EXPLORING THE RELATIONSHIPS AMONG THE PLACENTAL AND UMBILICAL MICROBIOMES, THE ORAL MICROBIOTA, AND ADVERSE PREGNANCY OUTCOMES

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ABSTRACT

KRYSTAL REYES VIRUET: Exploring the Relationships among the Placental and Umbilical Microbiomes, the Oral Microbiota, and Adverse Pregnancy Outcomes
(Under the direction of Flavia Teles, Steven Offenbacher, and Andrea Azcarate-Peril)

Aims: To compare the microbiomes of placenta and umbilicus samples from 44 women presenting periodontal diseases who experienced full term (FTB, n=22) and preterm births (PTB; <35 weeks, n=22), as well as, the level of maternal subgingival periodontal species in each group pre and post-partum. ***Materials and methods:*** Clinical and demographic data, subgingival plaque samples, as well as, placenta and umbilical cord tissues samples were obtained. The microbial content of the tissue samples were analyzed using 16rRNA sequencing, while subgingival biofilm samples were analyzed using checkerboard DNA-DNA hybridization method. ***Results:*** Most abundant phylum in both placenta and umbilicus samples was *Firmicutes*. Most abundant genera in both placenta and umbilicus samples was *Veillonella.* Some of the most common species detected were *Neisseria elongata*, *Pyramidobacter piscolens*, and *Gemella morbillorum.* ***Conclusion:*** PTB and FTB exhibit distinct umbilical microbiomes. PTB samples are more heavily colonized by oral pathogenic taxa, whereas FTB specimens had higher levels of commensal organisms.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
AL	Attachment level
APOS	Adverse Pregnancy Outcomes
BMI	Body Mass Index
BOP	Bleeding on probing
CAL	Clinical attachment level
CC	Correlation coefficient
CEJ	Cementoenamel junction
CI	Confidence Interval
CRH	Corticotrophin Releasing Hormone
DNA	Deoxyribonucleic acid
G-CSF	Granulocyte Colony Stimulating Factor
GI	Gingival Index
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon Gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin-1
IL-1β	Interleukin-1 Beta
IL-6	Interleukin-6
IL-8	Interleukin-8
LBW	Low Birth Weight

LPS	Lipopolysaccharide
NBW	Normal Birth Weight
NIH	National Institutes of Health
PCA	Principal component analysis
PCR	Polymerase chain reaction
PD	Probing depth
PG	Porphyromonas gingivalis
PGE-2	Prostagladin E2
PI	Plaque Index
PLBW	Premature Low Birth Weight
PPROM	Preterm premature rupture of membranes
PT	Preterm
PTB	Preterm Birth
OR	Odd Ratio
RR	Relative Risk
RNA	Ribonucleic acid
FTB	Full-term Birth
TLR	Toll-like Receptor
TNF-α	Tumor necrosis factor- alpha
UNC	University of North Carolina
US	Unites States

CHAPTER 1: ASSOCIATION BETWEEN PERIODONTAL DISEASE AND ADVERSE PREGNANCY OUTCOMES

Introduction

WHO defines preterm birth as babies born alive before 37 weeks of pregnancy are completed, with subcategories moderate-late preterm (32 to < 37 weeks), very preterm (28 to <32 weeks), and extremely preterm (<28 weeks). Worldwide an estimated 11.1% of all live births in 2010 were born preterm (14.9 million babies born before 37 weeks of gestation), with preterm birth rates increasing in most countries with reliable trend data. About 84% of preterm births occur after 32 weeks of gestation. Direct complications of preterm birth account for one million deaths each year, and preterm birth is a risk factor in over 50% of all neonatal deaths. [9]

Each year, preterm birth affects almost 500,000 infants born in the U.S. In an average week, over 8000 infants are born prematurely at less than 37 weeks' gestation. Blencowe and colleagues ranked the U.S. as the sixth country with the greatest number of preterm births in the year 2010[10] . The effects of preterm birth amongst some survivors may continue throughout life, impairing neuro-developmental functioning through increasing the risk of cerebral palsy, learning impairment and visual disorders and affecting long-term physical health with a higher risk of non-communicable disease.[55] Ultimately, the effects of prematurity are far-reaching: besides representing a significant struggle for the affected individuals and their families they also represent a challenge for the health care system and for the society as a whole.

