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The Role of Microbial Diversity in Female Reproductive Health: An Analysis of the Microbiome's Influence on Pregnancy Outcomes in Postparturient Mares

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THE ROLE OF MICROBIAL DIVERSITY IN FEMALE REPRODUCTIVE HEALTH: AN ANALYSIS OF THE MICROBIOME'S INFLUENCE ON PREGNANCY OUTCOMES IN POSTPARTURIENT MARES

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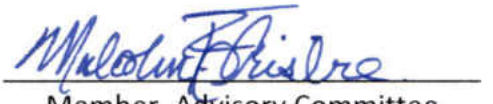

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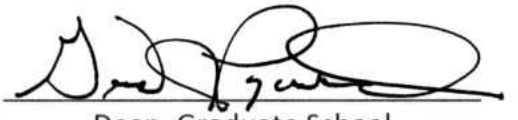

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GABRIELLA PUGH

Submitted to the Faculty of the Graduate School of
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in partial fulfillment of the requirements for the degree of

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DEDICATION

This thesis is dedicated to my parents, Samantha and Wayne Pugh, and grandparents, Charles and Carol Sanders.

To your unfailing love and devotion, and numerous sacrifices to help make my dreams a reality, for none of it was in vain.

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ABSTRACT

Birth is initiated by a programmed inflammatory response of the placenta and amniotic fluid carried out by maternal and fetal signals, between 37-42 weeks gestation for human females and 320-340 days gestation for mares. When the inflammatory response occurs prematurely, pre-term birth is initiated. Pre-term birth is most commonly associated with a maternal infection that consequently leads to infection of the placenta and amniotic fluid; however, the terms of infection are unclear, as microbiota has been discovered in placental membranes and amniotic fluid of healthy pregnancies. The objective of this study is to challenge the sterile womb hypothesis and investigate the relationship between microbiota and negative pregnancy outcomes, specifically pre-term birth. Blood, oral, fecal, uterine, and vaginal samples were collected from 58 postparturient mares by attending veterinarians at Hagyard Equine Institute in Lexington, KY, within 24-36 hours of foaling and before uterine lavage/infusion. Expected due date and date of delivery was noted for birth categorization as either pre-term or full-term. A foal is considered pre-term if born prior to 320 days gestation. Microbiota samples were prepared on sterile nasopharyngeal swabs, which were then used for 16S rRNA gene sequencing on Illumina MiSeq. Sequencing output was analyzed using QIIME 1.9 on VirtualBox. The abundance of Fusobacteria, across all samples, in mares that delivered pre-term (PT) was 2-fold higher than those who delivered full-term (FT). Moreover, Fusobacteria comprised 15% of PT vaginal samples in comparison to 4.9% FT vaginal. Nearly identical ratios are observed in uterine samples of PT and FT deliveries (15.5% and 4.4% respectively). Fusobacteria is a

common, nonpathogenic microbe of the oral cavity but can cause periodontitis in the case of overgrowth. Fusobacteria can also lead to negative pregnancy outcomes if transferred hematogenously through the placenta. However, this is not the case in the PT deliveries of this study, as Fusobacteria did not comprise even one percent of both oral and blood samples. On the other hand, Fusobacteria has been reported in the vaginal microbiome of negative reproductive health and pregnancy outcomes, such as bacterial vaginosis and pre-term births. These results support the hypothesis that vaginal microbiota may vertically ascend through the cervix and into the uterine cavity to proliferate and colonize. We suggest a cross talk occurring between vaginal microbiota and the uterine environment. Whether this interaction always leads to negative outcomes is unclear, although the inflammation of the placenta and amniotic fluid in response to such microbes may be a consequence of their uncontrolled proliferation.

Key words: microbiome, microbiota, vaginal, uterus, pre-term, birth, reproduction, equine

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CHAPTER 1

INTRODUCTION

Up until the early 2000s, health and disease had been primarily attributed to two major factors: genetics and environment. Understanding the human genome has and continues to be a crucial part of research science in discovering complex mechanisms that drive each system and how alterations to the genome, whether by random mutation or epigenetically driven, can lead to disease development. There was, however, an important component of the human body that was being ignored: the microbiota. Though its presence was known, not much thought had been given to the nonpathogenic microorganisms that inhabit every living animal.

Symbiotic relationships have been a driving force of evolution and species survival. Such relationships, in which all of the organisms involved benefit, is said to be mutualistic. The Oxpecker bird lands on rhinos or zebras and eats parasites living on their skin, in exchange for safe transportation. Bees aid in pollination of flowers when collecting nectar, from which they make food, leaving behind pollen from other flowers. However, perhaps the most complex and least understood of these is between the mammalian body and its “normal flora”, which live on and inside the body, and perform a variety of functions beneficial to mammals. These taxa of bacteria are a variety of different phyla, an estimated 10-100 trillion microbial cells that inhabit mucosal and epithelial areas of the body such as the gastrointestinal (GI) tract, skin, mouth, vagina, urogenital tract, and respiratory tract. These resident, commensal microbes are termed microbiota [1].

In 2001, Joshua Lederburg coined the term 'microbiome', to describe the genetic catalog of communal microbiota and its ecological relationship with the host [2]. Included in its definition are bacteria, yeast, fungi, and viruses, though this thesis will focus solely on bacterial microbiota [1, 2]. Since the launch of the National Institute of Health's (NIH) Human Microbiome Project (HMP) in 2008, research scientists have uncovered a plethora of evidence in association with the role of microbiota in health and disease. It has become clear that when microbiota are undisturbed in their desired niche, they perform beneficial functions for the host in exchange for nutrients and other components for survival. However, when these populations are disturbed and no longer in homeostasis with the host, overall health is often compromised.

In 2008, the NIH HMP reported approximate distributions of bacterial microbiota throughout the body as: 29% GI tract, 26% oral, 21% skin, 14% airways, 9% urogenital regions, 1% blood, and 0% in the eyes [2]. It was also reported by the MetaHit Consortium that the gut microbiome alone contains approximately 3.3 million nonredundant genes [1]. The human genome consists of an estimated 22,000 genes; therefore, this finding revealed that our own cells are dramatically outnumbered by the microbial cells on and inside of us, an estimate of a 10:1 microbe-host cell ratio [1, 3]. This means that for every single cell belonging to the host, there are 10 microbial cells, suggesting that we are actually 10% human and 90% microbial ecosystem. Since the microbial cells outnumber the host cells, humans must have evolved mechanisms

for dealing with such large populations of microbes to prevent their destruction by the immune system [4].

Several evolutionary theories have developed in regard to host-microbiota symbiosis. Foster et al. describes the host microbiome as an, 'ecosystem on a leash,' meaning that humans have developed mechanisms that allow them to keep microbiota under favorable conditions while keeping proliferation rates controlled [4]. First, the review discusses the problem with a diverse microbiota: natural selection will favor microbes that proliferate at rapid rates and will select against those that replicate slowly, regardless of the benefit that the slower-growing microbe may bring to the host. This suggests that, from an evolutionary theory perspective, microbes that invest in their own reproductive fitness outcompete those that do not. Therefore, it cannot rightfully be assumed that the host and microbial communities work together to benefit one another; rather, it is more reasonable to infer that the outcome of our health is dependent on the microbes that survive under selective pressures [4]. This may explain why particular microbiome characterizations correlate with particular disease states. Even with this information, it is still observed that the microbiota predominantly contains beneficial microbes, giving rise to the theory that the host possesses monitoring and targeting mechanisms as a means of providing its own selective pressure [4]. Given these two opposing theories, it remains unclear whether natural selection or host monitoring is the ultimate decision maker in choosing which microbes survive.

Throughout this thesis, the following topics will be discussed in regard to microbiota: functions, niche preferences, and interactions with the host. Outlined interactions with the host will primarily focus on those of the female reproductive tract, although gut microbiota functions are briefly reviewed as these microbes have proven to play a substantial role in overall health. Though much has been discovered about the microbiome's influence on human health, we have merely scratched the surface of the topic. Considering the ratio of microbiome DNA to host DNA, it is reasonable to suggest that until we understand the microbiome, we cannot fully understand the human genome [1].

CHAPTER 2

BACKGROUND

2.1 The Gut Microbiome

2.1.1 Overview

More than a quarter of our resident microbiome resides within the gastrointestinal tract, which is a key reason for why this particular area has been given a lot of attention in microbiome research. Gut microbes are known to aid in nutrient absorption and modulation of the immune response. Within the past decade, studies have suggested these microbes are responsible for the overall health of an individual, proposing new potential interactions with every system in the body. In support of this, it has been demonstrated that when the continual flux of gut microbiota is disrupted, disease susceptibility increases. These diseases range from cancers, autoimmune disorders, cardiovascular diseases, metabolic syndromes, to endocrine disorders. Although this study focuses on microbe-female reproductive interactions, it is important to note and appreciate the diversity of the functions of the gut microbiota, as it plays a significant role on reproductive health.

2.1.2 Nutrient Absorption and Immunomodulation

The large intestine (colon) is an ideal environment for anaerobes, both obligate and facultative [4]. Bacteria that reside here predominantly undergo anaerobic respiration and carry out fermentation, which is useful in the breakdown of indigestible carbohydrates, such as cellulose and pectin, into short fatty-acid chains [5]. These short fatty-acid chains can be metabolized as precursors for cholesterol

synthesis, utilized in the liver for gluconeogenesis, lipogenesis, and protein synthesis, and contribute to 3-9% of overall energy requirements of humans. Few microbial communities can be found in the stomach and small intestine due to the bactericidal activity of gastric acid [5]. However, as of 2009, the presence of *Lactobacillus, Veilonella,* and *Helicobacter* has been reported in the stomach at approximately 10^1 cells/gram of stomach contents [6]. Moving along the GI tract, abundance and diversity increase greatly. The duodenum contains approximately 10^3 cells/gram, the jejunum 10^4 cells/gram, and the ileum 10^7 cells/gram. In addition, the colon contains an estimated 10^{12} cells/gram. Although the composition and abundance of gut microbes fluctuates daily, there are certain phyla that remain fairly consistent. Bacteroidetes and Firmicutes are the predominant phyla (particularly in the large intestine), with smaller proportions of Proteobacteria, Actinobacteria, Cyanobacteria, Fusobacteria, Tenericutes, and Verrucomicrobia [6, 7]. When observing beyond the phylum level, variation in microbial communities increases. It is speculated that due to functional redundancy, different microbes capable of performing the same functions across phyla allow for variation on the genus and species level [7].

Bacterial populations differ greatly between mucosal/epithelial layers and the intestinal lumen. For example, commonly reported microbiota present in the mucosal and epithelial areas includes *Clostridium, Lactobacillus,* and *Enterococcus*, whereas typical microbiota of the intestinal lumen includes *Bacteroides, Bifidobacterium, Streptococcus, Enterobacteriaceae, Enterococcus, Clostridium, Lactobacillus,* and *Ruminococcus* [6]. Approximately 1-3 genera are shared between these areas.

Variation between intestinal environments is primarily due to the differing functions of the stomach, small intestine, and colon, as well as their divergent structures, which corresponds to the metabolic requirements of the residing microbiota [6].

Factors such as diet, antibiotic use, infection, sexual contact, and stress can lead to alterations of gut microbiota [5-7]. However, there are particular circumstances in which some factors do not have a significant affect. For instance, diet does not seem to influence composition or abundance of microbiota in pregnant women. Proteobacteria and Actinobacteria have been demonstrated to increase in the gut microbiome of pregnant women despite decrease in overall richness, which is defined as the number of different species in an ecological community [5]. During pregnancy, estrogen levels remain high in contrast to non-pregnant women, in which estrogen and progesterone levels peak then decrease at different periods of the menstrual cycle. This suggests that hormones may play a role in the regulation of microbial communities, or conversely. Furthermore, particular situations such as pregnancy, may lead to hormone interactions with microbes that outcompete that of dietary effects. Pregnancy, however, is the only circumstance known to date in which reduced dietary effects on gut microbiota composition have been observed. Several studies have produced a germ-free mouse model without metabolic defects that become obese after transfer of microbiota from obese mice [8-10]. Furthermore, when gut microbes of lean mice are transferred to obese mice, obesity is resolved, thus supporting the hypothesis that gut microbiota plays a role in maintaining a healthy body weight unless present in the wrong proportions. This means that certain gut microbes have differing

functions that affect the host inversely. For instance, Bacteroidetes are more efficient in lipid metabolism, whereas Firmicutes are not. A higher abundance of Firmicutes in ratio to Bacteroidetes has been associated with increased fat deposition and body weight, placing greater importance on proper nutrition that appeals to the individual's GI microbiota that inclines an individual toward a healthy weight [8, 9].

Commensal microbes are able to inhabit their host without causing disease pathogenesis because they are recognized by the host as nonthreatening, meaning the host has tolerance for microbiota. Gut microbiota, in particular, are able to enhance the innate immune response. Intestinal epithelial cells (IECs) and Paneth cells provide a barrier function by physically confining commensal and pathogenic microbes to the intestinal lumen [11]. Paneth cells prodyce additional immune responses by secretion of antimicrobial peptides (AMPs) and expression of nucleotide-binding oligomerization domain-containing protein 2 (NOD2), which initiates innate immune signaling in response to the cytosolic peptidoglycan fragment, muramyl dipeptide (MDP). Microbiota in the intestinal lumen can upregulate Paneth cell immune responses by activating Toll-like receptors (TLRs) on IECs and Paneth cells, inducing Reg3γ expression. Reg3γ is an islet-derived C-type lectin that is bactericidal against Gram-positive bacteria [11]. Furthermore, innate lymphoid cells enhance this function of gut microbiota through the production of the cytokine interleukin-22 (IL-22). IL-22 promotes IEC growth and Reg3γ expression. Additionally, microbiota-derived MDP can activate NOD2 on Paneth cells, which stimulates production of a subset of AMPs called defensins. Defensins function in host defense with activity against bacteria, fungi, and

many viruses [11]. Additional immune functions of gut microbiota include development of Foxp3^{+} T regulatory cells, stimulation of secretory IgA from mucosal plasma cells, shaping of gut associated lymphoid tissue (GALT), and stimulation of the immunomodulatory action of macrophages of the intestinal lamina propria [7].

2.1.3 Dysbiosis and Disease

Gut dysbiosis, a complete shift in whole populations of gut microbiota, has been associated with several diseases and conditions, including atherosclerosis, hypertension, obesity, colitis, Alzheimer's disease, and even psychiatric disorders [12-16]. Dysbiosis of the gut microbiota can be influenced by several factors, such as the use of broad-spectrum antibiotics, disease in which inflammation of the GI tract occurs, and poor nutrition [5, 14]. Interestingly, gut dysbiosis has been associated with disorders that alter reproductive functionality; the most commonly associated reproductive disorder being polycystic ovary syndrome (PCOS) [5]. As of 2016, the estimated prevalence of women diagnosed with PCOS was approximately 15% of women worldwide, making it the most common endocrine disorder for females [17]. This disorder is characterized by hyperandrogenemia (excess androgen production), obesity, insulin resistance, irregular menstrual cycles, and ovaries that develop multiple cysts. These cysts are categorized as either physiological (follicular and luteal cysts) or pathological (ovarian tumors) [18, 19]. Association between gut dysbiosis and PCOS suggests that a hormone imbalance may alter GI microbiota composition, or that the GI microbiota composition may cause a hormone imbalance.

Physiological cysts are solid or fluid-filled pockets in the ovary that typically become inflamed. Formation of cysts is most commonly a consequence of a follicle's failure to reach maturity, resulting in the inability to release an oocyte- termed 'anovulation' [18]. It is common for a physiological ovarian cyst to occasionally develop, but it is usually not life-threatening and resolves on its own. However, in women with PCOS, physiological cysts form with nearly every menstrual cycle, halting menstrual flow from occurring. This also makes it difficult, or impossible in some cases, for the female to reproduce successfully. A frequently observed secondary outcome of PCOS is endometriosis, which is the abnormal growth of endometrial cells outside of the uterus, most commonly on other organs within the pelvis [18]. Endometriosis can be life-threatening and dramatically decreases the individual's fertility. In both cases, it has been shown that the gut microbiota becomes altered, though it is unclear as to which circumstance is the cause and which is the effect [17, 19].

Gut microbiome alterations have been investigated in PCOS mouse models, which is achieved by administration of letrozole. Letrozole is a nonsteroidal aromatase inhibitor, preventing cleavage of testosterone into estrone; therefore, estradiol levels are reduced [17]. Kelley et al. reported that letrozole treatment resulted in decreased species abundance and decreased alpha and beta diversity (diversity within a sample and between sample types, respectively). They also observed that after one week of letrozole treatment, a shift in the gut microbiome occurs, largely effecting Bacteroidetes and Firmicutes. Given these results, it was concluded that steroid hormones might regulate the gut microbiome as aromatase activity has not yet been

described in bacteria. Since it was an aromatase inhibitor that was used, it was determined that PCOS leads to gut dysbiosis and not the other way around. Since this study was the first to demonstrate a hormone interaction with gut microbiota by manipulating endogenous testosterone, further studies are required to support or refute the conclusion that PCOS is the cause and gut dysbiosis is the effect [17]. An alternative explanation to consider is that either circumstance can occur first, leading to the other in a bidirectional manner.

In a study conducted by Lindheim et al., gut microbiome composition and barrier functions of the gut in 24 PCOS-diagnosed females with reproductive and metabolic defects were compared to that of 19 healthy women [19]. They discovered that the phyla Tenericutes and Bacteroidetes were significantly lower in stool samples of PCOS subjects and were associated with reproductive defects. Furthermore, their results revealed that, in some cases, gut barrier dysfunction and endotoxemia contribute to metabolic defects observed in PCOS patients. Similarly to the study by Kelly et al., they were unable to determine if gut dysbiosis lead to PCOS development or vice versa [19]. It may be useful to induce gut dysbiosis in a mouse model and monitor for any reproductive abnormalities that result. In addition, if PCOS development is not observed in these mice with gut dysbiosis, letrozole-induction of PCOS could be administered to observe how an already altered gut microbiome influences the severity of PCOS reproductive and metabolic defects.

In conclusion, the resident microbes of the GI tract play key roles in carbohydrate and lipid metabolism, nutrient absorption, immunomodulation, and

maintenance of the overall health of the body. It appears that in order for the health of the body to be maintained, the gut microbiota composition must be undisturbed. When composition is altered by any factor previously mentioned, overall health is compromised and disease susceptibility increases. When studying the microbiota composition of a particular body site, it is important to consider the gut microbiome as well, as it may have influence over the site of interest.

2.2 Organization and Functions of the Female Reproductive Tract

2.2.1 Overview

The female reproductive system is the organization of both internal sex organs and external genitalia that function in concert with one another. The progression of follicular development to ovulation and menstruation, or pregnancy, is generally well understood; however, little is known about how the microbiome influences pregnancy outcomes. The lack of knowledge is primarily as to whether the amnion, defined as the innermost membrane within the uterus that encloses the embryo/fetus, is truly a sterile environment. If the amnion is not sterile, what consequences does this bear on female reproductive health? More specifically, are pregnancy outcomes affected by the resident microbiota of the host?

2.2.2 Follicular Development: Humans versus Mares

The ovaries carry out two primary functions: gametogenesis and steroidogenesis [20, 21]. Gametogenesis is defined as the production of gametes, which is termed oogenesis for females. The term oocyte refers to a gamete in development and ovum refers to a mature gamete [20]. Steroidogenesis executed by

the ovary is the production of the appropriate sex steroid hormones, estrogen and progesterone [22]. This process occurs in both theca and granulosa cells of the ovary, which will be discussed later in this section. It is important to note that other hormones, such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are involved in oogenesis; however, it is the pituitary gland that produces and secretes these hormones [21, 22].

2.2.2.1 Folliculogenesis in Human Females

Ovaries contain structures called follicles, which house the oocyte and undergo extensive maturation steps influenced by both sex steroid hormones and gonadotropins until the oocyte develops into an ovum through a process known as folliculogenesis [21]. Follicle production occurs early in fetal development, as mitotic divisions increase the number of oogonia, later giving rise to primary oocytes. These initial follicles are termed primordial follicles and are present at birth where they will remain in a state of arrest until puberty [21]. There are three developmental states of ovarian follicles: primordial, growing (further classified into primary and secondary), and mature/Graafian [20]. The size of the follicle is typically indicative of its developmental state; however, the change in particular structures associated with the follicle is key to its identification [20, 21].

Primordial follicle assembly in the ovaries is not dependent on gonadotropin stimulation, as these follicles are developing during early fetal life, around the third month of development [21]. Once the ovary has reached maturity, primordial follicles are stored in the stroma of the cortex, beneath the tunica albuginea, which is the

external connective tissue covering the ovaries [20]. Structural changes become apparent once a primordial follicle becomes a primary follicle, which is identified as such when the flattened follicular cells surrounding the growing oocyte proliferate and become cuboidal. It is at this stage that the oocyte's protective barrier, termed the zona pellucida, is formed between the oocyte and adjacent follicle cells. The zona pellucida is an extracellular covering abundant in sulfated acidic glycoproteins (labeled ZP-1, ZP-2, and ZP-3), which function in spermatozoa binding and induction of the acrosome reaction, characterized by the release of enzymes to allow a single sperm cell to penetrate the zona pellucida [20]. The enzymes released in this reaction are primarily hyaluronidases, which act by catalyzing the degradation of hyaluronan- a chemical compound found at high concentrations in the extracellular matrix covering a mature ovum [20, 21].

The cells surrounding the primary oocyte rapidly proliferate into a stratified epithelium, termed the stratum granulosa [20]. Cells in the stratum granulosa become granulosa cells; these are the cells in which progesterone production occurs [22]. Two types of receptors can be found on the granulosa cell: the LH receptor and the FSH receptor, although FSH receptors are greater in abundance due to continual growth of the follicle [22, 23]. Due to proliferation of granluosa cells, a connective tissue layer forms from stromal cells that proximately enclose the follicle. This layer is termed the theca folliculi, which will further differentiate into the theca interna and theca externa [20]. Cells within the theca layers are termed theca cells and those within the theca interna possess a large number of LH receptors [23]. The external layer serves as an

outer layer of connective tissue barrier. Until this point, the follicle has been avascular; however, it is within theca cells that blood vessels begin to appear, which will later serve as a transportation route for the appropriate hormones. At this stage in development, the follicle is now classified as a secondary follicle [20].

