Meconium	24h Fecal	7d Fecal	42d Fecal	60d Fecal
Cellular Processes	0.032	0.053	0.015	0.020	0.033	0.032	0.036	0.021	0.019	0.027
Environmental Information Processing	0.130	0.145	0.138	0.124	0.112	0.121	0.175	0.126	0.109	0.123
Genetic Information Processing	0.176	0.162	0.245	0.210	0.207	0.187	0.168	0.189	0.221	0.214
Human Diseases	0.009	0.011	0.007	0.009	0.007	0.009	0.010	0.008	0.008	0.007
Metabolism	0.494	0.456	0.443	0.474	0.487	0.491	0.434	0.494	0.490	0.473
None	0.001	0.002	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.002
Organismal Systems	0.007	0.006	0.005	0.008	0.008	0.007	0.004	0.007	0.007	0.007
Unclassified	0.146	0.161	0.143	0.151	0.141	0.147	0.167	0.149	0.141	0.142
Cellular Processes	0.032	0.053	0.015	0.020	0.033	0.032	0.036	0.021	0.019	0.027

Table S4.5 Relative abundance of Kyoto Encyclopedia of Genes and Genomes from Predicted metagenomes from Holstein dam (n = 6) and calf (n = 6) microbiomes. Pathways are from 2nd hierarchical level.

Pathway	Placenta	Colostrum	Vagina	Oral	Cow Fecal	Meconium	24h Fecal	7d Fecal	42d Fecal	60d Fecal	*P*-value (Bonferroni)
Amino Acid Metabolism	0.106	0.099	0.076	0.098	0.101	0.103	0.084	0.096	0.095	0.095	3.11E-08
Biosynthesis of Other Secondary Metabolites	0.008	0.008	0.005	0.006	0.010	0.008	0.005	0.010	0.009	0.009	5.73E-06
Cancers	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	2.638
Carbohydrate Metabolism	0.096	0.087	0.105	0.093	0.099	0.096	0.094	0.109	0.101	0.100	7.78E-06
Cardiovascular Diseases	6.31E-05	0.000	2.03E-06	1.19E-05	6.57E-06	5.17E-05	0	1.97E-09	1.03E-06	1.02E-06	9.00E-05
Cell Communication	0	0	5.87E-08	2.2E-07	2.14E-08	2.98E-08	0	0	0	0	8.550
Cell Growth and Death	0.004	0.004	0.005	0.005	0.005	0.005	0.003	0.004	0.005	0.005	5.55E-08
Cell Motility	0.024	0.046	0.008	0.011	0.023	0.024	0.031	0.012	0.009	0.019	7.45E-07
Cellular Processes and Signaling	0.044	0.054	0.040	0.044	0.039	0.043	0.053	0.045	0.042	0.040	0.001
Circulatory System	0.000	0.000	7.88E-06	0.000	2.14E-05	0.000	2.48E-07	1.69E-07	3.18E-06	3.43E-06	2.38E-06
Digestive System	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001
Endocrine System	0.003	0.002	0.002	0.003	0.003	0.003	0.001	0.003	0.003	0.003	2.21E-06
Energy Metabolism	0.055	0.051	0.053	0.057	0.060	0.057	0.050	0.058	0.061	0.059	2.57E-06
Environmental Adaptation	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.258
Enzyme Families	0.019	0.021	0.019	0.018	0.021	0.020	0.021	0.021	0.022	0.022	0.002
Excretory System	0.000	0.000	7.8E-05	0.000	0.000	0.000	0.000	0.000	9.5E-05	0.000	0.398
Folding, Sorting and Degradation	0.025	0.024	0.031	0.031	0.026	0.025	0.023	0.025	0.028	0.026	1.12E-04
Genetic Information Processing	0.024	0.024	0.029	0.029	0.027	0.025	0.029	0.026	0.028	0.028	8.65E-08
Glycan Biosynthesis and Metabolism	0.019	0.021	0.023	0.027	0.027	0.025	0.025	0.032	0.031	0.026	0.012
Immune System	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	5.39E-07