Progesterone production also occurs in theca cells and is driven by conversion of ATP to cyclic AMP (cAMP) [22]. The secondary follicle continues to grow in size due to stimulation by FSH, growth factors, and recruitment of calcium ions until an antrum forms on one side of the oocyte. Antrum formation is the key characteristic that now classifies the follicle as a tertiary follicle [20]. Once the antrum becomes a fluid-filled cavity rich with hyaluronanic acid, termed follicular fluid, the follicle is designated as mature/Graafian. Upon initiation of ovulation, the Graafian follicle will rupture and release the oocyte through the oviduct (fallopian tube) by the fimbriae and into the uterus for fertilization [20]. The ruptured follicle will collapse into deep folds, giving rise to the corpus luteum, also termed the luteal gland [20, 22]. The theca interna and granulosa cells become luteal cells by luteinization, which causes the cells to dramatically increase in size and fill with lipid droplets [23]. Lutein cells within the corpus luteum establish a highly vascularized network derived from the theca interna cells. This vasculature will serve as a hormone transportation route for preparing the endometrium for implantation in the event of fertilization [23]. In the absence of fertilization and subsequent human chorionic gonadotropin (hCG), the corpus luteum will degenerate approximately 10-12 days after ovulation [20]. As the corpus luteum regresses, menstrual flow begins, which is a 3-7 day process. Menstrual flow is the

partial shedding of the endometrium's stratum functionale layer (the portion that becomes thicker throughout the ovulatory cycle) and bleeding as a result of destruction of the mucosal vessels [20, 21].

As previously mentioned, gonadotropins released from the anterior pituitary drive the follicles in both the growth/follicular phase and the luteal phase (Figure B1). During the follicular phase, FSH carries out two main functions: follicle growth and stimulation of estradiol production [22]. To begin this process, FSH will bind to FSH receptors on granulosa cells. Activation of FSH receptors causes rapid proliferation of granulosa cells for follicle growth. These new granulosa cells will express the enzyme aromatase, which is responsible for cleaving testosterone into estradiol. The source of testosterone comes from theca cells [22, 23]. Upon stimulation of LH receptors by LH, LDL will enter the cell and be used to synthesize pregnenolone followed by progesterone. Progesterone is chiefly responsible for preparing the internal sex organs for pregnancy, specifically the uterus, by stimulating secretory changes in the endometrium [22]. Progesterone can also be converted to 17-OH-Progesterone, which is then converted to androstenedione, the precursor to testosterone. Androstenedione can be readily cleaved by either aromatase or 17β-hydroxysteroid dehydrogenase (HSD). Cleavage by aromatase produces conversion to estrone, then to estradiol by 17β-HSD [22, 23]. On the other hand, cleavage of androstenedione by 17β-HSD causes conversion to testosterone, which will translocate to granulosa cells through the basement membrane to be cleaved by aromatase into estradiol. Estradiol

plays a key role in sexual maturation, meaning it is responsible for development of female sex characteristics such as breast development, and follicular maturation [22]. Aside from aiding in estradiol production, LH also plays a role in ovulation and choosing the dominant mature follicle [23]. During the midpoint of ovulation, a surge of LH will migrate to the follicle that has the most LH receptors expressed on the surface of theca cells, ultimately choosing it to achieve full maturation [24]. This surge of LH will signal luteinization of granulosa and theca interna cells, which will transition the follicle from the follicular phase to luteal. The luteal phase starts at day 14 of the menstrual cycle when ovulation is complete. It is during this phase that estrogen levels decrease sharply and progesterone dominates. This increase in progesterone is what leads to a thickening of the stratum functionale. The source of progesterone comes from granulosa lutein and theca lutein cells of the corpus luteum [24].

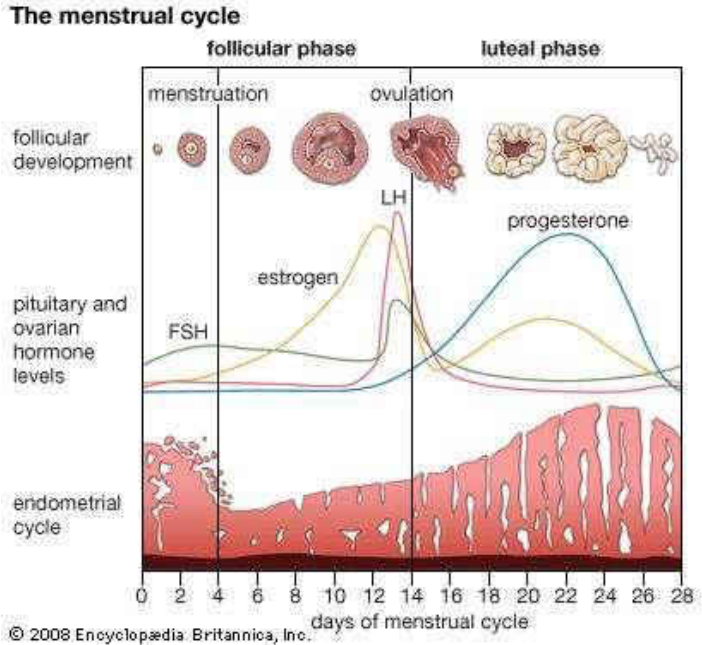


Figure B1. The Human Female Menstrual Cycle. Divided into two phases: follicular and luteal. The follicular phase occurs during days 4-14 and involves the maturation of an ovarian follicle until oocyte release (ovulation). During the follicular phase, estrogen and FSH are the dominant hormones. The luteal phase (days 12-14), is characterized by a surge of LH and signals the final maturation into a Graafian follicle and release of oocyte at ovulation. The corpus luteum develops and primarily produces progesterone to thicken the endometrium for possible implantation. (*From:* Encyclopedia Brittanica, Inc. 2008). *Source(s):* Clayton, S. G. Menstruation (2018), https://www.britannica.com/science/menstruation

It is important to note that during follicular development, several follicles are developing simultaneously within the ovary. It is the surge of LH at mid-ovulation that chooses which follicle will release its mature oocyte [24]. The remaining follicles will undergo atresia, a process mediated by apoptosis of granulosa cells [20]. Primordial and primary atretic follicles will shrink and eventually disappear after repeated apoptosis. Large, growing atretic follicles degenerate in the same fashion; however, if they contain a mature oocyte, the oocyte itself will be delayed in its destruction as it is no longer sensitive to atresia stimuli of the follicle. Once menstruation has reached completion, primordial follicles will repeat the process of follicular development. Unlike males, females have a predetermined gamete quantity, approximately 400 oocytes [20]. When this reserve is depleted, the female will no longer ovulate and menstruate, marking the stage of menopause [25].

2.2.2.2 Folliculogenesis in Mares

Like human females, follicular and oocyte development occurs in early fetal life and is independent of gonadotropin stimulation [26]. During this period, at approximately 70 days of gestation, primordial germ cells proliferate and meiotically divide until around 150 days of gestation, when they are arrested in prophase I as primary oocytes. At this time, oocytes are developing into primordial follicles, which will occur throughout fetal life so that thousands of follicles are present within the ovaries upon birth. The period in which primary oocytes proliferate, divide, arrest, and subsequently develop into primordial follicles is termed the preantral stage [26].

For mares, there are four classifications of follicular development: primordial, primary, secondary, and antral [26]. Primordial, primary, and secondary follicles develop during the preantral stage. Maturation of a primordial follicle to a primary follicle is initiated by the proliferation of somatic cells surrounding the primordial follicles, similar to when flattened follicular cells surrounding primordial follicles, in humans, proliferate and become cuboidal, marking the transition of the follicles from primordial to primary. Proliferation of surrounding somatic cells occurs during post-natal life, stimulated either by atresia or a surge of LH. Classification of primary and secondary follicles is solely based on size, with a 0.1mm diameter categorized as primary and 0.2mm classified as secondary [26].

Developing follicles enter the antral stage upon formation of an antrum, which occurs when the follicles are approximately 0.3mm in diameter [27]. Once antral follicles reach a diameter of 2mm, their growth and the subsequent selection of a dominant follicle is determined through follicular waves, which are under the systemic control of gonadotropins and the local control of insulin-like growth factor-1 (IGF-1), sex steroid hormones (estrogen and progesterone), inhibins, activins, and vascular endothelial growth factor (VEGF) [26]. Follicular waves are defined as the simultaneous development of several growing follicles at a common growth rate, which is typically 2-3 mm in diameter per day and occurs during estrus. Follicular waves are categorized as either major or minor waves [27]. Minor waves are characterized by smaller, subordinate follicles (< 30mm diameter) in the absence of a dominant follicle. Minor waves occur outside of breeding season to ensure the mare undergoes anovulatory

estrous cycles [26, 27]. Major waves produce a dominant ovulatory follicle, with 1-2 waves (primary and secondary) occurring around the middle of the estrous cycle [26]. IGF-1 initiates deviation of the dominant follicle by upregulating other growth factors, which in turn will lead to increased diameter of the follicle that has the most receptors for the growth factors, meaning it binds more ligand than the other follicles and will, therefore, become the dominant ovulatory follicle. A unique characteristic of the mare estrous cycle is that ovulation occurs at high levels of progesterone [26]. This differs from that of human female ovulation, which occurs in the presence of high estrogen concentrations.

2.2.3 The Estrous Cycle Varies Between Mammals

In humans, the process of follicular development, ovulation, and subsequent shedding is termed menstruation. In other mammals, such as mares, baboons, and mice, this cycle is termed the estrous cycle [28]. As this study involves reproduction in mares, it is important to understand the differences in cycles between humans and horses, and a commonly used laboratory model- the CD1 female mouse. For most mammals, such as the mouse and the baboon, there are four stages of the estrous cycle, from primary follicle development to shedding of the endometrial mucosal layer: metestrus, diestrus, proestrus, and estrus [28].

In mice, metestrus defines the period in which the primary follicle is undergoing changes to become a secondary follicle and is influenced by a rise in progesterone levels. During diestrus, the primary follicle has matured into the secondary follicle and progesterone levels decrease as estrogen production increases.

Proestrus is the complete maturation into a Graafian follicle, as estrogen levels sharply decrease and progesterone dominates for a brief period. It is at this stage that ovulation occurs, which occurs on day four or five [28]. Upon absence of fertilization of the ova, the corpus luteum regresses and the lining of the endometrium begins to shed, beginning the final stage of estrus. Estrus is characterized by a sharp increase in estrogen during the dark cycle (night time) and a return to basal levels in the morning as estrus reaches completion. It is important to distinguish between estrous cycle and estrus; estrous cycle refers to the complete menstrual cycle, whereas estrus refers to menstruation (endometrial mucosal layer shedding). In humans, menstruation normally lasts 3-7 days; however, this is quite short-lived for a mouse, as shedding only lasts for approximately 12-hours [28]. Defining menstruation changes when discussing the estrous cycle of the mare.

The mare is described as a polyestrous mammal, meaning it undergoes ovulatory estrous cycles seasonally [27]. The mare experiences ovulatory estrous cycles during a particular season in contrast to monthly repetition for humans and weekly repetitions for mice. Seasonal breeding is a selective pressure that acts to prevent the mare from delivering during harsh, cold weather [29]. Ovulatory estrous cycles occur during the months of May to October, lasting approximately 22 days each, with 5-7 days of this period being in estrus, during the spring and summer breeding season. A feature of the mare's estrous cycle that makes it unique from the human and mouse is that it is positively phototropic, meaning that it is directly initiated and controlled by the photoperiod, particularly during long days [27]. Seasonal breeding is

determined by the duration of exposed light (i.e. the length of days), dividing the estrous cycle into four phases: spring transition period, ovulatory/breeding period, autumn transition period, and winter anestrus.

The increased exposure of light during the transition from winter to spring marks the end of winter anestrus and the beginning of the spring transition period, which will last approximately 2 months. The mare's pineal gland is stimulated by the release of serotonin due to nerve impulses generated from light captured by photoreceptors on the retina [27]. Increased exposure to light during spring and summer leads to a decrease in melatonin production, signaling the release of GnRH from the hypothalamus to the adenohyphosis, most commonly referred to as the anterior pituitary; here, FSH and LH are produced and then discharged to the ovaries [27, 29]. During this period FSH levels dominate and the production of LH, inhibin, insulin-like growth factor, and estradiol-17β (E2) are deficient, preventing the formation of preovulatory follicles [27]. Inhibin, and E2 work together to induce negative feedback on FSH; however, in low concentrations, negative feedback is inhibited, allowing FSH to remain in high concentrations. Due to the lack of LH synthesis, there is no production of a dominant follicle, resulting in the simultaneous regression of the minor follicular waves. Minor follicular waves are only produced during the spring transition period, with many non-ovulatory follicles developed, ranging from 6-21 mm in diameter [27].

The estrous cycle of a mare is defined as the period between two consecutive ovulations; this lasts approximately 18-22 days. Unlike other mammals, the mare's

estrous cycle is divided into two stages: estrus (follicular phase) and diestrus (luteal phase) [27]. During the follicular phase, estrogen, luteinizing hormone, and inhibin levels peak, whereas, progesterone, FSH, and prostaglandin F2α (PGF2α) levels remain at baseline levels (Figure B2). Estrus refers to the period in which the mare is in 'heat,' meaning she is sexually receptive to the stallion's genitals and is ready to receive and transport sperm for fertilization of the oocyte. Estrus lasts approximately 5-7 days, and the rise in estrogen- in particular, the peak at around day 5- influences sexual behavior in the mare [27]. As mentioned previously, the increasing levels of inhibin and luteinizing hormones, which peak between days 5-7 of estrus, are responsible for deviation of the dominant follicle and atresia of subordinate follicles. Once deviation of a dominant follicle reaches completion, ovulation occurs and estrogen levels return to baseline while progesterone levels rise, marking the beginning of diestrus [27]. Once the dominant follicle ruptures and releases the mature oocyte, estrogen levels rise to the same level as that of progesterone, to aid in formation and maturation of the corpus luteum, which functions in the same ways as that of the human female. In the absence of implantation, the corpus luteum regresses and menstruation occurs in the final days of diestrus [27]. Mare menstruation differs from that in human females as mares do not bleed during this period. Instead, they absorb their endometrial slough and may also excrete it, which is advantageous to mares as bleeding may attract predators [29].

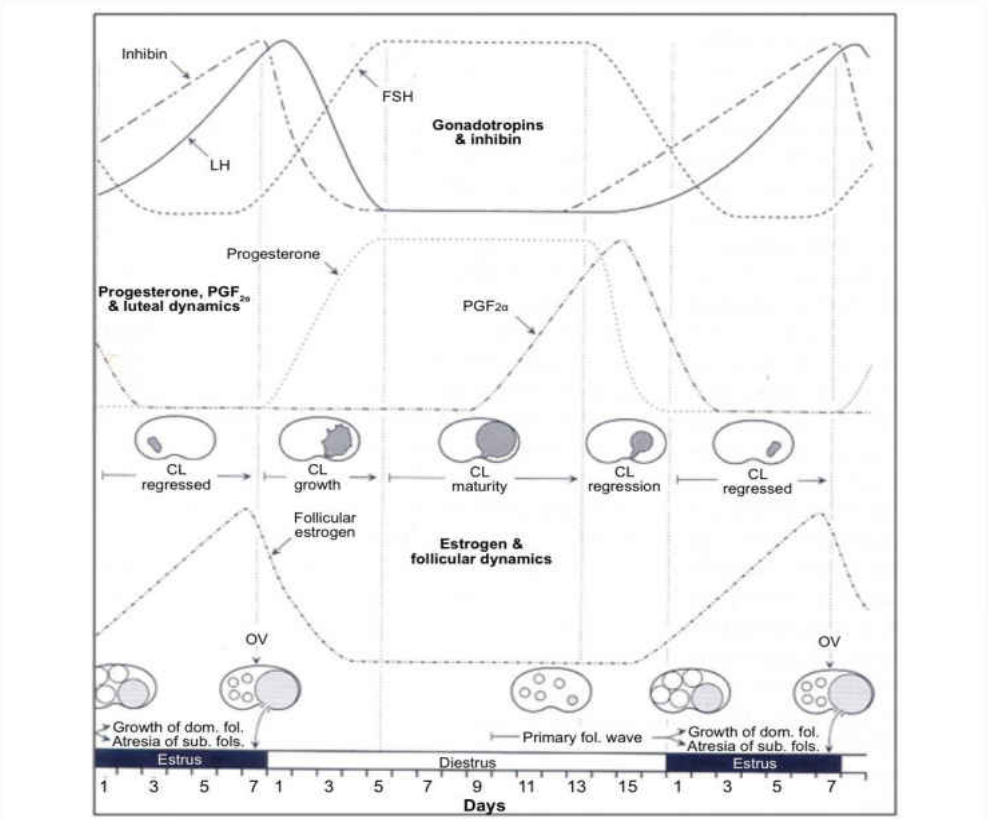


Figure B2. The Mare Estrous Cycle. Divided into two phases: estrus and diestrus. Estrus occurs between day 1-7 and is characterized by secondary follicular waves up until the deviation of a dominant follicle. During this period, estrogen levels are high and progesterone is low. Ovulation marks the beginning of diestrus, in which progesterone levels rise and will continue to remain high until the corpus luteum regress (around day 13), while estrogen returns to baseline. In the final days of diestrus, primary follicular waves occur and estrogen slow increases as progesterone decreases *Source(s): Journal of Steroids & Hormonal Science* 2013). *Source:* Saute, K. & Gardon, J. C. A review of the estrous cycle and the neuroendocrine mechanisms in the mare. *J Steroids Horm Sci* 4(115), 1-8, doi:10.4172/2157-7536.1000115 (2013).

2.2.4 The Uterus, Placenta, Vagina, and Microbiota: Implications in Pregnancy

The uterus has one major responsibility: to provide a suitable, secure environment for a fetus throughout development until delivery, usually 37-40 weeks of gestation for humans and 320-340 days of gestation for mares [29, 30]. For human females, the uterus is prepared throughout menstruation for implantation of an embryo by increasing the thickness of the endometrial layer [20]. If implantation is successful, the thick endometrium will remain until after delivery and the placenta will

begin to develop [20]. The placenta is a temporary organ made up of both maternal and fetal tissues, derived from maternal endometrium and the fetal chorionic sac [31]. Chorion cells within the placenta will produce hCG and other luteotropins to allow the corpus luteum to remain [20, 31]. By maintaining the luteal gland, estrogen and progesterone will continue production and prevent degeneration of the endometrium [31].

The placenta functions as the region for nutrient and gas exchange between mother and fetus, while also providing blood supply to fetal circulation [31]. The function of nutrient and gas exchange is carried out by syncytium, a fused multinucleate trophoblast layer on the surface of the villi within the intervillous space [32]. The syncytium is also responsible for protecting the fetus from blood-borne pathogens, though how it does so is poorly understand [32]. It is important to note that this structure, the syncytium, is present in human females; however, placental structure varies greatly between mammals. Although the placenta of pregnant mares is diffuse and cotyledonary like that of human females, the presence of syncytium has not been reported [33, 34]. In a non-pregnant woman, the cervical canal acts in conjunction with the uterine peristaltic pump for sperm transport [35]. During pregnancy the cervical mucus plug forms, which acts as a permeability barrier to limit passage of potentially pathogenic bacteria from the vagina [35, 36]. Recent studies have indicated that the cervical mucus plug may not be completely impermeable to ascending bacteria from the vagina and, with help from the uterine peristaltic pump, may allow transport of bacteria into the intrauterine cavity [35, 36].

Until recently, it was thought that the upper female reproductive tract did not possess a microbiome; however, we now know that one component, the uterus, does. Studies that have subjected endometrial tissue isolates to 16S rRNA gene sequencing in women with and/or without uterine abnormalities have identified distinct microbial communities [37, 38]. Verstraelen et al. harvested endometrial tissue and mucus of nineteen women without uterine abnormalities [37]. They targeted the 16S rRNA gene hypervariable V1-2 region by barcoded Illumina MiSeq paired-end sequencing and found 15 bacterial phyla that were present in all samples. Furthermore, it has been shown that this uterine microbiome persists during pregnancy [30]. These findings have led the field of microbiome research to question the widely accepted sterile womb hypothesis, which holds that the fetus develops within a sterile amnion. To ascertain that the amnion is sterile is also to assume that the placenta is naturally free of microbes and that microbes present within placental structures occur as a result of infection. An additional assumption of this hypothesis is that the fetus will not be exposed to maternal microbiota until passage through the birth canal [30].

These ideas have been challenged, however, since 1927, when Harris and Brown discovered bacteria in the amniotic fluid of cesarean section deliveries. This discovery provided strong evidence that vaginal deliveries may not be the newborn's initial exposure to microbes [30]. Doyle et. al conducted a study in 2014, in which they determined that microbial communities were present in placental membranes regardless of mode of delivery [39]. In addition, the authors correlated certain microbial communities with pre-term births and others with full-term births. This study

revealed a greater relative abundance of the *Enterobacteriaceae* family and the genera *Fusobacterium, Streptococcus, Mycoplasma, Aerococcus, Gardnerella*, and *Ureaplasma* in the pre-term samples, most of which are found in the vaginal microbiota of individuals with bacterial vaginosis. It was also noted that these communities were absent in the full term (FT) placentas. Furthermore, they discovered that bacteria were present in all placentas of both vaginal and caesarean births, supporting the idea that the fetus is exposed to microbiota prior to delivery [39]. Doyle et. al describes that labor is initiated by a scheduled inflammatory response in both the placenta and amniotic fluid, which is influenced by maternal and fetal signals. Based on this description, an unscheduled inflammatory response is defined as pre-term birth (PTB) initiation [39].

PTB can be classified as either very pre-term (V-PTB) or just pre-term. V-PTB, for humans, is defined as delivery prior to/at 28-weeks gestation and PTB is defined as delivery between 28-32 weeks gestation [30]. For mares, PTB is defined as delivery prior to 320 days gestation [34]. A common cause of PTB is infection, either exogenous or endogenous, which is subsequently able to pass the placental membrane and infect the fetus [30]. In fact, 90% of PTB cases in mammals are due to an infectious agent that causes severe inflammation of the placenta and amniotic fluid (placentitis and amnionitis) [30, 39]. In 2013, Stout et. al hypothesized that negative pregnancy outcomes may arise from bacterial communities residing in the maternal basal plate of the placenta. The results revealed Gram positive and negative intracellular bacteria in 27% of placental basal plates with a significant association in pre-term births [40].

However, bacterial communities residing in the placenta may not always be indicative of placentitis. A study conducted by Queiros da Mota et al. observed that 28 out of 73 culture-positive placenta samples were positive in the presence of chorioamnionitis (intra-amniotic infection) and the remaining 45 cases did not, indicating that microbial communities in the placenta are less likely a consequence of an infectious agent and that microbiota may naturally transfer to the placenta during pregnancy to perform beneficial functions [41]. Aagaard et al. characterized 320 placental microbiome samples of healthy pregnancies and found that the most common microbes are Firmicutes, Tenericutes, Proteobacteria, *Bacteroides*, and *Fusobacteria* [42]. The results imply that the placenta might possess microbiota that are transient colonizers and that bacteria within the placenta may not always lead to negative pregnancy outcomes.

In contrast to the sterile womb hypothesis, Perez-Munoz et al. proposed that the infant gut microbiome is established in utero via hematogenous transfer of maternal oral and gut microbiota [43]. The sterile womb hypothesis argues that the gut microbiome of vaginally delivered babies resembles the maternal vaginal microbiome and C-section babies have gut microbiota similar to that of the maternal skin microbiota. In contrast, the in-utero colonization hypothesis states that maternal oral and gut microbes hematogenously transfer to the placenta throughout gestation. These microbes are subsequently transferred to the fetus via the umbilical cord, where they will begin initial colonization before birth [43]. This proposal was derived from results of studies in which microbiota found in placental membranes and amniotic fluid

were also found in the oral cavity and colon, some of which had no associated pregnancy complications and others linked with negative pregnancy outcomes such as PTB and miscarriage [44-46].

To investigate how the microbiome of the uterus impacts health of pregnancies, Moore et. al compared uterus samples of virgin and pregnant heifers [47]. In uteri of both virgin and pregnant heifers, three phyla were found in similar abundance: Bacteroidetes, Firmicutes, and Proteobacteria, which also happen to be common phyla of the GI tract across a majority of mammalian species. In addition, the investigators discovered that the same microbes present in the virgin and pregnant uteri are also microbes associated with post-partum uterine disease, a common cause of infertility in cattle due to pathogenic bacteria persisting in the uterus after delivery. Based on these results, Moore et. al hypothesized that post-partum uterine disease is most likely a result of the overgrowth of resident microbes upon pregnancy and the heifers' immune response to the overgrowth. Overall, Moore et. al concluded that a resident microbiome of the uterus is established by the time of reproductive maturity [47]. Consistent with this study and several others, microbial communities found in the uterus are also present in the vagina and gut. Therefore, Moore et al. proposed that microbiota from the vagina and cervix migrate via intrauterine ascension, where they will subsequently colonize the uterus. The investigators also concluded that the uterine microbiome is maintained in a quiescent state in the virgin and pregnant uterus until influenced by events, in which mechanisms utilized by the body to maintain microbiota composition are disrupted [47]. The results of this study suggest

that negative pregnancy outcomes may be a result of the loss of ability for the host to keep resident microbiota under controlled growth.

Much of the literature that encompasses uterine microbiome studies also focus on the vaginal microbiome of only pregnant subjects, non-pregnant subjects, or both. Due to discoveries of similarities between the uterine and vaginal microbiome, it stands to reason that microbes present in the vagina should be considered when investigating the microbiome's influence on the uterus and pregnancy. This is especially true when considering the vertical ascension route of vaginal microbiota to the uterus during sexual maturity and/or to the amnion during pregnancy. A general consensus among vaginal microbiome studies is that, in comparison to the GI tract, the vagina is low in biodiversity in terms of both alpha- and beta diversity [48, 49]. These studies are also in agreement that a healthy vaginal microbiome is comprised mostly of *Lactobacillus* (approximately 20 species reported) and is dominated by one to two of the four most common *Lactobacilli* species: *L. crispatus, L. gasseri, L. iners,* and *L. jensenii* [49, 50]. It is hypothesized that *Lactobacilli* protect the vagina from colonization by pathogenic microorganisms through the production of lactic acid, and by outcompeting them for nutrients and epithelial cell receptors [49]. There are circumstances, such as bacterial vaginosis (a polymicrobial disease due to bacterial overgrowth in the vagina) and the variation of vaginal microbiota composition in women of different geographical locations, in which *Lactobacilli* are in smaller abundance and anaerobic bacteria dominate [49]. The most common anaerobes reported under these circumstances are *Prevotella, Megasphaera, Gardnerella*

vaginalis, Sneathia, and *Atopbium vaginae.* Nevertheless, composition of vaginal microbiota is subject to change, depending on hormonal changes, age, sexual activity, and overall reproductive health [49, 50].

Once pregnancy is established, it is observed that the individual's vaginal microbiome composition is more stable and less diverse [49]. This is primarily attributed to the dominance of estrogen; therefore, hormone fluctuation is dramatically decreased [51, 52]. It is also noted that the presence of anaerobes in the vagina decrease during the time of gestation. The rise of estrogen may favor *Lactobacilli* survival as this results in increased vaginal glycogen deposition [49]. Though the vaginal microbiome composition tends to remain constant throughout pregnancy, as mentioned previously, the gut microbiome changes frequently, independent of diet. The most commonly reported bacterial species colonizing the vagina in association with PTB deliveries are *Ureaplasma urealyticum, Mycoplasma hominis, Bacteroides spp., Gardnerella vaginalis,* and *Fusobacterium nucleatum* [49, 50]. As mentioned previously, defects in the cervical mucus plug play a key role in the intrauterine ascension of vaginal bacteria.

After reviewing the literature, we are left with three main questions regarding the uterine microbiome: (1) Is the amnion exposed to microbiota? If so, in what stage of pregnancy is this exposure initiated and why? (2) Why and how does the host maintain a resident uterine microbiome upon pregnancy? (3) How do these resident microbes benefit the host prior to reproduction, and what is their influence on both mother and fetus during pregnancy? The host-microbe interactions with female

reproductive organs is still far from understood; additional studies are required to confirm that the uterus houses its own unique microbiota, as well as what this implies with pregnancy and female reproductive health.

2.2.5 Objectives of Study

Although associations have been established between microbiota and pregnancy outcomes, what is left to understand about how microbiota effects pregnancy greatly outweighs what is known to date. The objective of this study is to investigate the intrinsic differences between the oral and reproductive microbial environments of post parturient mares who either foaled at or before full term. In addition, we seek to further understand the relationship between microbiota and pregnancy outcomes, more specifically, pre-term birth. We hypothesized that either a lack or large abundance of a particular phylum/phyla of bacteria in the uterus will be observed in the mares of our equine model who deliver pre-term. Furthermore, we assume that this lack/presence of microbiota associated with pre-term births will either not be present or present in small quantities of the uteri in mares who deliver at full-term.

CHAPTER 3

EXPERIMENTAL METHODS

3.1.1 Equine Model and Sample Collection

Surveys were conducted of 58 post parturient (after labor) mares, and their foals, by attending equine veterinarians from the Hagyard Equine Medical Institute in Lexington, KY**.** These surveys included the following information about the mare: name of mare, date of sample collection, farm in which mare was housed, veterinarian obtaining samples, date of mare foaling, original expected due date, inquiry of any antimicrobial treatment within the last month of gestation, location of foaling, and circumstances of foaling (normal or dystocia and how dystocia was resolved, if applicable). The following information was gathered about the foal: alive or dead at birth, whether resuscitation or similar emergency treatment was required at time of birth, a list of any congenital abnormalities present, presence and severity of flexure tendon contracture, and a list of other abnormalities present at birth or within the first 24-48 hours. These questions provide sufficient information as to the conditions of the mare and her foal pre- and post-delivery, which can be used for correlation purposes during data analysis. The following samples were collected from the mare by the attending veterinarian within 24-36 hours of foaling and before uterine lavage/infusion: swab from uterus or cervix, swab from vaginal vault, 2-5 ml of uterine fluid, blood sample, fecal sample, and oral swab. Upon collection, the samples were placed in biohazard bags and transported on ice to the Hagyard Laboratory, and

eventually shipped for sequencing preparation to the Oakley Laboratory at Eastern Kentucky University in the Department of Biological Sciences.

3.1.2 Sample Preparation and Sequencing

Once samples were received, they were stored in -20°C and -80°C (dependent on available storage room in either unit). Samples were then organized in order by their corresponding survey. Each mare was labeled consecutively "Sample #" on biohazard bag, survey, and appropriate preparation tubes for simplicity. A table and key were created throughout preparation to ensure proper labeling and identifying which, if any, specific samples from each mare were not collected at Hagyard for data analysis for future additional projects. The key is described as follows: B (blood), US (uterine swab), OS (oral swab), VS (vaginal swab), UF (uterine fluid), and FM (fecal matter). An 'X' was placed under any column in which a sample was not present for that mare. All microbiome samples were labeled in consecutive numerical order (1-332) on preparation tubes and in the table next to the appropriate mare's name.

Microbiome samples were thawed at room temperature for 30 min-1 hr, depending on severity of frozen state. Sterile microcentrifuge tubes were labeled appropriately to sample information in the collection table. All collected swabs (oral, uterine, and vaginal) were aseptically transferred directly from collection tube to preparation tube. Blood samples were centrifuged for 5 minutes at 1,300 xg and a subsequent aliquot of 300µl was placed in the corresponding preparation tube, followed by a sterile nasopharyngeal swab.

The aliquot and swab procedure was repeated for the uterine fluid collection, but these samples were not centrifuged. The aliquot was to serve as a backup in the event that the amount on the swab was not sufficient for sequencing. Finally, the fecal collection was prepared last as it takes the longest to thaw and needs to be thawed completely. Since the external regions of the fecal collection most likely contains microorganisms that are not present in the mare's gastrointestinal tract, the collection was split open and a nasopharyngeal swab was scraped to the inside of the fecal matter. An additional small amount of the solid sample was isolated using sterile, disposable tweezers was placed in the preparation tube with the swab, for the same reason for the fluid aliquots. All prepared samples were stored at -20°C and shipped on dry ice to the Gilbert Laboratory in Chicago, IL for Illumina MiSeq 16S rRNA gene sequencing.

3.1.3 Data Analysis

All data analysis of microbiome MiSeq files was executed through QIIME 1.9 on VirtualBox. Sequence reads were quality filtered (0.5 max errors and 151 trunclen), dereplicated, filtered of singletons, OTU clustered, filtered of chimeras, and mapped to OTUs using drive5 usearch 8.1. Greengenes database was used to assign taxonomy and align sequences with 97% identity. Samples exhibiting counts of ≤ 1.0 sequence/sample was filtered from OTU tables prior to taxa summary. Relative abundance of taxa was reported in percentages by the summarize_taxa.py python script in QIIME, which calculates relative abundance by dividing the counts in a particular OTU by the total number of sequences observed in the sample. Alpha

diversity nonparametric t-distribution based on 999 Monte Carlo permutations, at a depth of 180 sequences/sample was executed on the final biom files, as 180 was the minimum number of counts observed. The Shannon diversity index metric used to assess alpha diversity. Jack-knifed beta diversity at an even 100 replicates was executed on final biom files for weighted UniFrac distance matrices, which were used to make Emperor PCoA plots. Significance for relative abundance of summarized taxa reported by QIIME was assessed with a Linear Discriminant Analysis (LDA) using LEfSe (LDA Effect Size), developed by the Huttenhower Lab at Harvard University, on the web server Galaxy.

CHAPTER 4

RESULTS

Illumina Miseq output analysis in QIIME reported 2,634,820 sequencing reads, with the maximum number of OTU counts in a given sample being 24,531. Figure 1 shows relative abundance of a phyla summary across all sample types. A greater prevalence, in similar amounts, of Fusobacteria is detected in vaginal, uterine swab, and uterine fluid samples in comparison to the others (4.9%, 8.6%, and 8.2%, respectively). Figure 2 shows the same phyla summary categorized by term, showing an increase in Fusobacteria in PT samples in comparison to FT samples (6.1% vs 3.1%). To investigate the relationship between PT samples and increased Fusobacteria prevalence, genus summaries and corresponding proportions were produced for each sample type and term (Figures 3-8). PT uterine swabs share 12 genera with PT vaginal swabs (Figure 9), 12 genera with PT uterine fluid samples (Figure 10), 7 genera with PT oral swabs (Figure 11), 10 genera with PT blood samples (Figure 12), and 3 genera with PT fecal samples (Figure 13). FT uterine swabs share 14 genera with FT vaginal swabs (Figure 9), 14 genera with FT uterine fluid samples (Figure 10), 8 genera with oral swabs (Figure 11), 9 genera with FT blood samples (Figure 12), and 2 genera with FT fecal samples (Figure 13).

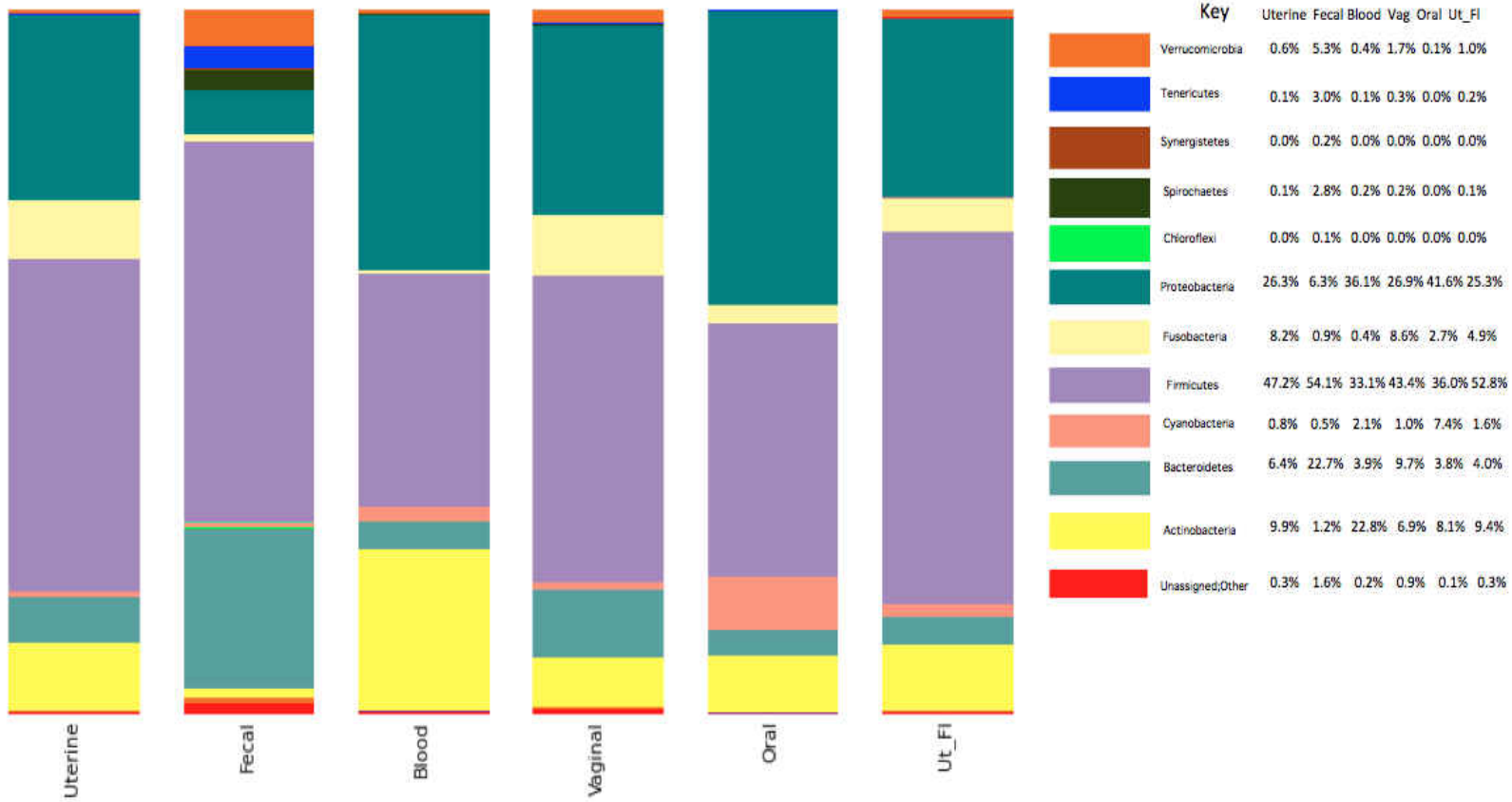


Figure 1. Phyla Summary Across All Samples by Sample Type. Phyla summary across all samples based on 97% identity on Greengenes taxonomy database. The same phyla are present in each sample, with the exception of Chloroflexi, Synergistes, and Spirochaetes. A greater abundance of Fusobacteria is detected in uterine, vaginal, and uterine fluid (8.2%, 8.6%, and 4.9% respectively. Fecal samples appear to be more diverse in comparison to other samples.

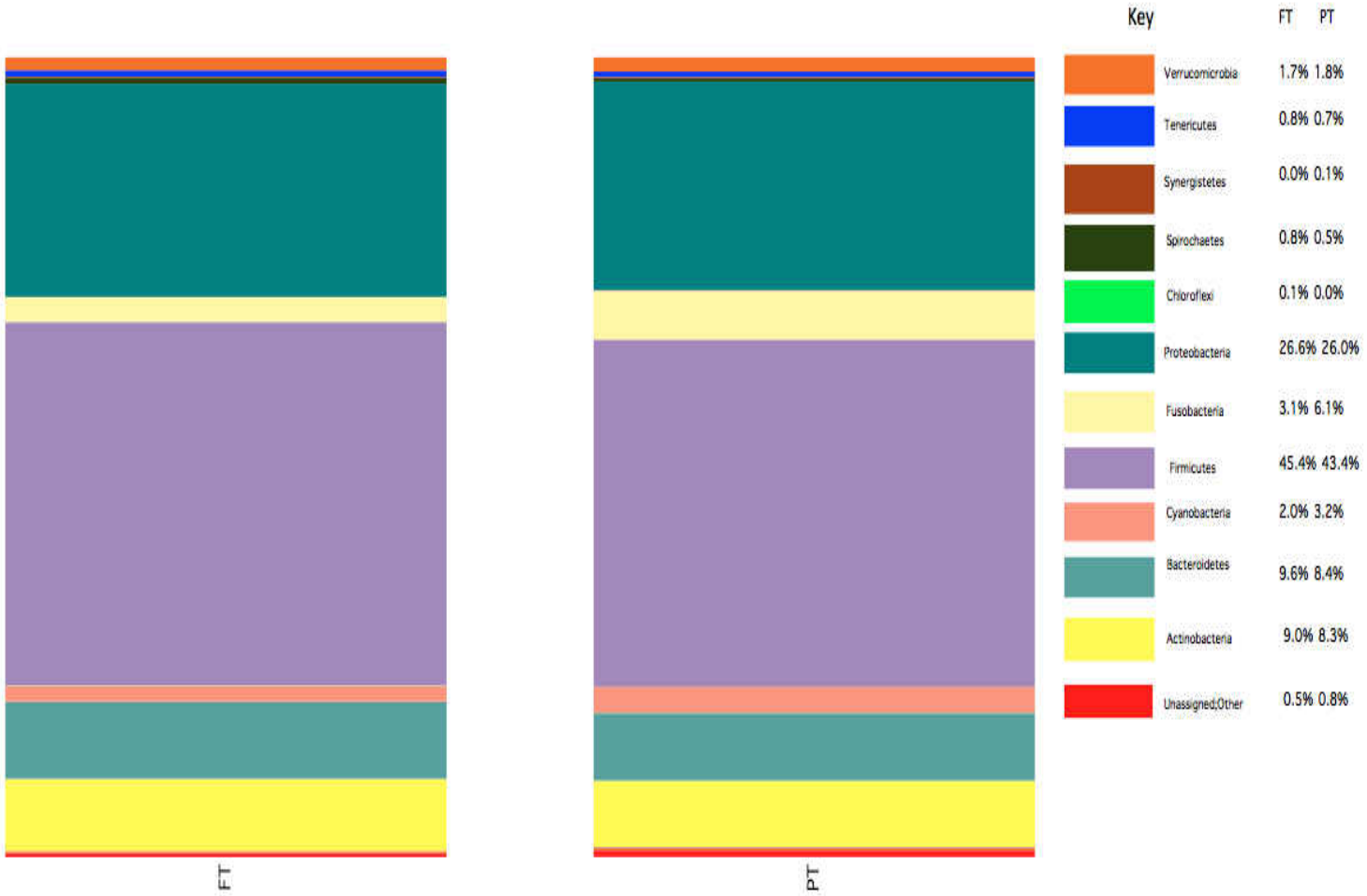


Figure 2. Phyla Summary Across All Samples by Term. The same phyla are present in pre-term (PT) and full-term (FT) samples, with the exception of Chloroflexi and Synergistes. A greater abundance of Fusobacteria is detected in PT samples versus FT samples.