Immune System Diseases	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.084
Infectious Diseases	0.004	0.005	0.003	0.003	0.003	0.004	0.006	0.004	0.004	0.004	5.53E-09
Lipid Metabolism	0.035	0.034	0.027	0.030	0.028	0.033	0.029	0.029	0.026	0.025	2.50E-04
Membrane Transport	0.108	0.114	0.123	0.108	0.094	0.100	0.147	0.109	0.096	0.108	0.001
Metabolic Diseases	0.000	0.000	0.001	0.000	0.001	0.000	0.000	0.001	0.001	0.001	0.013
Metabolism	0.025	0.024	0.023	0.022	0.025	0.025	0.028	0.025	0.022	0.023	3.68E-05
Metabolism of Cofactors and Vitamins	0.043	0.038	0.040	0.045	0.045	0.044	0.041	0.044	0.048	0.044	7.08E-05
Metabolism of Other Amino Acids	0.018	0.019	0.014	0.016	0.015	0.017	0.015	0.016	0.015	0.015	6.19E-06
Metabolism of Terpenoids and Polyketides	0.021	0.017	0.014	0.017	0.017	0.019	0.014	0.017	0.018	0.016	0.001
Nervous System	0.000	0.001	0.000	0.000	0.001	0.000	0.000	0.001	0.001	0.001	2.21E-06
Neurodegenerative Diseases	0.002	0.003	0.001	0.003	0.001	0.002	0.001	0.001	0.000	0.000	2.08E-07
Nucleotide Metabolism	0.036	0.029	0.047	0.041	0.042	0.038	0.034	0.041	0.046	0.044	1.81E-05
Poorly Characterized	0.052	0.058	0.049	0.054	0.048	0.052	0.057	0.052	0.048	0.050	2.94E-05
Replication and Repair	0.078	0.071	0.109	0.093	0.093	0.083	0.074	0.086	0.102	0.098	2.75E-06
Sensory System	0	0	0	2.14E-09	0	0	0	0	0	0	20.693
Signal Transduction	0.020	0.030	0.013	0.015	0.016	0.018	0.026	0.015	0.011	0.013	2.85E-08
Signaling Molecules and Interaction	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.204
Transcription	0.024	0.024	0.026	0.023	0.026	0.024	0.028	0.025	0.025	0.026	0.427
Translation	0.048	0.041	0.077	0.063	0.061	0.053	0.042	0.052	0.064	0.063	1.55E-05
Transport and Catabolism	0.003	0.002	0.001	0.003	0.004	0.003	0.002	0.004	0.003	0.002	1.30E-05
Xenobiotics Biodegradation and Metabolism	0.035	0.027	0.013	0.020	0.016	0.026	0.018	0.016	0.014	0.014	6.43E-06

5.1: Conclusions

5.1.1: INTERPERATIVE SUMMARY

The objectives of this dissertation were: 1) to examine differences in phenotypes related to reproductive physiology in virgin Holstein heifers based on outcome of first insemination, 2) to examine associations between uterine microbial composition and fertility related phenotypes, insemination outcome, and season of breeding, and 3) to examine the influence of the maternal microbiome on calf gut development during the pre-weaning phase. I observed that phenotypes related to pregnancy maintenance and the uterine microbiome differed in virgin Holstein heifers based on first insemination outcomes. I also observed multiple multiparous Holstein dam microbiomes could be predicative of calf fecal microbiome development.

Traits related to pregnancy maintenance, like serum progesterone concentration and corpus luteum volume, were greater in heifers that did become pregnant than those that did not become pregnant. Reduced progesterone concentration before ovulation could influence dominant follicle selection and result in poorer quality oocytes being ovulated. Determining a threshold for progesterone that predicts heifer insemination outcomes could lead to more accurate selection for heifer fertility and be utilized to aid in breeding decisions.

Minority taxa in the heifer uterine microbiome contributed to differences in microbial community structure based on insemination outcome, while majority taxa contributed to differences in microbial community structure based on season of breeding. Of the taxa that were associated with heifer insemination outcomes, *Lactobacillus* and *Gardnerella* may be of the most interest, as there are also associations with these taxa and fertility in other species. Future experiments could examine the abundance of these species in the uterus of lactating cows, where poor reproductive performance and pregnancy loss is greater. Additionally, research needs to be

conducted to determine if these bacteria are causing poor fertility due to some mechanism of interaction or if they just tend to increase in abundance in animals with poor fertility, serving as a biomarker. This will decide how this research can be applied on a dairy farm. If bacteria are causative and either aid or inhibit fertilization, we could modify the uterine bacterial community composition to improve reproductive performance. However, if bacteria are just indicative of cow fertility, they could be used as markers to predict fertility, providing an additional phenotype that could be incorporated into existing fertility models and improve their accuracy.

Genera abundance in the maternal reproductive, colostrum, oral, and fecal microbiomes were predictive of genera abundance in the calf fecal microbiome from birth until weaning. Examining maternal and calf microbiomes between healthy and sick calves could lead to using these microbiomes as health biomarkers in cattle. While further research is needed examining factors that influence these phenotypes and microbiomes, our results indicate that they might be able to be utilized to improve dairy cattle reproduction and calf health.

Overall, the microbiome in the reproductive tract could serve as a new phenotype that plays an important role before, during, and after pregnancy. The key to how we can use this microbiome data relies on determining which bacteria are causative to changes in the cow and which are just associative. Future reproductive microbiome research should begin to steer away from examining just changes in microbial population structure and instead include how the microbiome or key bacteria in the microbiome interacts with the reproductive tract. Instead of just identifying who is there, host-microbiome interaction research will reveal why certain bacteria can more easily colonize in the reproductive tract and how they might influence fertility.