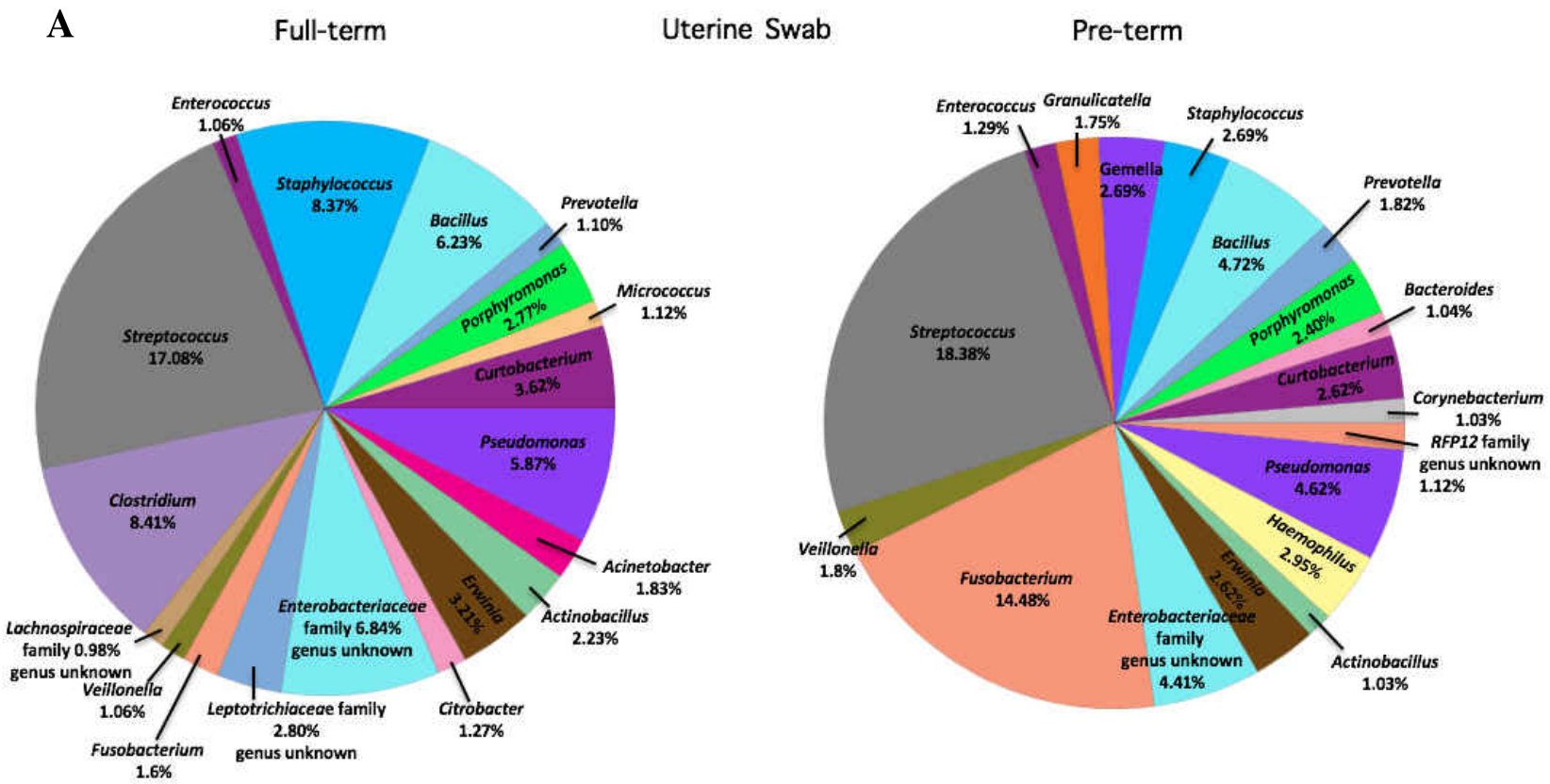
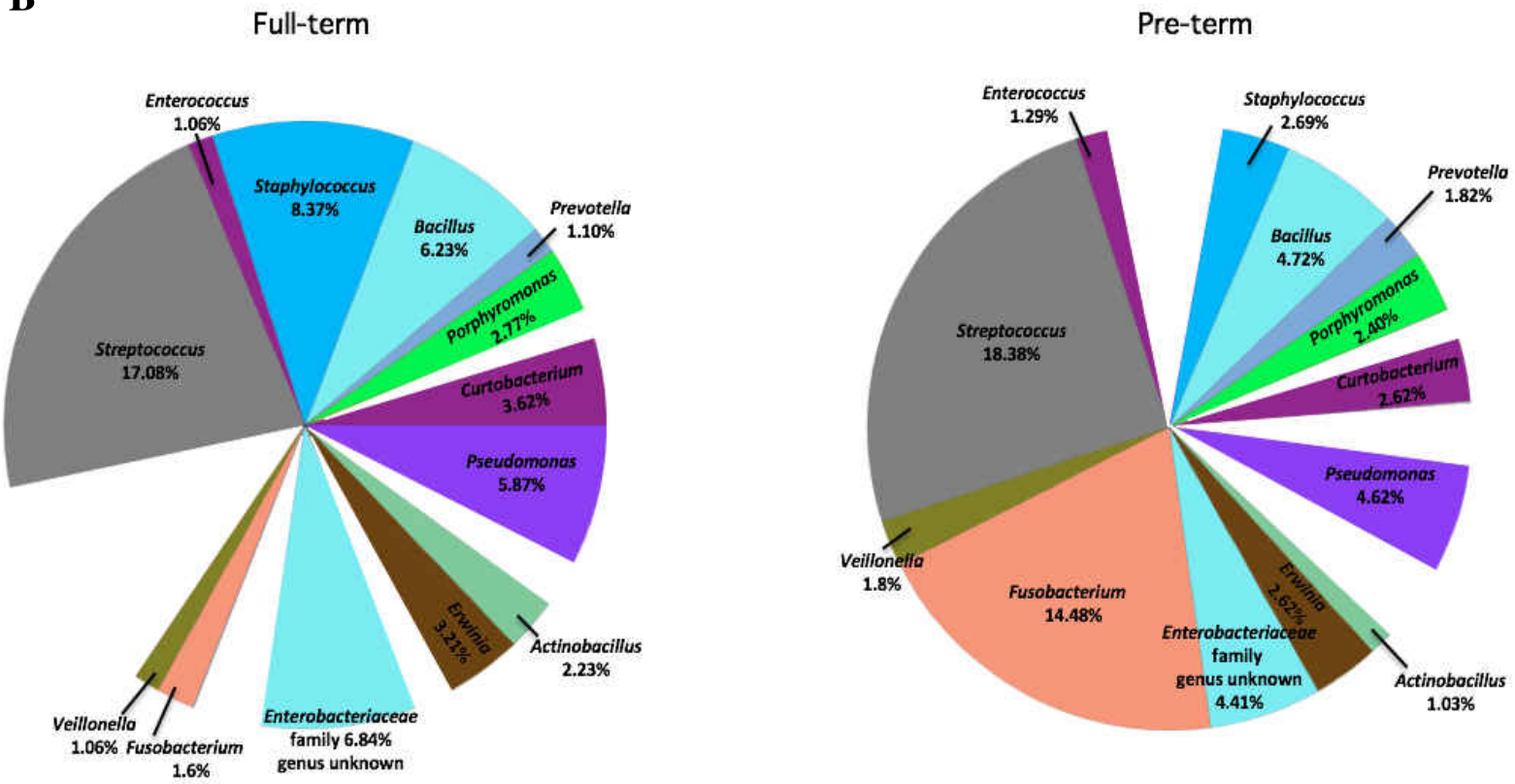


Figure 3. Genera Summary of Uterine Swabs by Term. **A)** Complete relative abundance summary of genera identified in FT (left) and PT (right), of which share 13 out of 19 (68%) reported genera. Largest observable differences in abundance of shared genera is in *Fusobacterium* (1.6% and 14.48%) and *Staphylococcus* (8.37% and 2.69%). **B)** Relative abundance with differences removed (*Granulicatella, Gemella, Micrococcus, Bacteroides, Corynebacterium, RFP12* family, *Haemophilus, Acinetobacter, Citrobacter, Leptotrichiaceae* family, *Lachnospiraceae* family, and *Clostridium*).

Figure 3 (Continued)

B



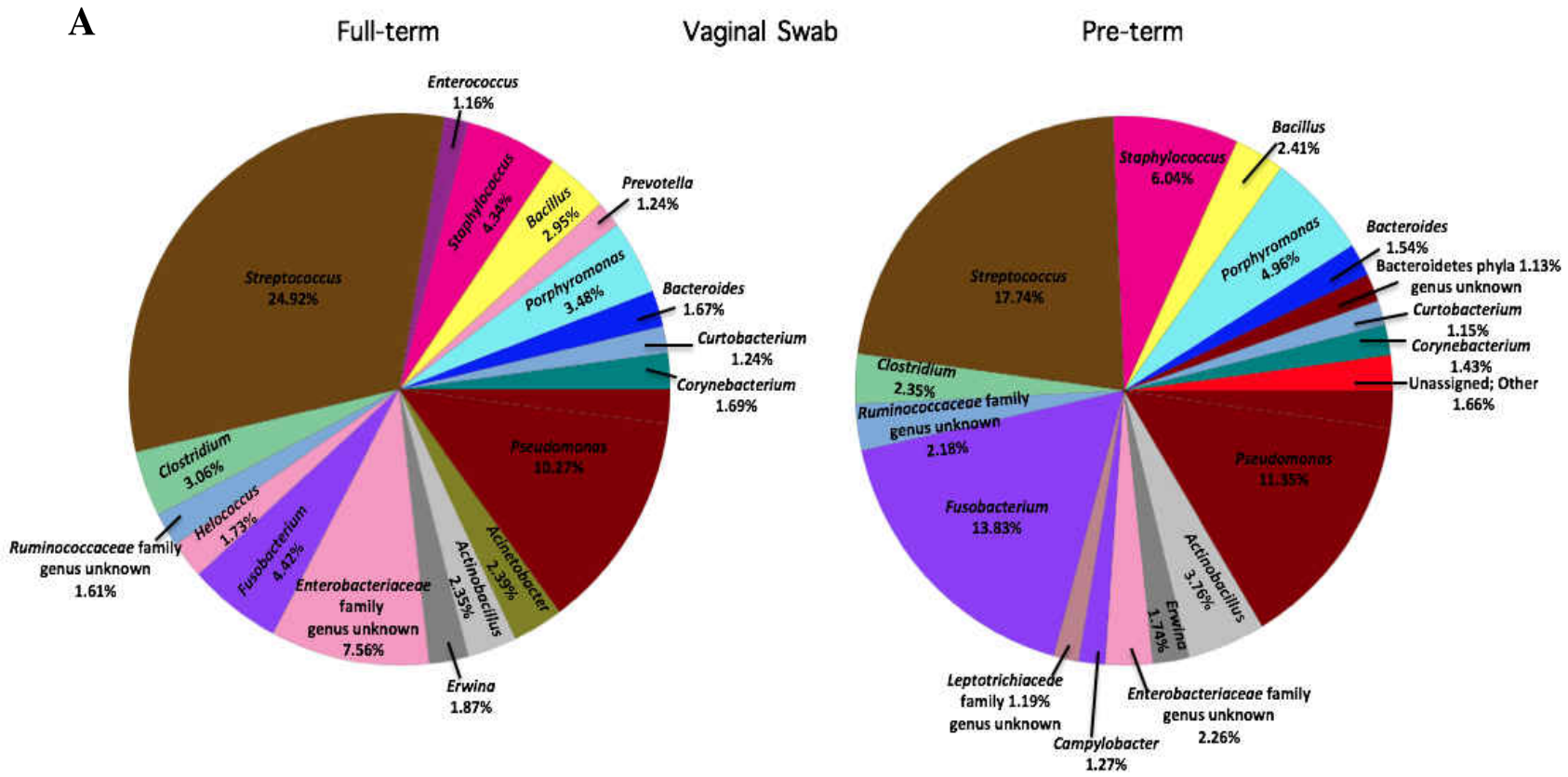
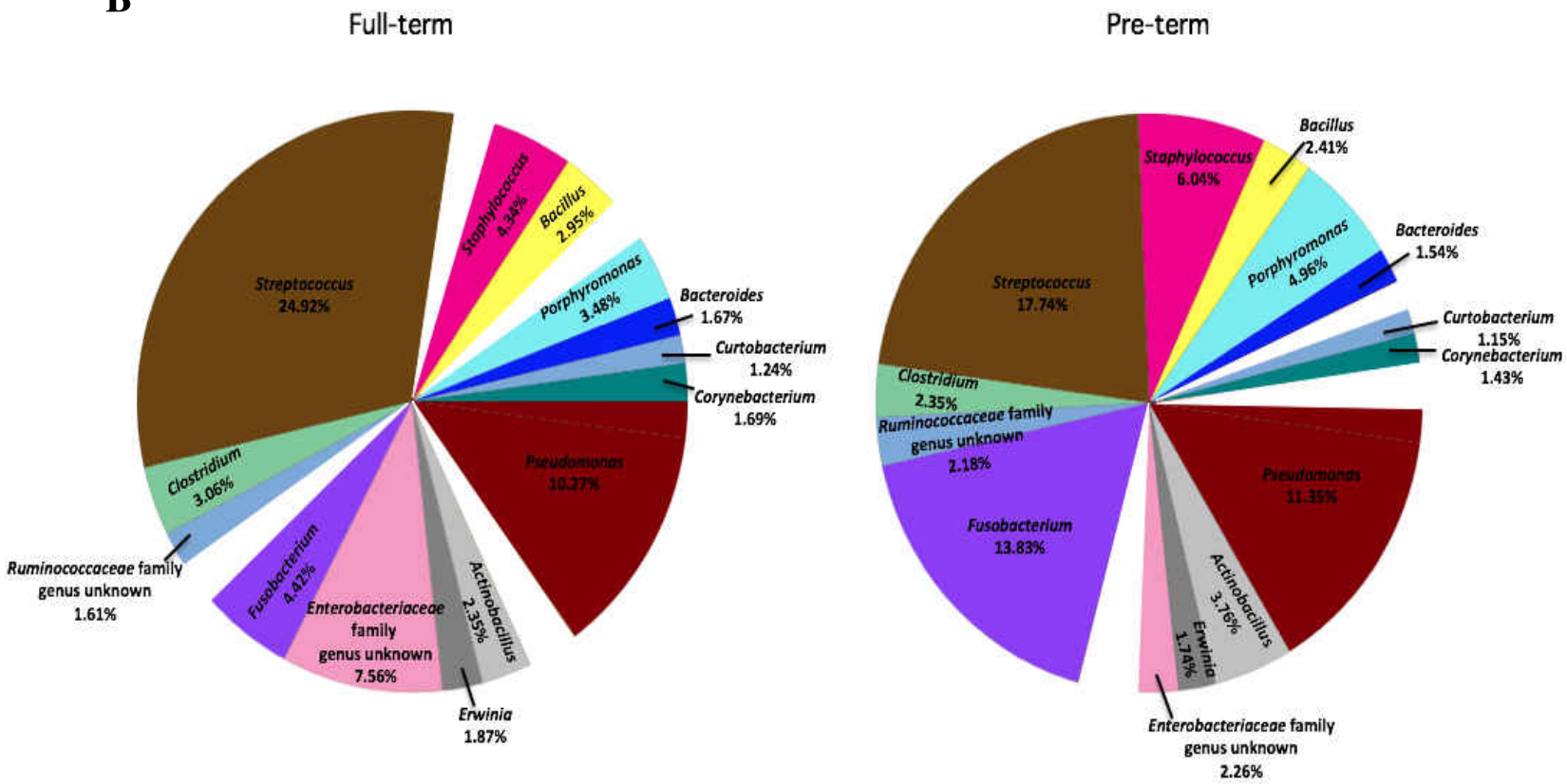


Figure 4. Genera Summary of Vaginal Swabs by Term. **A)** Complete relative abundance summary of genera identified in FT (left) and PT (right), of which share 14 out of 19 (74%) reported genera. Largest observable differences in abundance of shared genera is in *Fusobacterium* (4.42% and 13.83%) and *Enterobacteriaceae* family (7.56% and 2.26%). **B)** Relative abundance with differences removed (*Enterococcus, Prevotella,* Bacteroidetes phylum, Unassigned: Other, *Acinetobacter, Campylobacter, Leptotrichiaceae* family, and *Helococcus*).

Figure 4 (Continued)

B



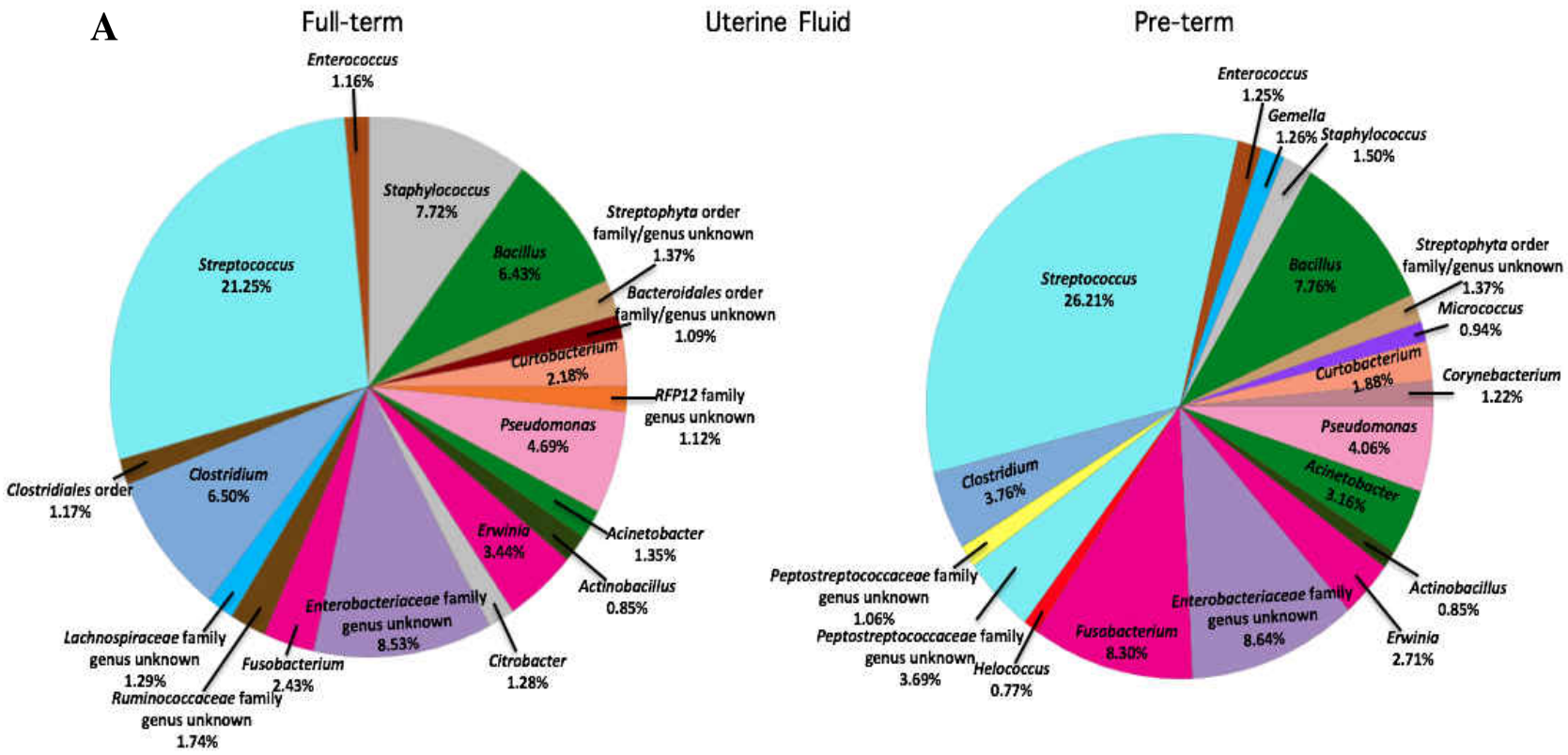
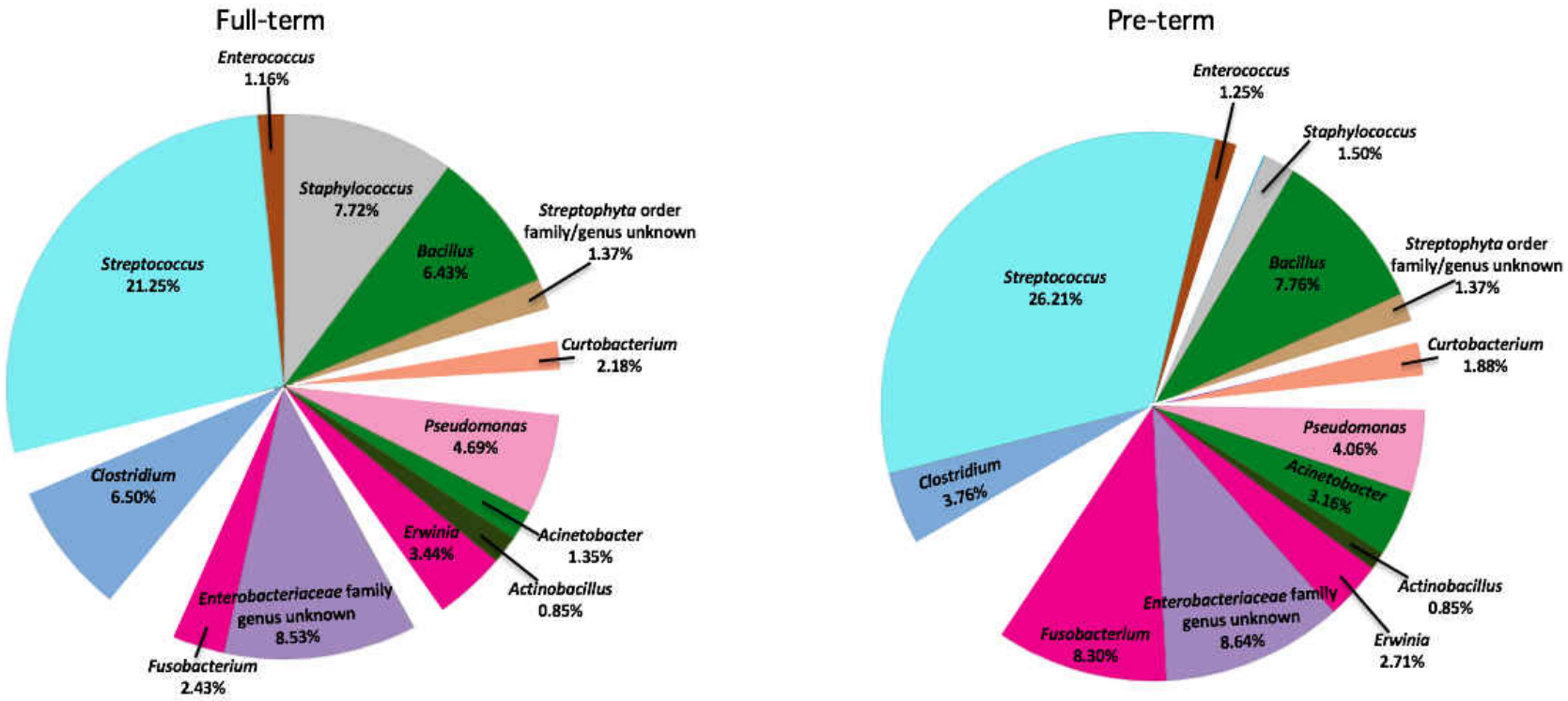


Figure 5. Genera Summary of Uterine Fluid by Term. **A)** Complete relative abundance summary of genera identified in FT (left) and PT (right), of which share 13 out of 19 (68%) reported genera. Largest observable differences in abundance of shared genera is in *Fusobacterium* (2.43% and 8.30%) and *Staphylococcus (*7.72% and 1.50%). **B)** Relative abundance with differences removed (*Bacteroidales* order, *RFP12* family, *Gemella, Micrococcus, Corynebacterium, Citrobacter, Ruminococcaceae* family, *Lachnospiraceae* family, *Clostridiales* order, *Helococcus,* and *Peptostreptococcaceae* family.

Figure 5 (Continued)

B



A

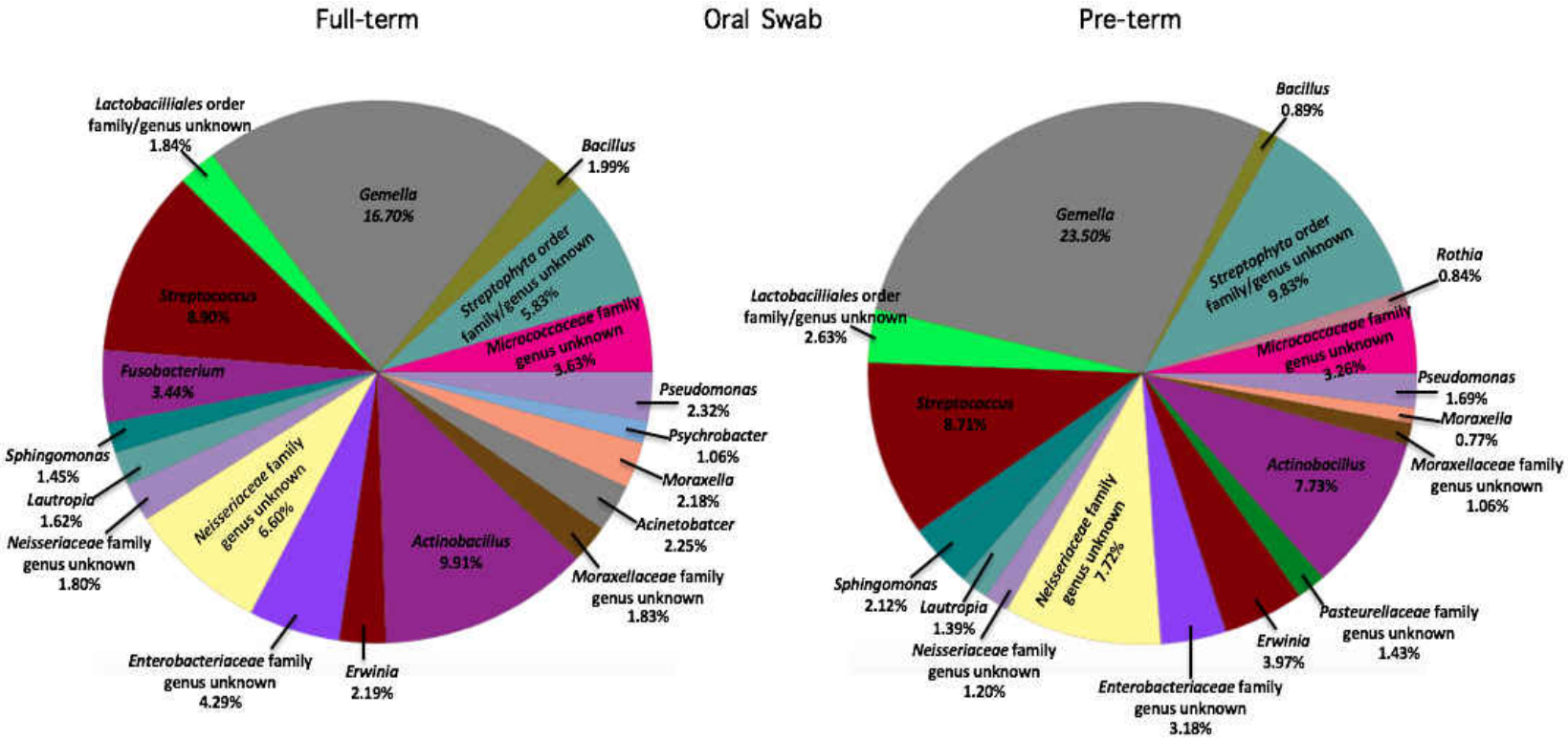
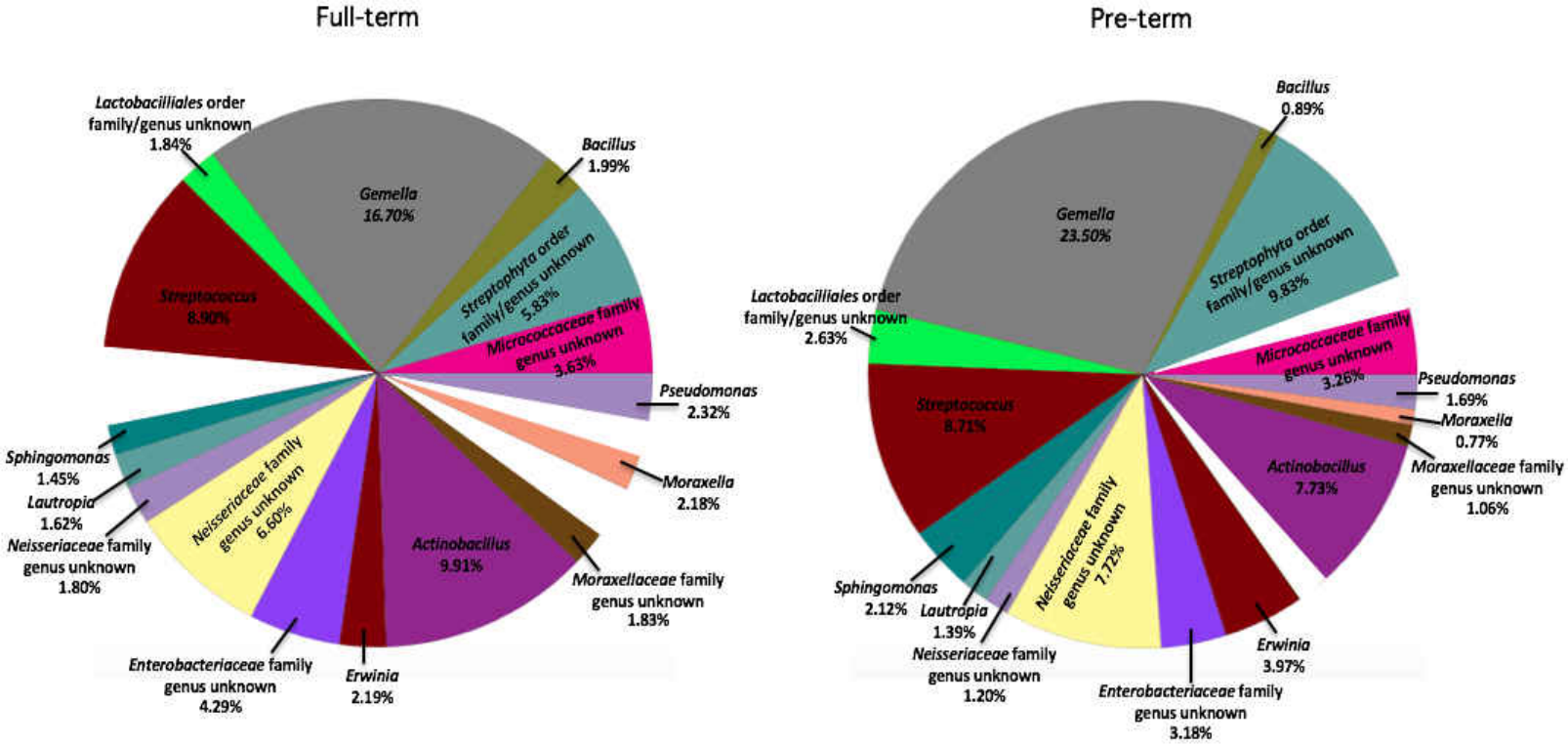


Figure 6. Genera Summary of Oral Swabs by Term. **A)** Complete relative abundance summary of genera identified in FT (left) and PT (right), of which share 16 out of 19 (84%) reported genera. Largest observable differences in abundance of shared genera is in *Streptophyta* order (5.83% and 9.83%). **B)** Relative abundance with differences removed (*Fusobacterium*, *Psychrobacter*, *Rothia, Acinetobacter,* and *Pasteruellaceae* family).

Figure 6 (Continued)

B



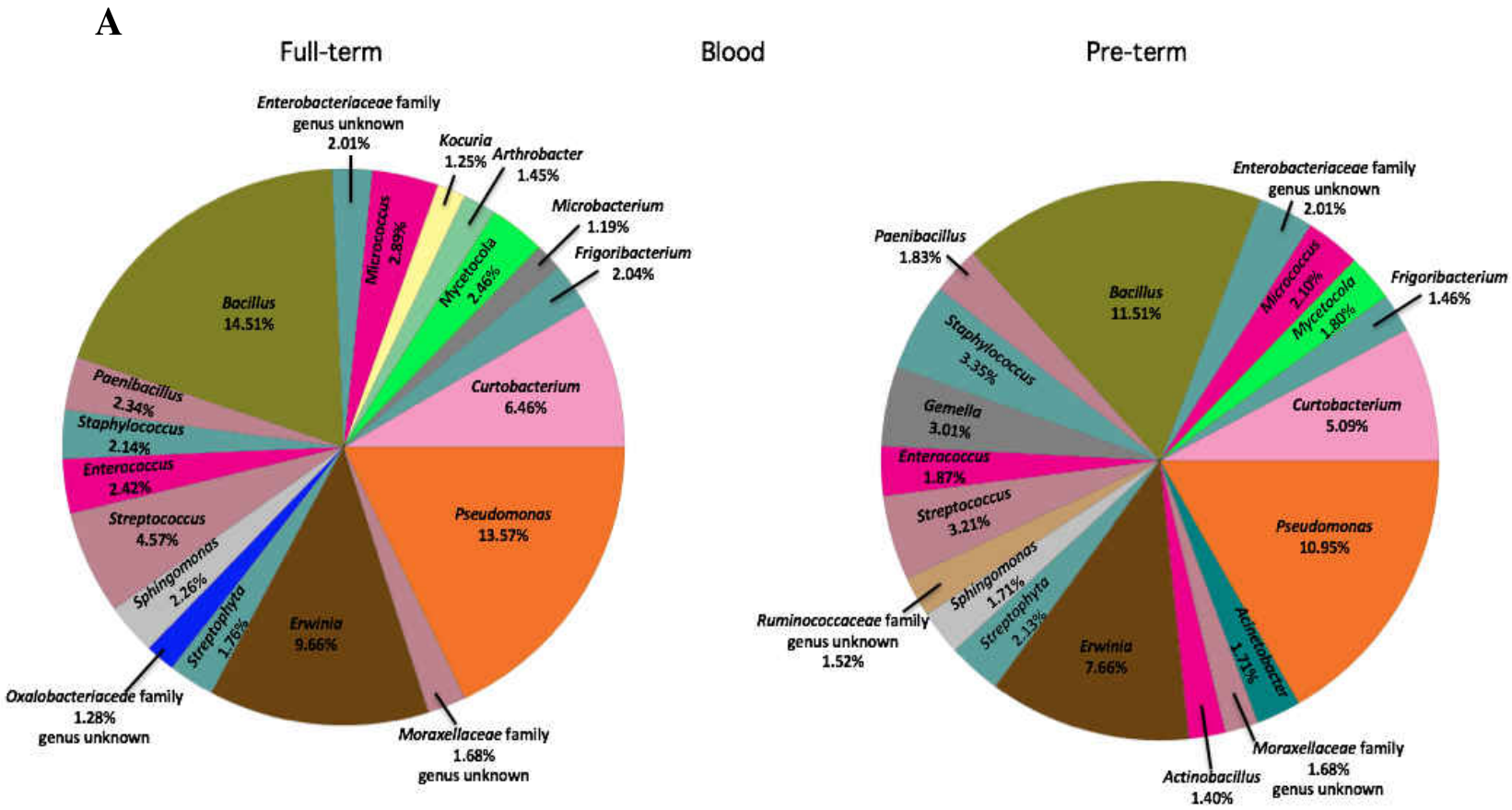
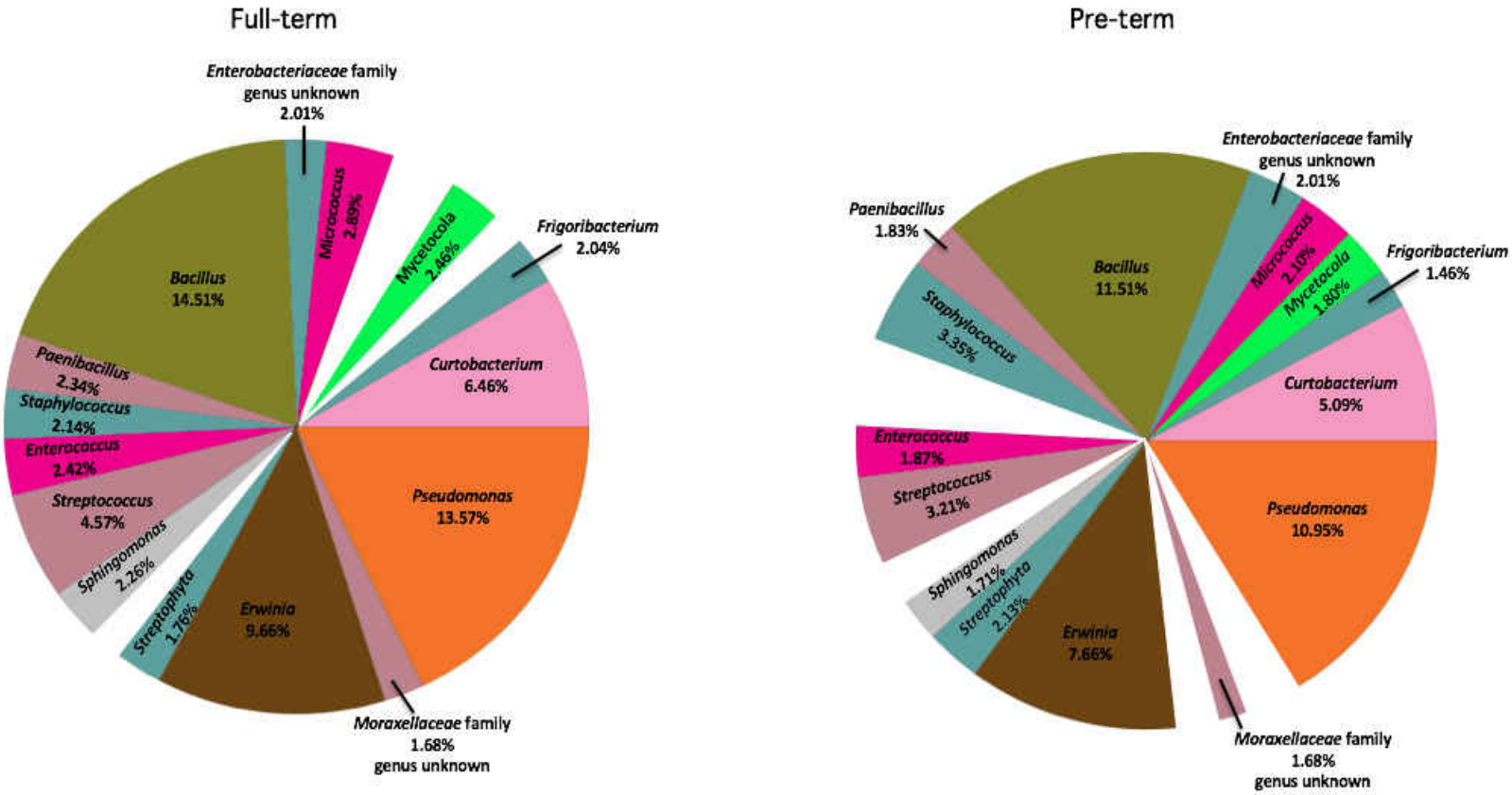


Figure 7. Genera Summary of Blood Collection by Term. **A)** Complete relative abundance summary of genera identified in FT (left) and PT (right), of which share 15 out of 19 (79%) reported genera. Relative abundance is approximately the same between FT and PT (no large observable differences). **B)** Relative abundance with differences removed (*Arthrobacter, Microbacterium, Acinetobacter, Actinobacillus, Oxalobacteriaceae* family, *Ruminococcaceae* family, and *Gemella*).

Figure 7 (Continued)

B



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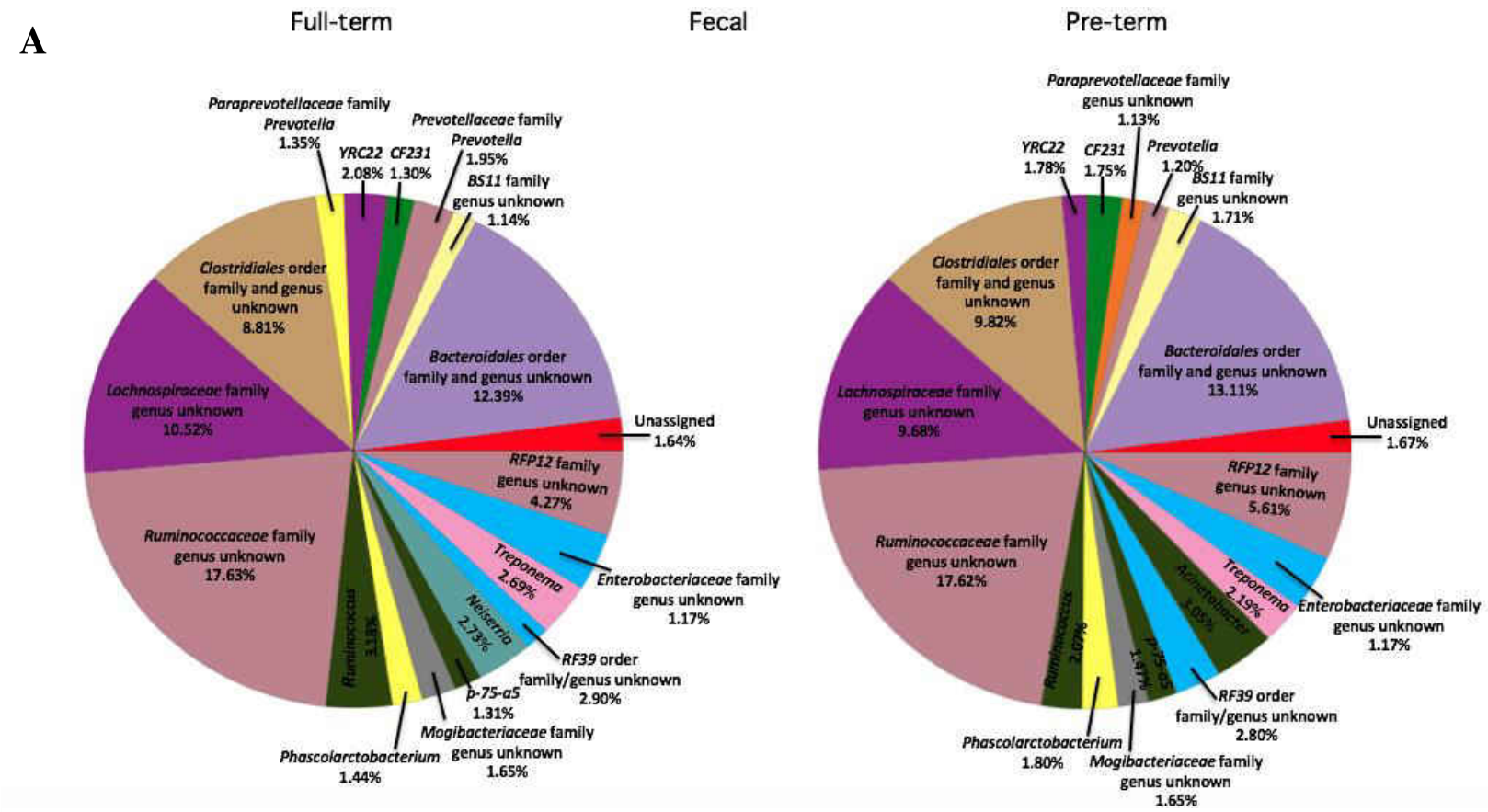
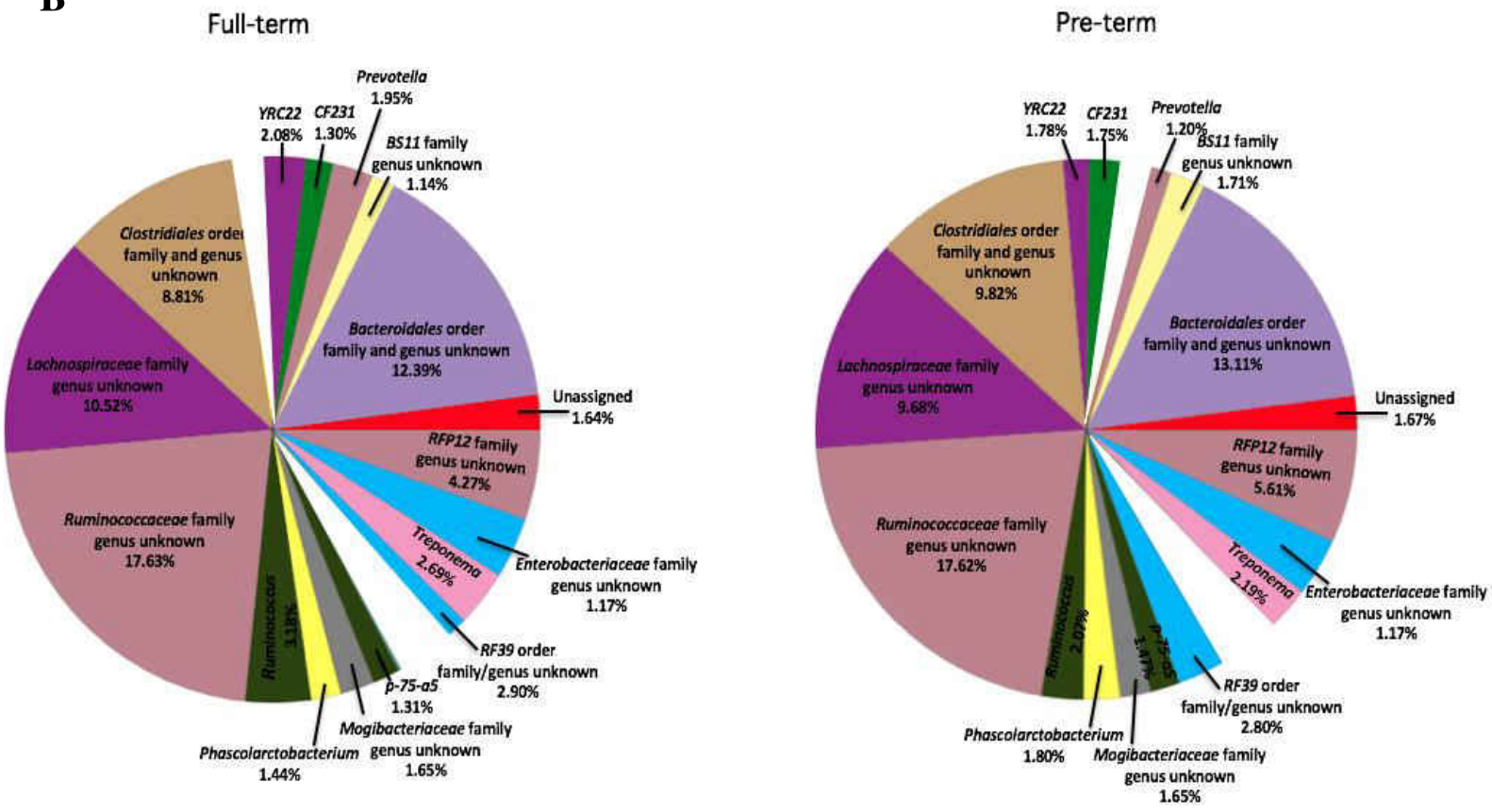


Figure 8. Genera Summary of Fecal Collection by Term. **A)** Complete relative abundance summary of genera identified in FT (left) and PT (right), of which share 17 out of 19 (89%) reported genera. Relative abundance is approximately the same between FT and PT (no large observable differences). **B)** Relative abundance with differences removed (*Paraprevotellaceae Prevotella, Paraprevotellaceae* family unknown genus, *Acinetobacter,* and *Neisseria*).

Figure 8 (Continued)

B



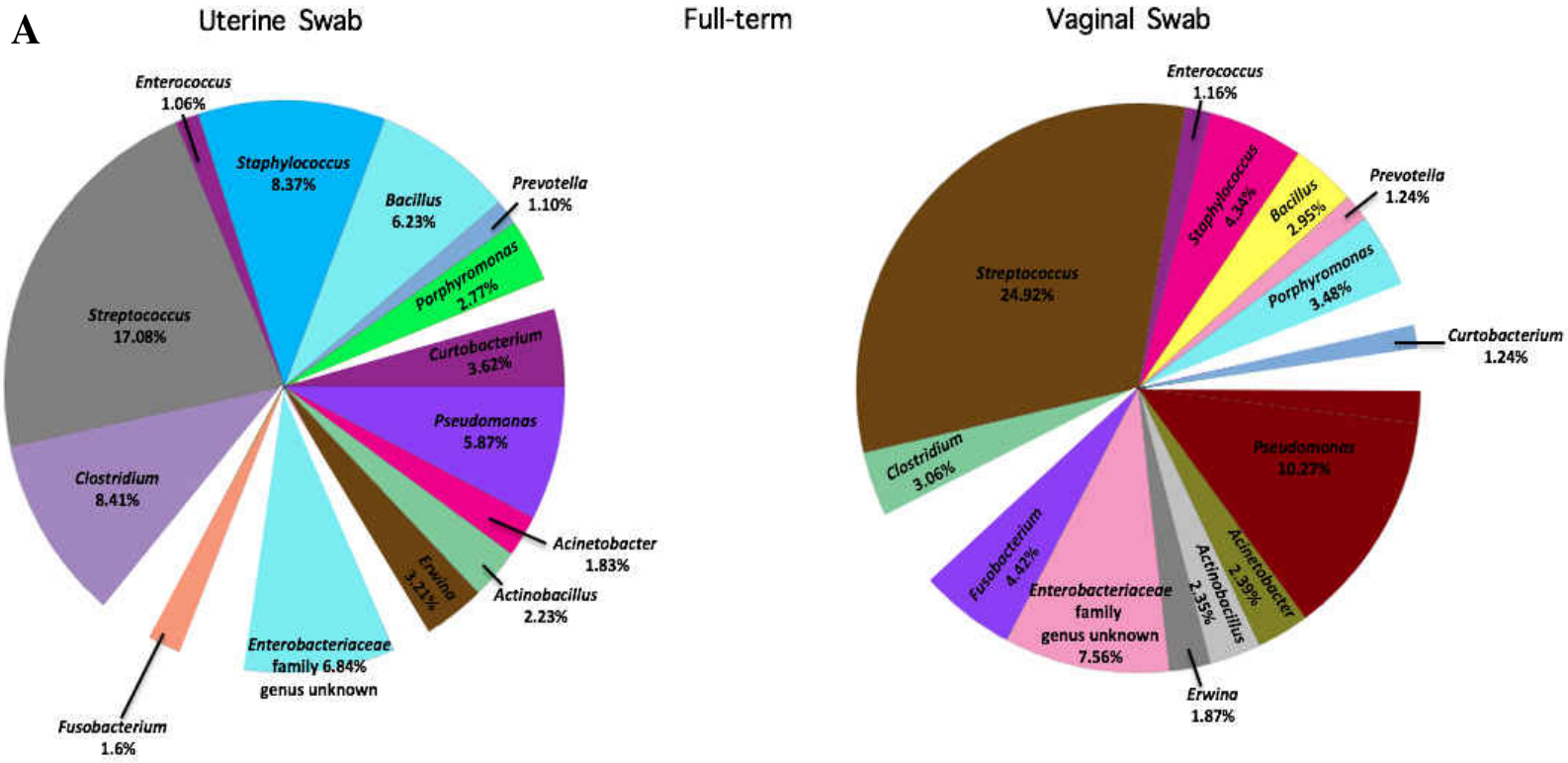
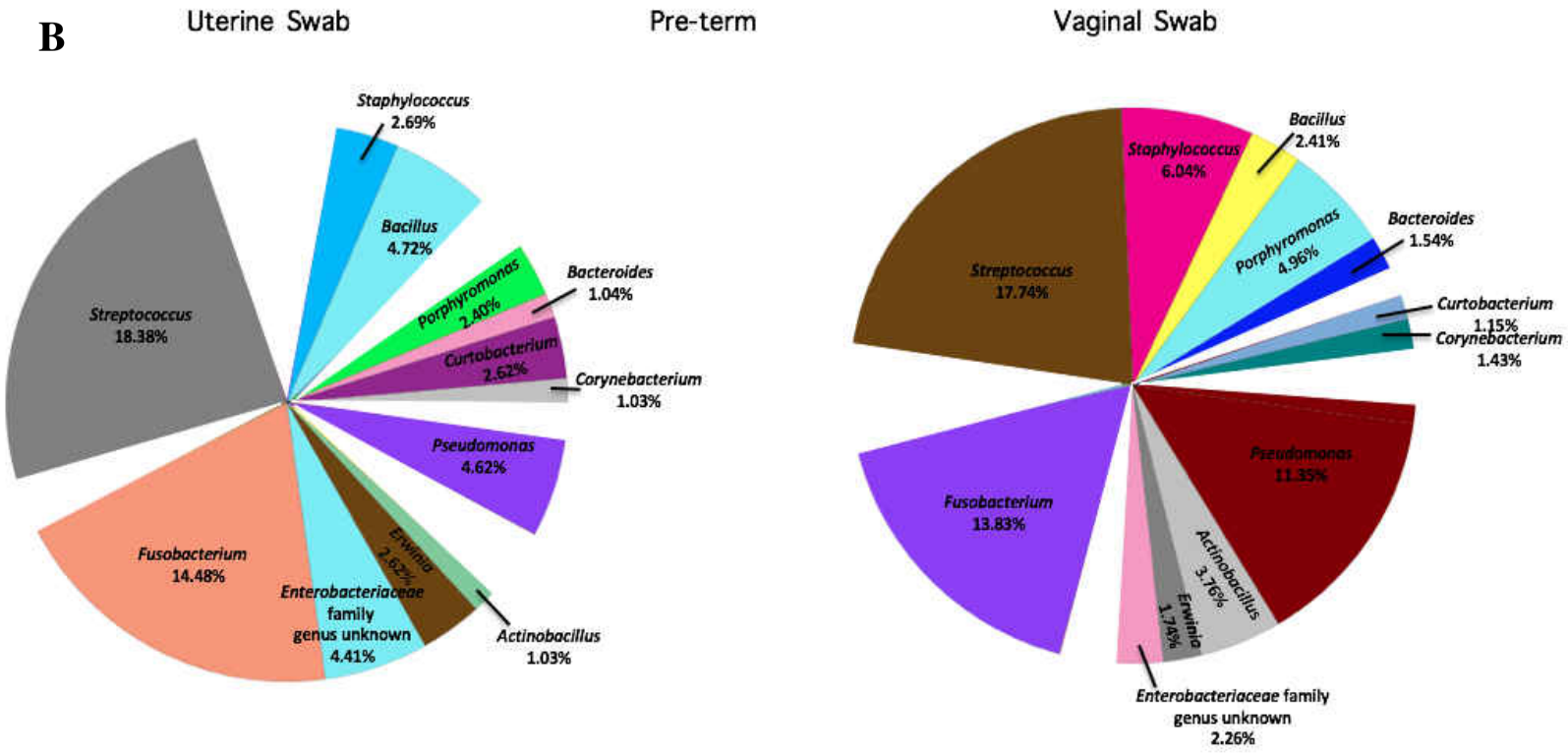


Figure 9. Genera Summary of Uterine and Vaginal Swabs by Term. **A)** Relative abundance summary of shared genera identified in FT samples (14 out of 19, 74%). **B)** Relative abundance summary of shared genera identified in PT samples (12 out of 19, 63%). *Fusobacterium* abundance is much higher in PT uterine and vaginal swabs in comparison to FT (14.48% and 13.83% PT vs. 1.6 and 4.42% FT. *Clostridium* is present in FT samples but is not present (< 0.5%) in PT.

Figure 9 (Continued)



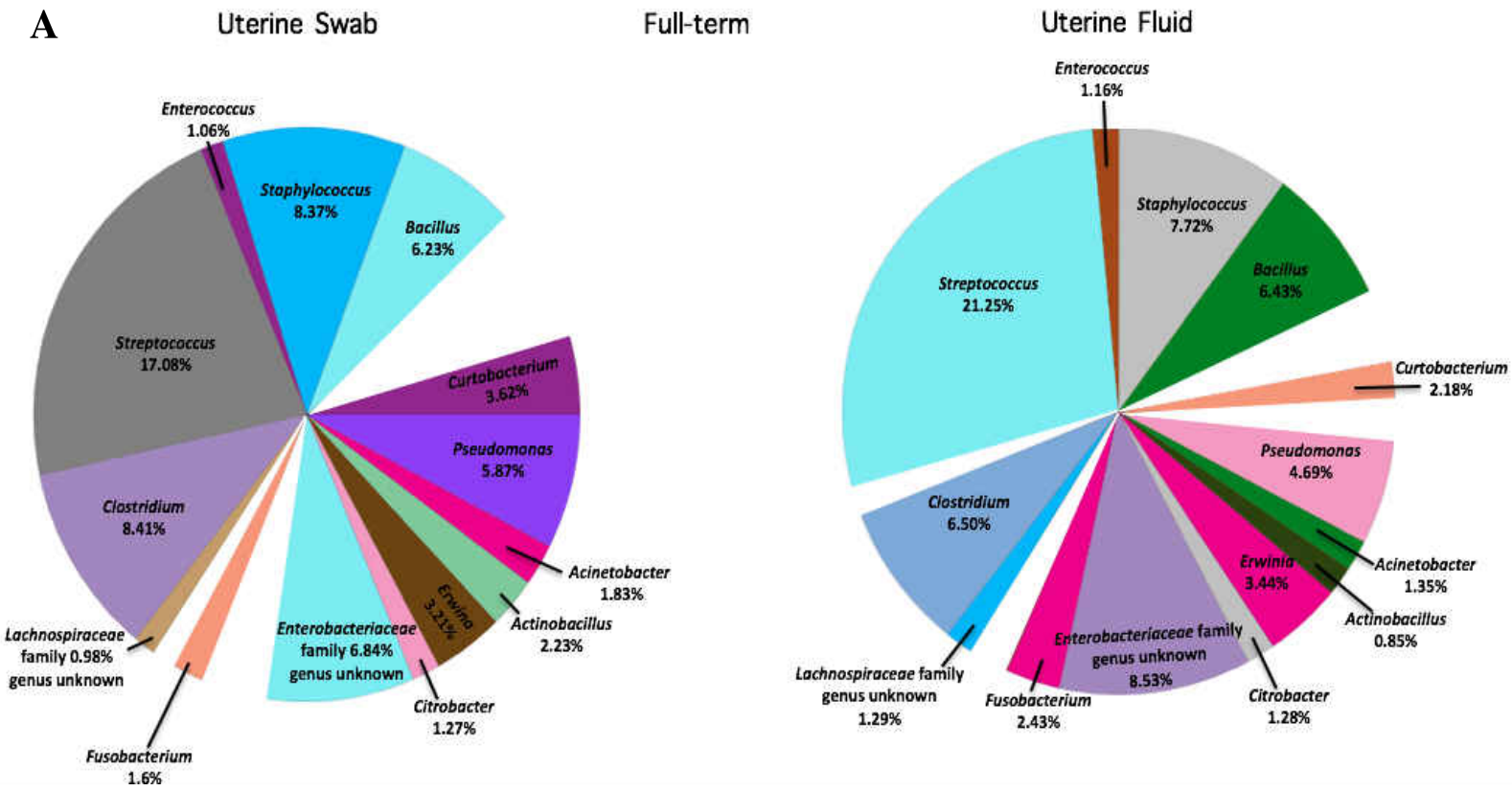
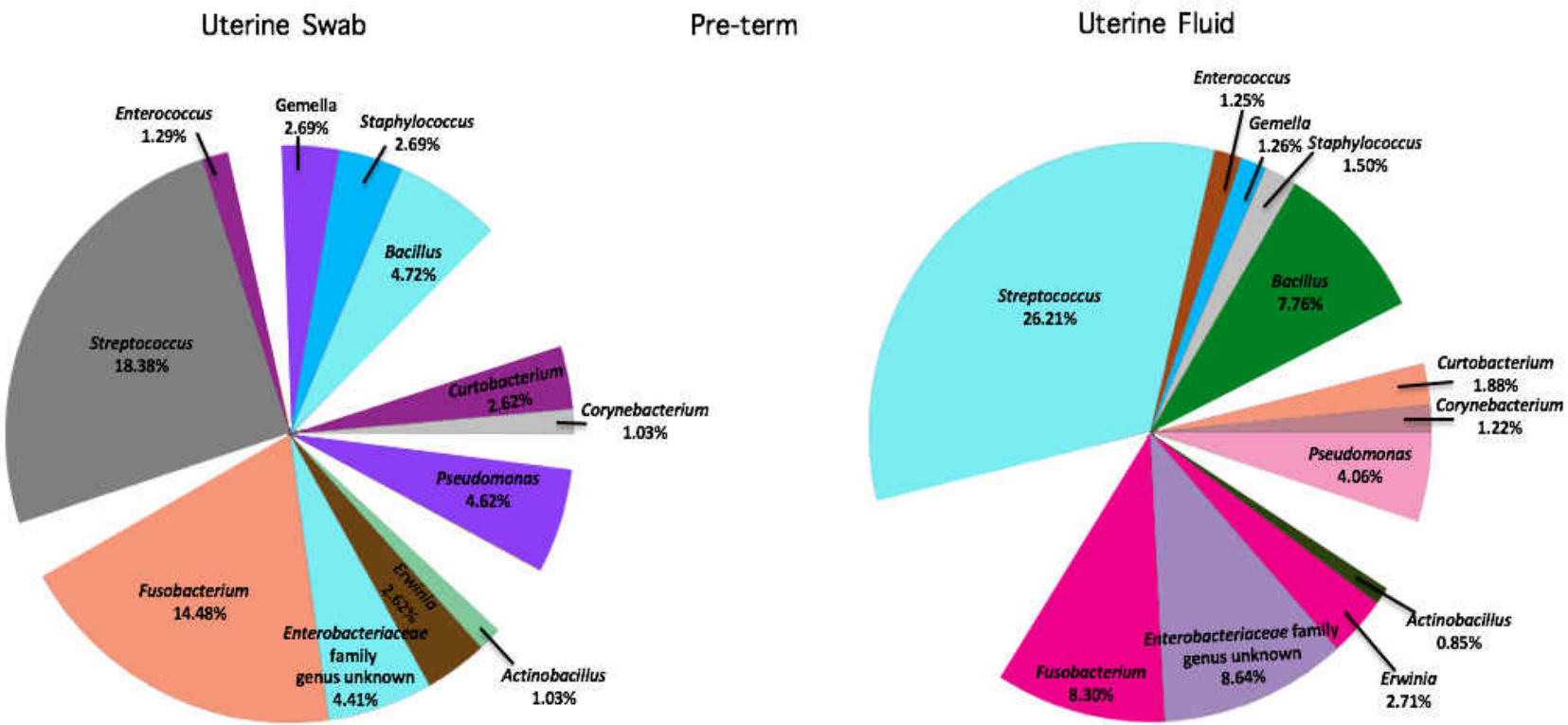


Figure 10. Genera Summary of Uterine Swab and Uterine Fluid by Term. **A)** Relative abundance summary of shared genera identified in FT samples (14 out of 19, 74%). **B)** Relative abundance summary of shared genera identified in PT samples (12 out of 19, 63%). *Fusobacterium* abundance is much higher in PT uterine swab and vaginal uterine fluid in comparison to FT (14.48% and 8.30% PT vs. 1.6 and 2.43% FT). *Clostridium* is present in FT samples but is not present (< 0.5%) in PT. *Gemella* is present in PT samples but is not present (< 0.5%) in FT samples.

Figure 10 (Continued)

B



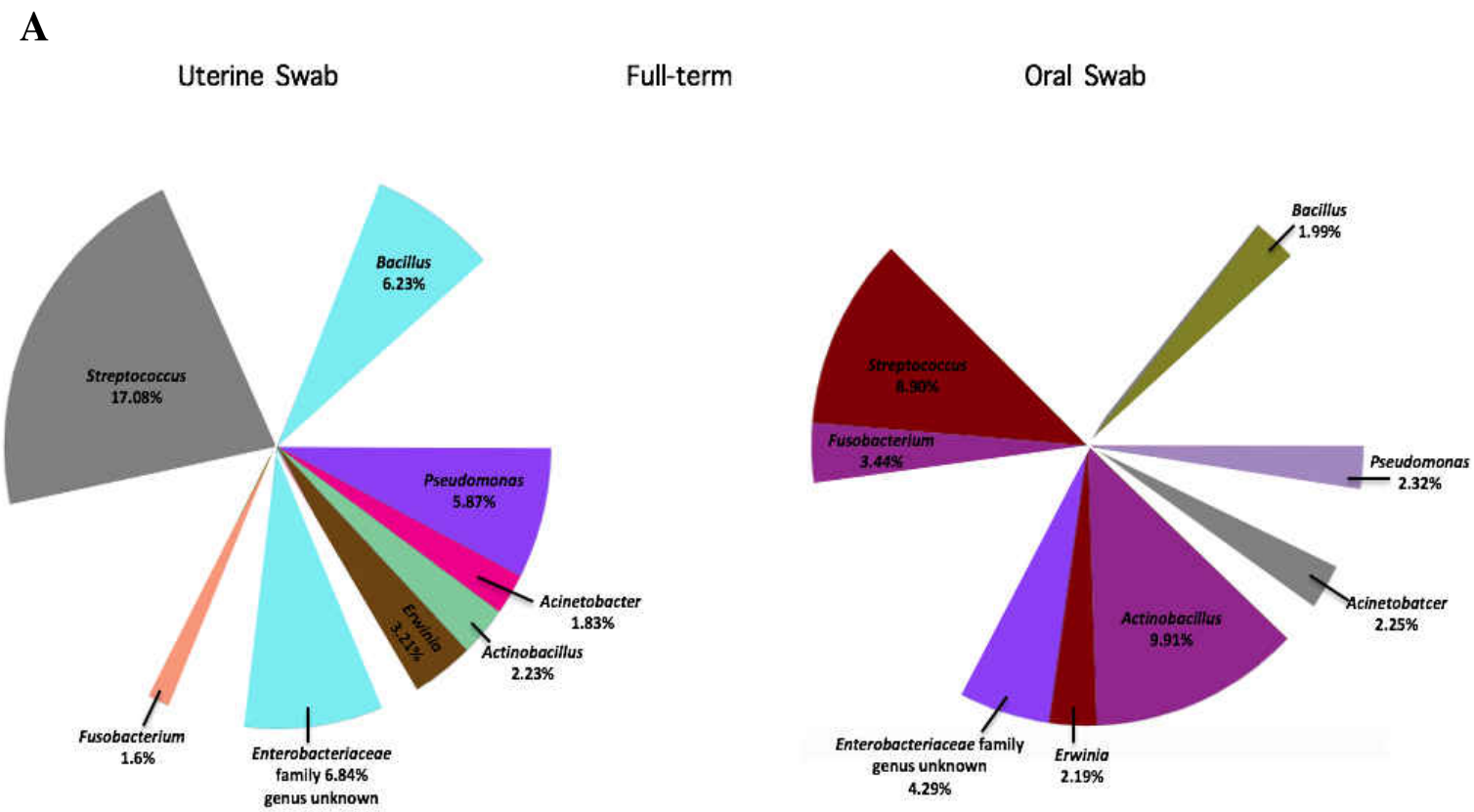
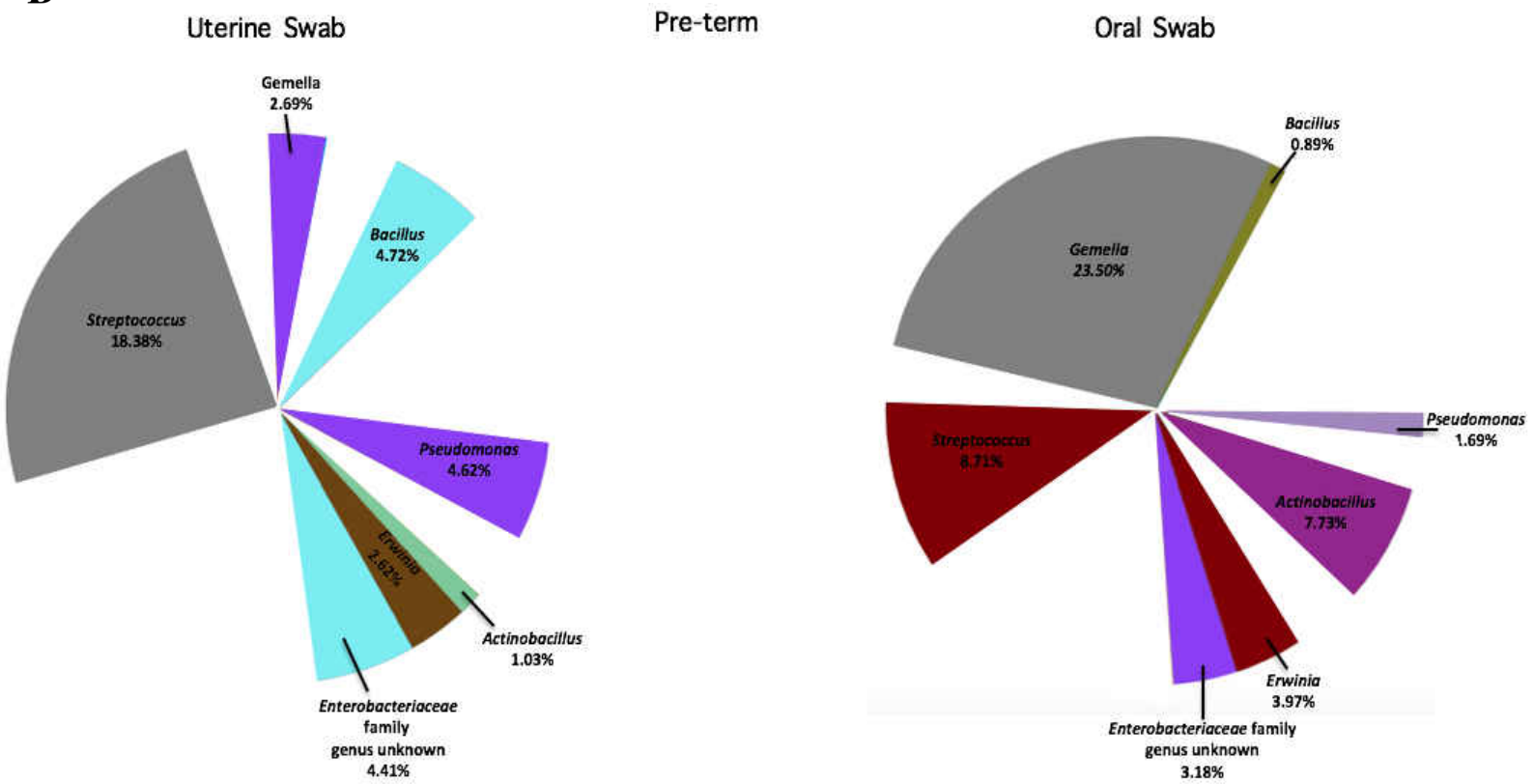


Figure 11. Genera Summary of Uterine Swab and Oral Swab by Term. **A)** Relative abundance summary of shared genera identified in FT samples (8 out of 19, 42%). **B)** Relative abundance summary of shared genera identified in PT samples (7 out of 19, 37%). *Fusobacterium* abundance is reported at low relative abundance in FT uterine swab and oral swab samples (1.6% and 3.44%) and is not present (< 0.5%) in PT. *Gemella* is present in PT samples, primarily dominant in the oral swabs, but is not present (< 0.5%) in either FT samples.

Figure 11 (Continued)

B



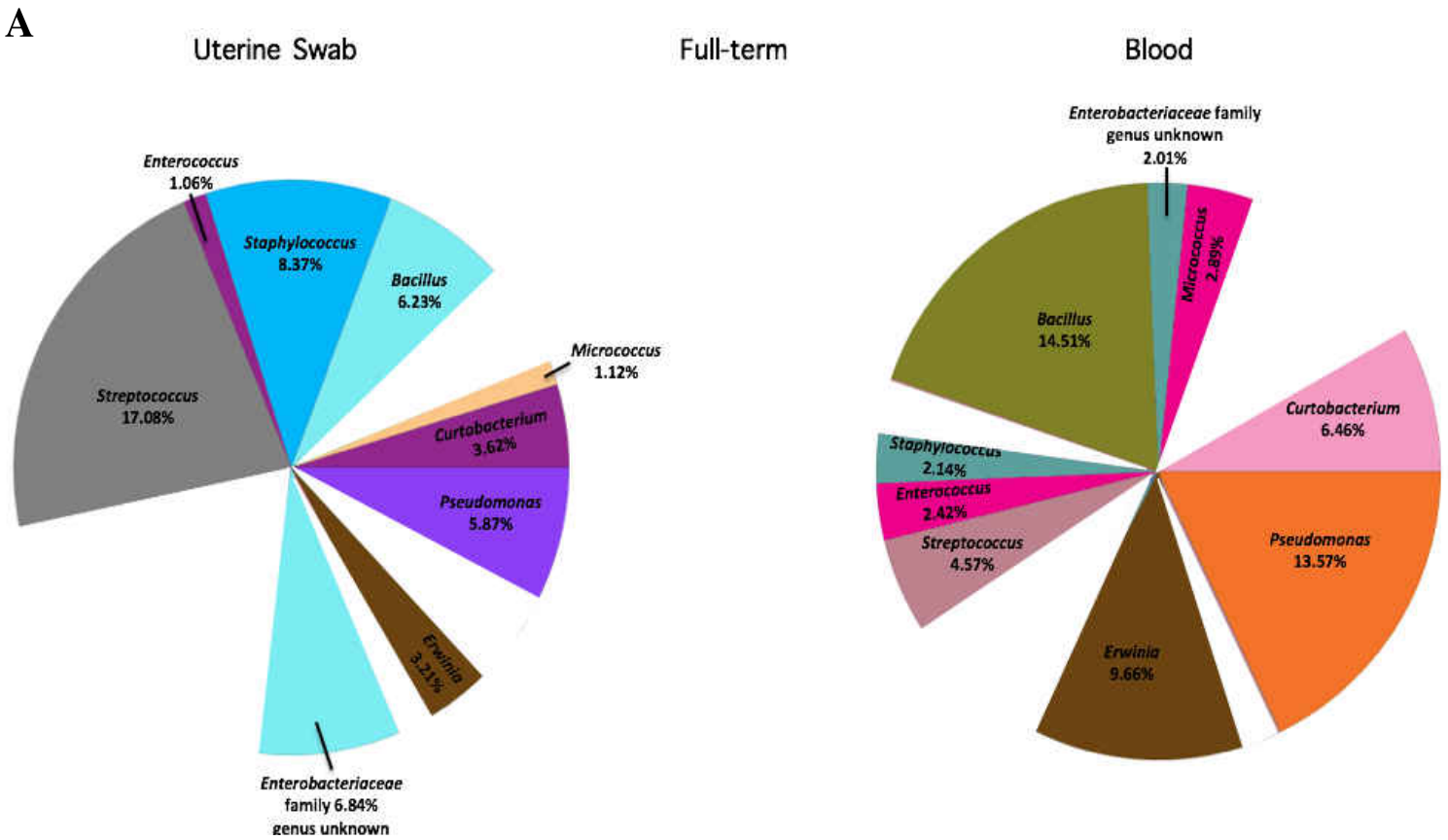
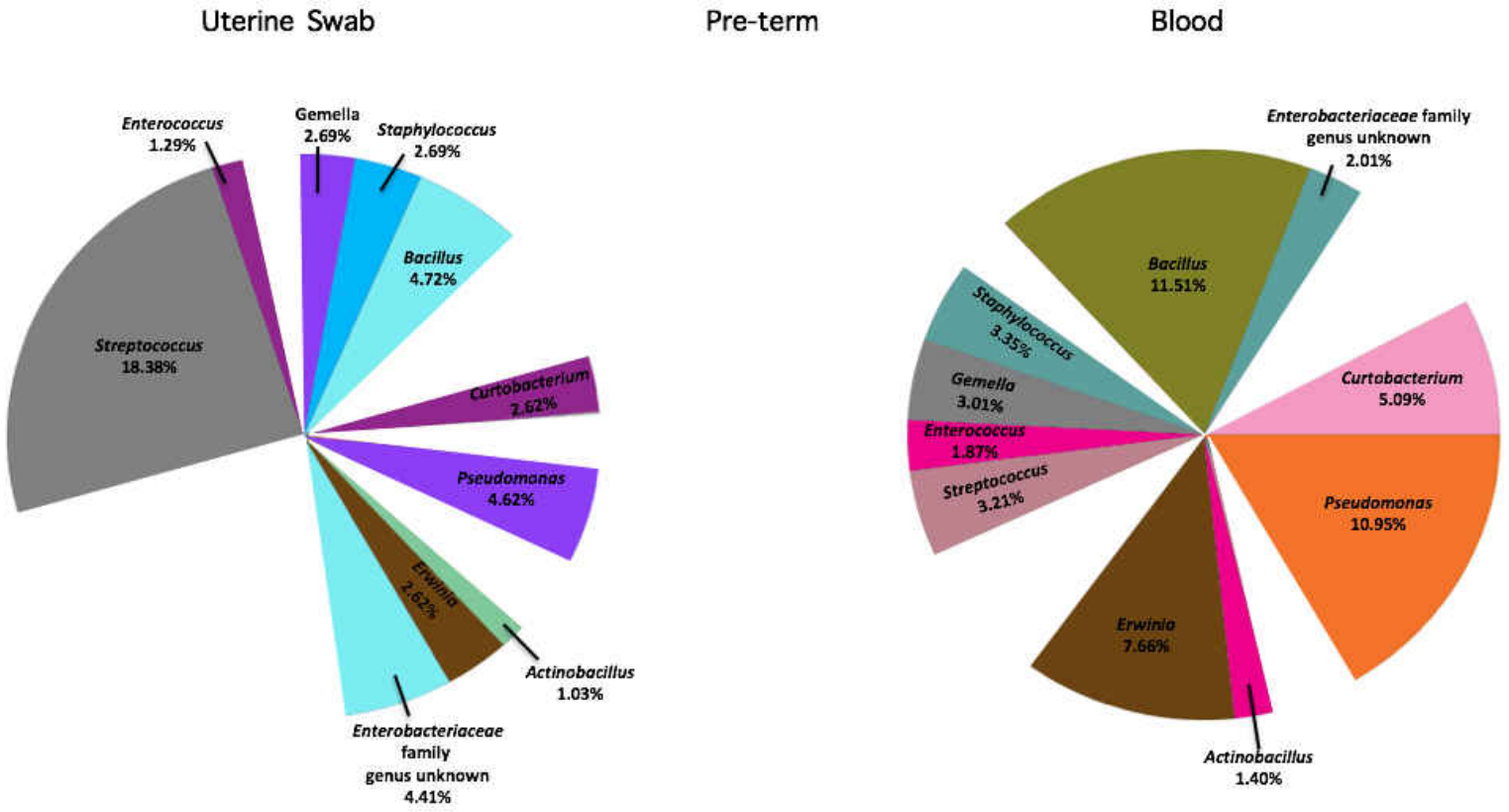


Figure 12. Genera Summary of Uterine Swab and Blood Collection by Term. **A)** Relative abundance summary of shared genera identified in FT samples (9 out of 19, 47%). **B)** Relative abundance summary of shared genera identified in PT samples (10 out of 19, 53%). *Gemella* is present in PT samples but is not present (< 0.5%) in FT samples. Most reported genera (except *Actinobacillus*, *Gemella*, and *Enterococcus*) are not similar in terms of abundance percentage in both FT and PT samples.

Figure 12 (Continued)

B



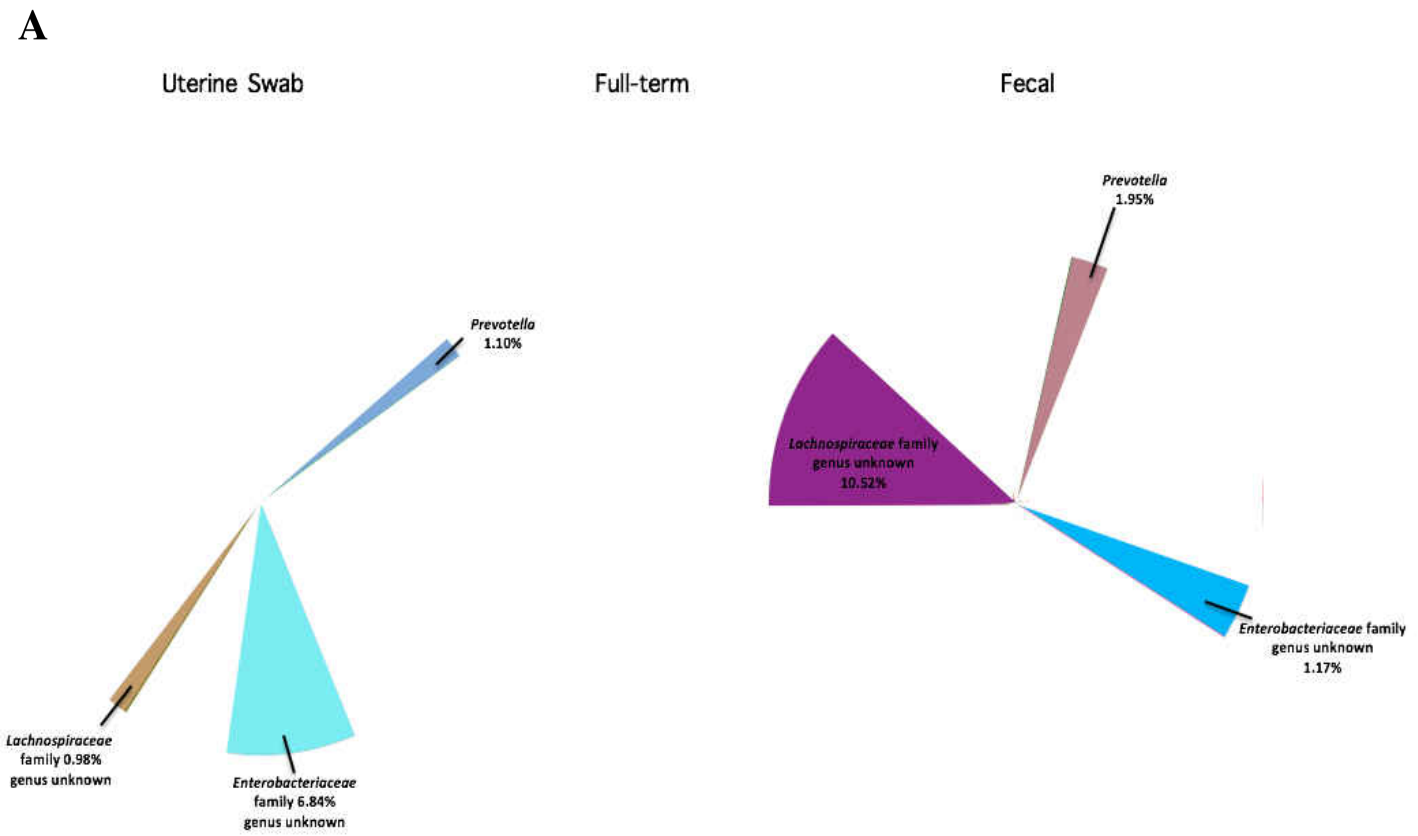
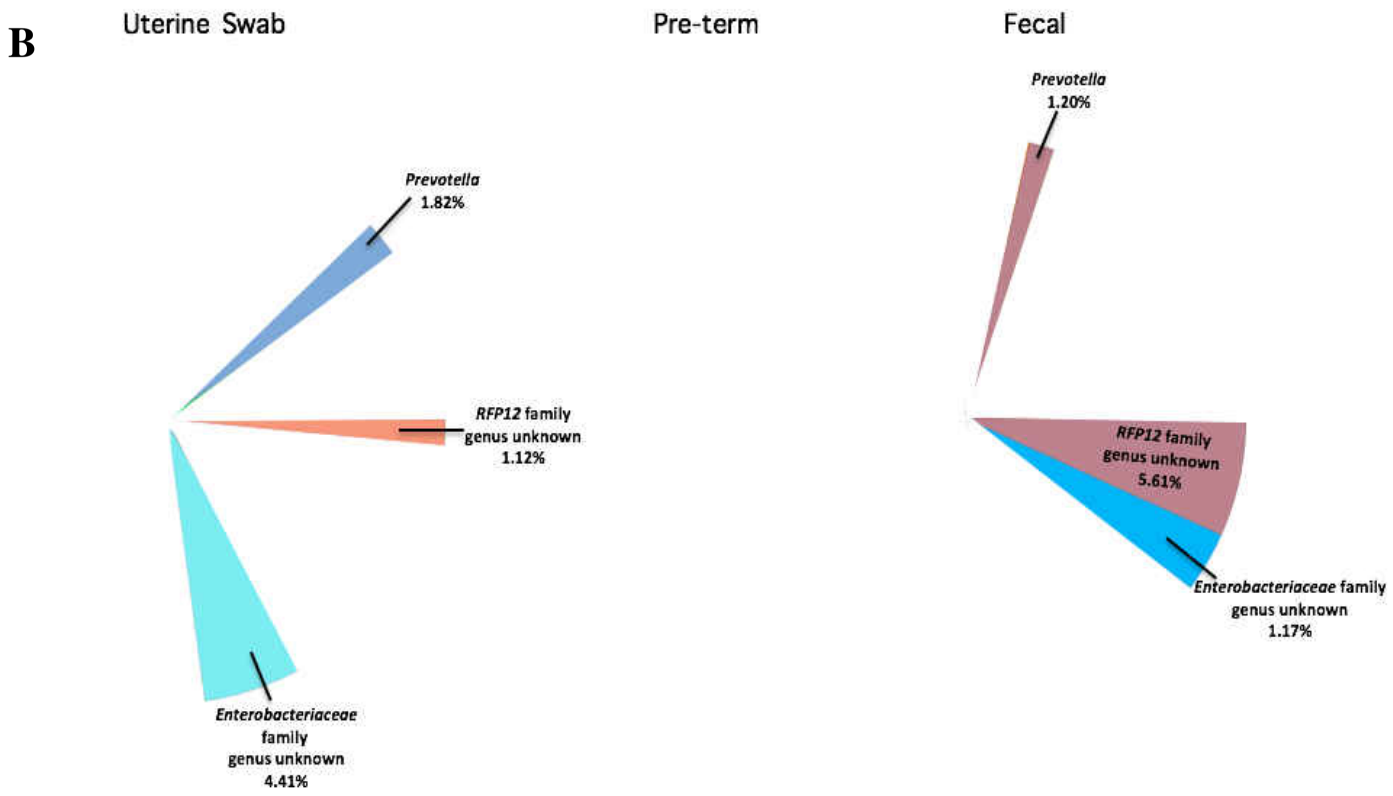


Figure 13. Genera Summary of Uterine Swab and Fecal Collection by Term. **A)** Relative abundance summary of shared genera identified in FT samples (3 out of 19, 16%). **B)** Relative abundance summary of shared genera identified in PT samples (3 out of 19, 16%). Uterine swabs (FT and PT) are most dissimilar to fecal collection (FT and PT).

Figure 13 (Continued)



Alpha diversity was assessed by the Shannon Diversity Index for consistency purposes to ensure that the diversity reported in each sample is consistent with that of the literature. Diversity is highest within cecum and blood samples, and lower in the remaining sample types (Figure 14). $P < 0.05$ (fecal and blood vs uterine fluid, vaginal, oral, and uterine swabs), $P > 0.05$ (fecal vs blood), and $P = 1.0$ (oral swabs vs. uterine fluid, vaginal, and uterine swab samples). Weighted jackknifed beta diversity at an even 100 replicates is represented in PCoA plots by sample type and term (Figures 15 and 16).

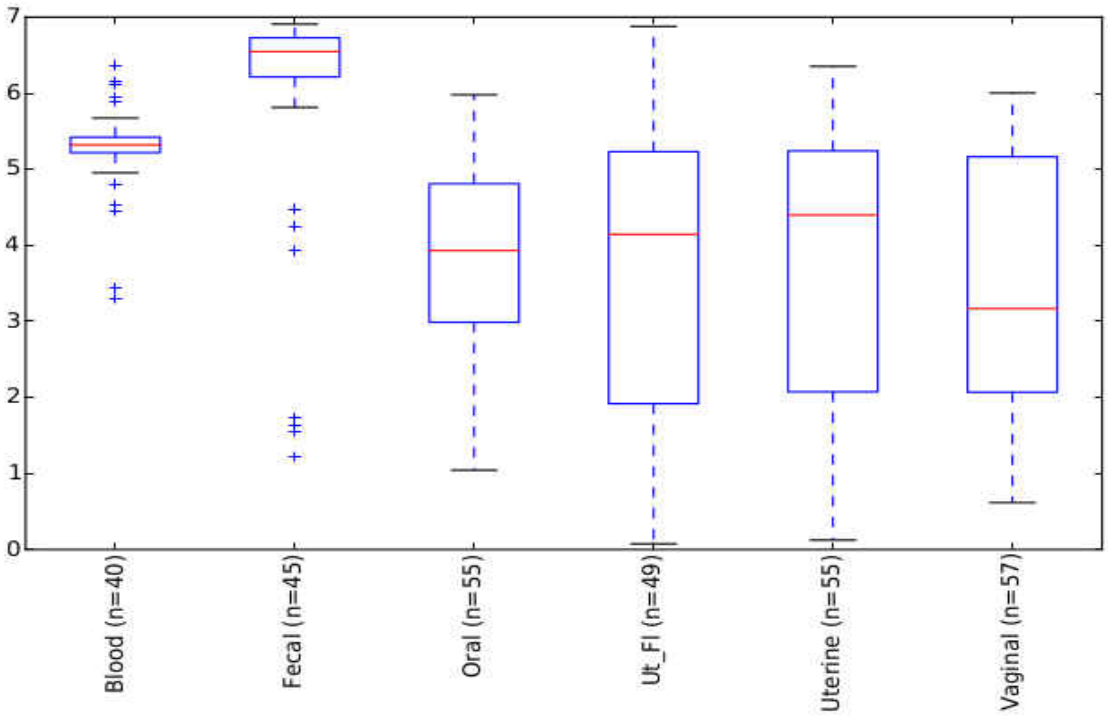


Figure 14. Shannon Diversity Index by Sample Type. Diversity (abundance and evenness) is highest within the fecal and blood samples. Diversity within oral swabs are not significantly different from that of uterine fluid, vaginal swabs, and uterine swabs. P-values for fecal samples= 0.015 against uterine fluid, vaginal, oral, and uterine swabs; 0.09 against blood samples. P-values for blood= 0.015 against uterine fluid, vaginal, oral, and uterine swabs. P-values for oral swabs= 1.0 against uterine fluid, vaginal, and uterine swab samples.

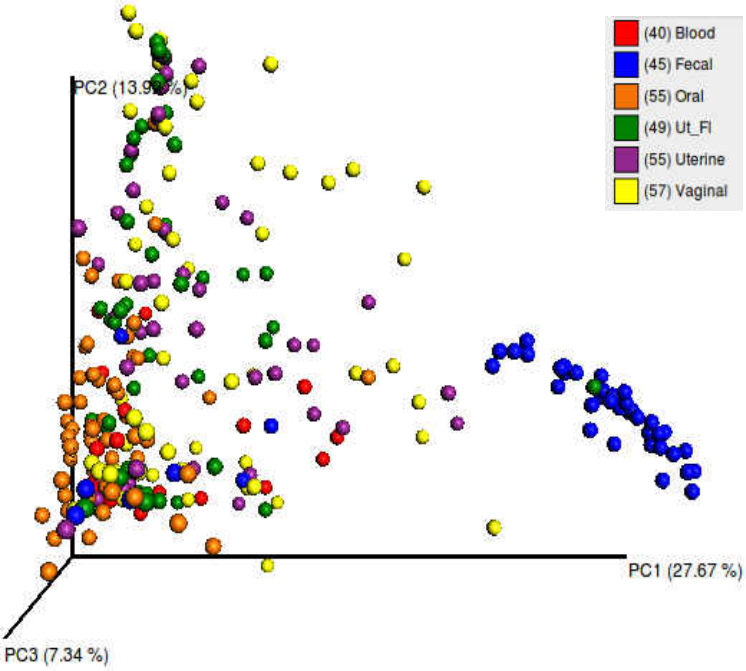


Figure 15. PCoA Plot Representation of Weighted Jackknifed Beta Diversity by Sample Type. Fecal samples have greater variance (diversity) of OTUs but fewer OTUs are shared between other sample types. Vaginal, uterine, and uterine fluid samples exhibit a clustering pattern (shared OTU abundance) as variance within these samples increases. Oral swabs and blood have lower variance but share OTUs with all other sample types.

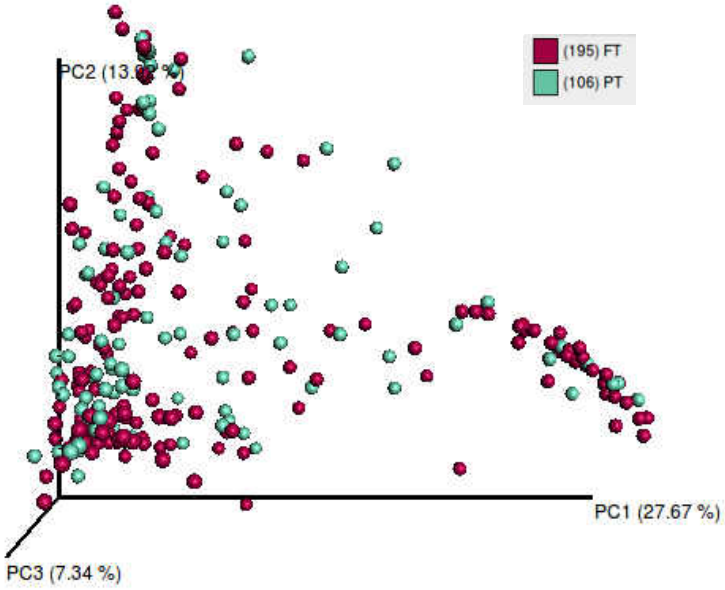


Figure 16. PCoA Plot Representation of Weighted Jackknifed Beta Diversity by Term. The same clustering patterns as seen in Figure 5, represented by term. The middle of the plot is representative of FT and PT points (OTUs) that have differing variance and do not exhibit a strong clustering pattern. The points are primarily vaginal, uterine scrape, and uterine fluid (refer to Figure 15).

LEfSe reported significantly abundant genus between FT and PT uterine swab samples (Figure 17). Significant abundant features reported for PT uterine swab: *Fusobacterium, Gemella,* Unassigned (genus designated as 'Other'), *Granulicatella, Geothrix, RFN20, 41B, RF16, Exiguobacterium, Actinomycetales* order (genus unknown), *Peptostreptococcus, Desulfovibrio, Mobiluncus,* Cyanobacteria phylum (genus designated as 'Other'), *Clostridiales* order (genus designated as 'Other'), and *Rhodocyclaceae* family. Assigned approximate LDA scores for each feature is as follows (respective to reported PT list): 4.5, 3.7, 3.7, 3.5, 3.5, 3.3, 3.2, 3.2, 3.1, 3.0, 2.9, 2.8, 2.7, 2.7, and 2.6. Significant abundant features reported for FT uterine swab: *Lachnospiraceae* family (genus designated as 'Other'), *Rhizobiaceae* family, *Lachnospiraceae* family (genus unknown), and *Rathyibacter*. Assigned approximate LDA scores for each feature is as follows (respective to reported FT list): 2.5, 2.6, 2.7, and 3.5. Note that Figure 17 shows negative values for the assigned LDA scores for FT abundance. This is because of the order of the numerator and denominator, which is determined alphabetically, when calculating effect size; therefore, absolute values can be used when interpreting the scale of the logarithmic LDA score.

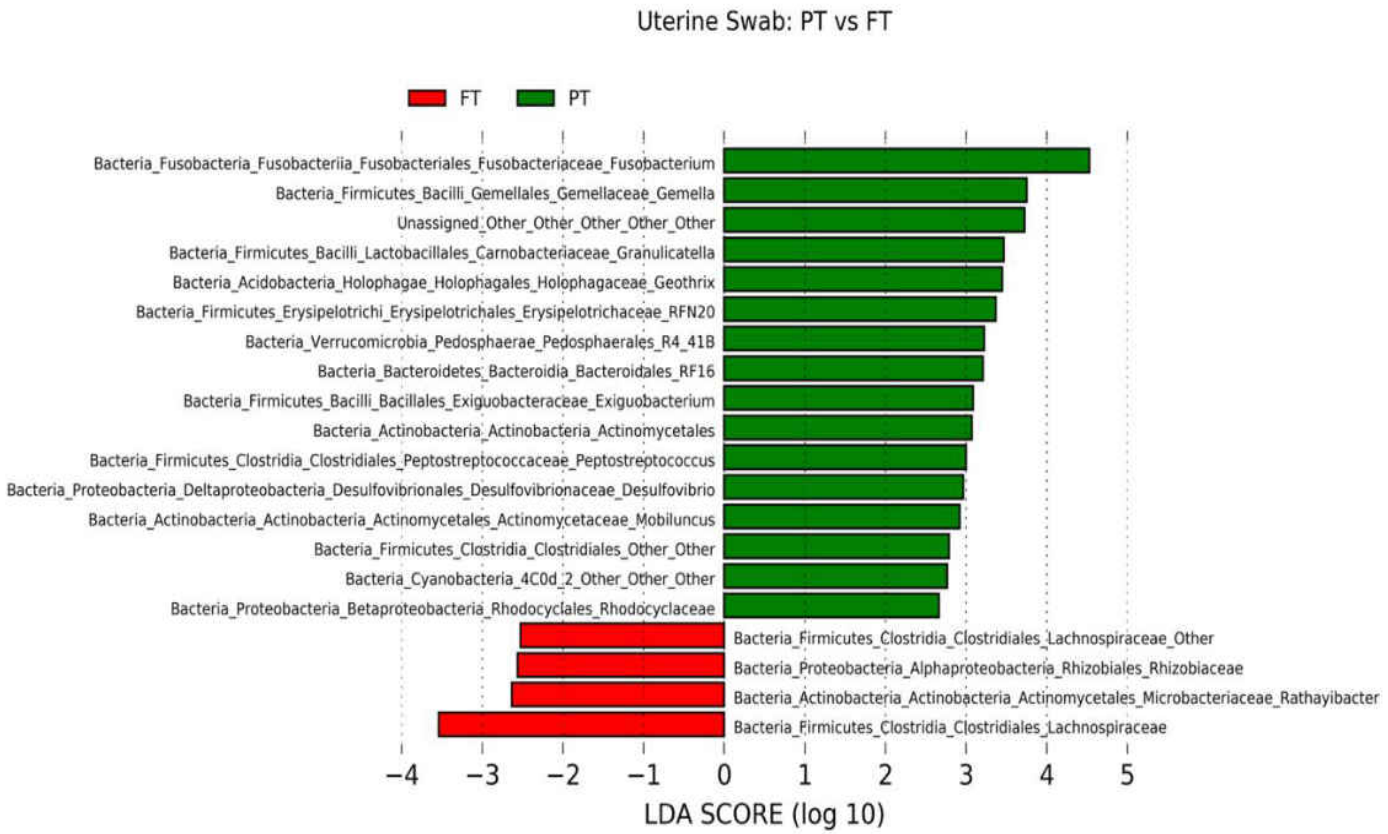


Figure 17. LEfSe Significance Analysis of Uterine Swab Relative Abundance by Term. Significantly abundant features are reported in descending order of abundance significance between PT and FT uterine swab samples. Most significantly abundant features for PT (LDA score ≥ 3): *Fusobacterium, Gemella,* Unassigned, *Granulicatella, Geothrix, RFN20, 41B, RF16, Actinomycetales,* and *Peptostreptococcus.* Most significantly abundant feature for FT: *Lachnospiraceae* family.

CHAPTER 5
DISCUSSION

5.1.1 Discussion Overview

Composition and proportion of bacteria detected in uterine swab and uterine fluid samples strongly overlaps with vaginal swab samples, supporting the vertical ascension route of vaginal microbiota through the cervix and to the uterus. A noticeable increase in prevalence of the phylum Fusobacteria is observed in uterine swab, uterine fluid, and vaginal samples, as well as in PT samples versus FT samples. Further assessment of this difference on a genus level reveals the highest abundance of *Fusobacterium* in uterine swab, vaginal swab, and uterine fluid PT samples. Interestingly, an inverse relationship is observed between the abundance of *Clostridium* and *Fusobacterium*. For instance, in FT uterine swab samples, *Clostridium* makes up approximately 8.41% of the overall genus composition, while *Fusobacterium* comprises approximately 1.6%. Meanwhile, in PT uterine swab samples, *Clostridium* is detected to be at less than 0.5%, explaining why there is not a designated spot for it on the pie chart; however, *Fusobacterium* was calculated to make up approximately 14.48% of the overall composition. A similar trend is seen in FT/PT uterine fluid and vaginal swab samples. In previous studies, *Clostridium* is typically associated with negative pregnancy outcomes such as pre-term birth or even fetal death, across several models (human, swine, mice, etc.). In this study, it appears that *Clostridium* may provide a protective effect by outcompeting pathogenic microbes, such as *Fusobacterium,* unintentionally keeping the growth of these organisms in check.

Hematogenous transfer of organisms from other body sites, across the placenta, cannot be completely ruled out in this study. Although uterine swab samples shared fewer groups with blood samples (approximately 10), the genus *Gemella* is only detected in PT uterine swab, uterine fluid, blood, and oral swab samples with greatest abundance in the oral swabs (23.5%). It is important to note that the causative agents of periodontal disease that have also been isolated in the placenta, reside in the subgingival spaces of the mouth. In this study, those microbes were likely not isolated due to method of sample collection. The oral swab collection consisted of a swab of the buccal regions, not between the subgingival spaces. Therefore, it is possible that *Gemella* sp. in the oral cavity, and even the *Fusobacterium* sp. detected in the uterine swab/fluid samples, transferred via blood vessels in the gums and across the placental membrane. The only genus that was detected in the PT uterine swab samples that was not shared/detected with any other sample type was *Granulicatella* at a small relative abundance of 1.75%.

An LDA reported markedly different results than the relative abundance reports. According to this analysis, *Clostridium* is not significantly abundant in FT uterine swab samples over PT uterine swab samples, and several taxa are reported to be significant in PT uterine swab samples that were not reported in the relative abundance charts. What is consistent between the reported LDA of summarized taxa and relative abundance is the abundance of *Fusobacterium, Gemella,* and *Granulicatella* being significantly more abundant in PT uterine swab samples compared to FT, with the largest score assigned to *Fusobacterium,* consistent with the reported

relative abundance. *Clostridium, Fusobacterium, Gemella,* and *Granulicatella* will be described throughout the remainder of this chapter to assess mechanisms behind how these microorganisms influence pregnancy outcomes. Furthermore, possible explanations will be explored for why *Fusobacterium* was reported at low relative abundance in the oral genus summaries.

5.1.2 *Fusobacterium*: Mechanisms of Pathogenesis

The phylum Fusobacteria consists of a group of gram-negative, non-spore forming, obligate anaerobes that typically inhabit the oral cavity as a commensal; in fact, Fusobacteria species are one of the most frequently detected bacteria in both healthy and diseased oral cavities [53, 54]. Furthermore, the genus *Fusobacterium* is the second most recurrently isolated anaerobic bacteria in human and animal microbiota [55]. Fusobacteria, more specifically *Fusobacterium nucleatum*, has been identified as a causative agent of most periodontal disease cases and has been associated with negative pregnancy outcomes (still birth, preterm birth, spontaneous abortion, and early-onset neonatal sepsis), GI disorders, skin ulcers, Lemierre's syndrome, etc. [53-58]. *F. nucleatum* is the most commonly identified Fusobacterium sp. in a wide variety of diseases due to the virulence factors it possesses. *F. nucleatum* has the abilities to coaggregate with other species to enhance disease progression and invade a variety of mammalian cells and host molecules via adhesins [53-55]. Adhesins are surface proteins that function in the attachment of bacterial cells to specific host-cell receptors/substrates or, in some cases of *F. nucleatum* pathogenesis, to salivary macromolecules, immunoglobulin G, and extracellular matrix proteins [53, 55]. *F.*

nucleatum has a tropism for several cell types including epithelial and endothelial cells, polymorphonuclear leukocytes, natural killer (NK) cells, erythrocytes, fibroblasts, and monocytes [53, 55].

F. nucleatum's ability to coaggregate with other bacteria is one of the reasons why it is often detected in mixed-community infections [55]. It does so by recognizing surface molecules on other species either by adhesins (Fap2, RadD, and aid1) or other outer membrane proteins (OMPs) [53, 54]. Coaggregation of *F. nucleatum* with other species of bacteria is the key first step to plaque formation in the oral cavity, usually in the subgingival spaces. The adhesin and invasin identified as FadA is the most well-studied *F. nucleatum* virulence factor [59]. FadA becomes an active complex (FadAc) when its two forms bind to one another, intact pre-FadA and secreted mature FadA (mFadA), of which is an absolute requirement for *F. nucleatum* to bind to and invade host cells [53]. FadAc attaches to cadherins, which are calcium-dependent adhesion molecules responsible for forming adherens junctions to bind host cells together [59]. Another well-studied virulence factor of *F. nucleatum* is Fap2, abbreviated for fusobacterial apoptosis protein [54]. In a 2015 study conducted by Coppenhagen-Glazer et. al, the authors characterized Fap2 as a galactose-inhibitable adhesin after observing the inhibition of hemagglutination (a mode of attachment conserved across many pathogens) in a strain of *F. nucleatum* by the addition of D-galactose, showing that Fap2 is galactose-sensitive/inhibitable. Furthermore, Coppenhagen-Glazer et. al discovered that Fap2 also plays a role in coaggregation after noting that three hemagglutination-deficient mutant *F. nucleatum* strains failed to coaggregate with

Porphyromonas gingivalis but retained coaggregation ability with *Streptococcus sanguins.* To observe the role of Fap2 in placental colonization of *F. nucleatum*, since FadA had been previously described as the only major virulence factor in adverse pregnancy outcomes, the investigators injected wild-type *F. nucleatum* and the hemagglutination-deficient mutant strain into the tail veins of CD1 female mice. They observed that the Fap2 mutant strain reduced placental colonization of *F. nucleatum* 2-fold [54].

The most frequently reported method by which *Fusobacterium* results in negative pregnancy outcomes is by hematogenous transfer from the oral cavity in pregnant women with periodontitis [53-55]. In 2004, Han et. al studied the effects of dental bacteremia, induced by *F. nucleatum,* on pregnancy outcomes. Seventy-two hours after injecting *F. nucleatum* into the tail vein of pregnant mice, the investigators observed cases of preterm birth and fetal death at term [60]. In a similar study conducted by Stockham et. al in 2015, three aims were investigated: 1) determine if hematogenous transfer of *F. nucleatum* from the oral cavity to the murine placenta and subsequent adverse pregnancy outcomes is strain specific, 2) utilize an oral gavage murine model of periodontitis to observe if increased severity of adverse pregnancy outcomes is influenced by induced periodontitis, and 3) compare immunological changes of the induced periodontitis pregnant murine model to intravenous injection of different *F. nucleatum* subspecies and correlate these changes to any adverse pregnancy outcomes that arise. The authors observed that hematogenous transfer from the oral cavity to the placenta was strain specific as *F. nucleatum* was the only

Fusobacterium sp. that colonized the placenta. Furthermore, inducing periodontitis did not further increase the severity of adverse pregnancy outcomes and there were no significant immunological changes between pregnant control mice and pregnant inoculated mice [56].

In this study, we did not observe *Fusobacterium* reported in relative abundance of PT oral swabs. but it was reported in similar abundance in PT vaginal swabs. *Fusobacterium* has been identified in cases of bacterial vaginosis and urinary tract infections, which can lead to negative pregnancy outcomes [61]. Interestingly, we observed a rather high biodiversity in all vaginal swab samples. Although, alpha diversity was reported as being low in vaginal swabs, several different genera were identified, which usually is not the case for human females. As discussed in Chapter 3, *Lactobacillus* sp. dominates the vaginal microbiome, with some variation, and remains dominant throughout pregnancy due to increased estrogen levels. *Lactobacillus*'s role in creating an acidic pH environment in the vagina exerts protection against obligate anaerobes by providing an unsuitable environment for their survival. A disruption in Lactobacillus dominance of the vagina is often the result of bacterial vaginosis [61]. In both FT and PT vaginal samples of mares, *Lactobacillus* does not dominate, and diversity appears to be high- despite the tendency of diversity in the vagina to decrease during pregnancy. These results, however, have been found to be true for humans and laboratory mice- mares live in a much different environment and have a vastly different diet; therefore, it is not surprising to observe a unique diversity in an equine model in comparison to mouse models and human studies. Due to the lack of

studies using equine models for microbiota characterization, we do not currently know what communities constitute a healthy vaginal microbiome for mares; therefore, we cannot definitively state which organisms are resident microbiota and which are pathogenic transient colonizers, as we do not have sufficient comparison.

5.1.3 *Clostridium*: Mode of Competition

Clostridium is a Gram-positive, obligate anaerobe genus under the phylum Firmicutes [62]. Many *Clostridium* sp. are responsible for causing serious disease, such as *Clostridium perfringens* and gas gangrene, *Clostridium difficile* and diarrhea, *Clostridium tetani* and tetanus, *Clostridium botulinum* and botulism, and *Clostridium sordellii* and toxic shock syndrome in post-partum women [63-67]. On the other hand, several *Clostridium* sp. are found to be part of a normal and healthy resident microbiome [3]. For example, although *C. difficile* can create infection within the GI tract, it is also part of the normal gut microbiome in small abundance because other populations dominate and outcompete it; in fact, *C. difficile* infections are most commonly a result of antibiotic-induced gut dysbiosis [11]. *Clostridium* has also been identified as part of a normal vaginal microbiome in less than 10% of women; therefore, it is not entirely unusual to find *Clostridium* sp. in a healthy woman, especially since these characterizations vary geographically [5, 30].

Clostridium has the ability to produce antimicrobial peptides (AMPs) termed bacteriocins [68, 69]. Bacteriocins function to aid the bacterium secreting it in outcompeting other bacteria similar to it. In essence, *Clostridium* will sense bacteria that are in close proximity, of which metabolize the same nutrients as it does for

survival [69]. All *Clostridium* sp. ferment butyrate and amino acids serine and threonine, while others can utilize tryptophan, histidine, arginine, alanine, methionine, leucine, asparatate, lysine, and/or phenylalanine, suggesting that amino acid fermentation is strain-specific [70]. All strains of *Fusobacterium* ferment butyrate and use glutamate, histidine, and aspartate for energy metabolism. In addition, *Fusobacterium nucleatum* utilizes glutamine and lysine for anaerobic fermentation in order to produce sufficient energy for glucose transport in the event of intracellular molecule biosynthesis [55]. Here, we propose that because *Clostridium* and *Fusobacterium* utilize much of the same amino acids for energy metabolism, *Clostridium* could potentially secrete bacteriocins to reduce or eliminate the growth of *Fusobacterium* in the uterus of a healthy mare. When *Clostridium* growth is reduced, possibly due to a dysbiosis in the uterus, *Fusobacterium* is able to outcompete it, resulting in placentitis and amnionitis, and eventual PTB. It was noted that *Clostridium* was reported in similar proportion in the vaginal swab samples as the uterine swab and uterine fluid samples. Considering the intrauterine ascension route of vaginal microbiota, the same relationship proposed between *Clostridium* and *Fusobacterium* could also be occurring in the mare's vagina.

5.1.4: *Gemella* and *Granulicatella*:

Gemella is a gram-positive, obligate anaerobe genus under the phylum Firmicutes [71]. It is a normal constituent of the oral cavity, upper GI tract, and vaginal microbiome in select populations [71]. *Gemella* is not frequently reported as cause for disease in clinical cases as it is primarily an opportunistic pathogen when mucosal

membranes are compromised, such as the lung environment in cystic fibrosis patients [71]. Because it has a preferred niche for mucosal areas, it is not surprising that this genus of bacteria was found in the uterine fluid, as this sample type is primarily a collection of the cervical and endometrial mucus. However, *Gemella* was detected in PT samples, but not in FT samples, suggesting that this bacterium may not be part of a microbiome in a healthy mare's reproductive tract. Additionally, it is important to note the small relative abundance reported in the uterine fluid and uterine swab samples because it does not appear to be an overgrowth and therefore, may not be contributing to the initiation of PTB. Lastly, *Granulicatella* was previously designated as nutritionally variant streptococci (NVS), but is now described as 'streptococci-like' due to a few distinct metabolic differences [72]. This bacterium has been described as a mouth and urogenital tract commensal; in terms of pathogenesis, it rarely causes disease. *Granulicatella* sp. has primarily been detected as an opportunistic pathogen in immunocompromised patients, most commonly causing infective endocarditis in the elderly, and has been reported in a few pre-term birth cases of mothers diagnosed with urinary tract infections [72, 73]. This genus is a fastidious, Gram-positive, facultative anaerobe under the phylum Firmicutes [73]. Much like *Gemella, Granulicatella* was detected in small relative abundance, only in the PT uterine swab samples; therefore, it does not appear to be an overgrowth and is unlikely to have initiated infection within the pregnant uterus.

CHAPTER 6
CONCLUSIONS & FUTURE DIRECTIONS

6.1.1 Conclusions

Mares may establish a uterine microbiome from vaginal microbiota during sexual maturity. In the event of pregnancy, stability in the flux of microbiota contributes to a healthy pregnancy; however, when these communities are disrupted, pathogenic microorganisms are able to colonize, proliferate rapidly, and infiltrate other body sites. In this study, it appears that a reduction in *Clostridium* populations in the upper and lower female reproductive tract results in the overgrowth of other microorganisms, such as *Fusobacterium* sp. However, this result must be taken with caution and further analyzed, as a linear discriminant analysis did not report *Clostridium* abundance to be significant in FT versus PT uterine swabs. Since the *Lachnospiraceae* family (under the *Clostridia* class) was reported significantly abundant in FT uterine swab samples with the highest LDA score, it may be more accurate to hypothesize that *Clostridia* exert a protective effect in the uterus during pregnancy, rather than the more narrow, specific identification of the *Clostridium* genus.

Due to the large increase in *Fusobacterium* in PT uterine swab and vaginal swab samples, as well as being reported as the most significantly abundant feature over FT uterine swabs, we conclude that this microbe is the infectious agent initiating pre-term birth, though the exact mechanism by which *Fusobacterium* initiates such an event in these mares is unknown. Before a correlation can be established between the relationship of *Clostridium* sp. and the female reproductive tract of mares, further

experimentation is required. We have shown here that *Fusobacterium* can lead to negative pregnancy outcomes in mares much like in humans and laboratory mouse models, suggesting that mechanisms of pathogenesis in this bacterium is ubiquitous across various mammalian species. This study provides evidence against the sterile womb hypothesis and contributes to the field preliminary results on the influence of microbiota on pregnancy outcomes in mares. We have characterized the uterine and vaginal microbiome in post-parturient mares, which has not been done before; therefore, the results of this study could prove to be useful in the prenatal care of pregnant mares. There is one study to date that characterized the uterine microbiome of non-pregnant mares, but only beta diversity is reported, of which shows a moderate diversity within the uterus [74]. The taxonomy of these microbes is unclear; therefore, we do not have any baseline comparison for our results.

6.1.2 Future Directions

To continue assessment of the microbiome's influence on pregnancy outcomes in an equine model, the previously described methods should be repeated; however, a routine vaginal and oral swab collection throughout the gestational period may be a useful additional measure. Observing the vaginal microbiome, throughout pregnancy, could provide insight into the stability, or instability, of a particular mare's vaginal microbial communities and may help predict pregnancy outcomes like pre-term birth. This is important for the field of equine care because mares that give birth pre-term one time, tend to carry out a habit of always delivering pre-term and the cause for this is unknown. Understanding the mechanisms behind this and creating diagnostic

measures to prevent such negative outcomes could provide a substantial boost for the advancement of veterinary medicine and the equine industry. Because hematogenous transfer of microbes has been reported in cases of pre-term birth and fetal death, routine oral swab and blood collection may also be a useful predictive measure in conjunction with routine vaginal swab assessment. The oral swab should be a collection from the subgingival spaces in order to isolate and identify bacteria involved in dental bacteremia and periodontal disease.

Another problem that needs to be addressed is the lack of knowledge pertaining to the characterization of the normal, healthy vaginal microbiome in mares. If possible, repetitive assessment of vaginal swab collections of healthy, non-pregnant mares could result in a sufficient characterization and could be used for future comparative analyses. Furthermore, knowing a mare's vaginal microbiome prior to pregnancy, could provide insight into potential pregnancy outcomes that particular mare may be susceptible to, allowing for proper preventative care to be administered. In closing, although the results of this study are not currently generalizable to humans, further experimentation with the recommended additions may provide translational results in the future.

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