Pathogenesis & Risk Factors of Pre-term Birth

Preterm births can be classified into two main categories: spontaneous births or provider-initiated preterm birth. Spontaneous preterm births can be further subdivided into preterm lab with intact membranes or preterm premature rupture of membranes (PPROM). Provider-initiated preterm birth can be due to maternal or fetal indications. In these cases, labor can be induced or infant can be delivered via caesarian section. [24]

Spontaneous preterm labor can be initiated by different mechanisms, which include immunologically mediated processes, infection and inflammation, uterine overdistension, utero-placental ischemia or hemorrhage, stress, among others. [56] Multiple maternal and fetal features have been linked to preterm birth, among these are pregnancy history, current pregnancy characteristics, maternal demographic characteristics, infection, uterine contractions and cervical length, nutritional status, psychological characteristics, adverse behaviors, and biological and genetic markers. [25]

Maternal race seems to influence the risk for preterm birth. In the US, preterm birth rates in black Americans range from 16-18%, while in white Americans rates range from 5-9%. Black mothers are three to four times more likely to have very early preterm birth than women from other races.[21, 23] Low educational and socioeconomic status, as well as, low and high maternal age are among other maternal demographic features linked to preterm births.[24]

Short inter-pregnancy interval increases the risk for preterm birth, with intervals of less than 6 months increasing the risk by more than a two-fold[60]. Maternal nutritional status has been associated to increased pre-term risk. Women with low pre-pregnancy BMI have an increased risk for spontaneous preterm birth.[30] Obese mothers have a higher risk for babies with congenital anomalies, as well as, the development of gestational diabetes and pre-eclampsia.[23]

Women with previous preterm births have a 2.5 fold increased risk in the following pregnancy; the risk being inversely proportional to gestational age of previous pregnancy.[37] Multiple gestations have 10 times increased risk of preterm birth than single gestation, approximately 60% of twins are born prematurely.[11, 24] Placenta previa is another condition that has been significantly associated with increased risk for preterm delivery and postpartum complications[71]. Certain medical conditions can lead to maternal complications which in turn can lead to increased risk of preterm birth, these include: diabetes, hypertension, asthma, and thyroid disorders. [24] Mothers suffering from depression have a <2-fold preterm birth risk.[31, 50]

Other risk factors are severe psychological stress and excessive physical work. [39,67] One of the proposed mechanisms for the role of stress in preterm births seems to be related to endocrine and inflammatory/immune responses coordinated by placental corticotropin releasing hormone.[67] High levels of C-reactive protein, a known inflammatory marker, seem to be associated with preterm birth.[24, 52]

Lifestyle habits such as smoking and alcohol use during pregnancy have been associated with increased PTB. Studies have associated smoking with multiple pregnancy complications such as fetal growth restriction, stillbirth, placental abruption, spontaneous abortions, ectopic pregnancies, placenta previa, and PTB.[15] Smoking relative risk of PTB ranges from 1.2-1.6 and the association seems to be stronger for very preterm births (<32 weeks of gestation).[15, 63]

The relative risk for preterm birth in mothers consuming alcohol during the pregnancy seems to be dose-dependent. Women who consumed four to six drinks per week during pregnancy had relative risk of 1.15 (95% confidence interval: 0.84-1.57) and those who consumed seven or more drinks per week had a relative risk of 1.77 (95% confidence interval:

0.94, 3.31), however, women consuming three drinks per week or less did not appeared to have an increased risk of PTB.[2]

The pathogenesis of preterm birth while complex, it appears to be driven by microbial colonization and invasion.[26] Studies have suggested that intrauterine infection could be the cause of 25-40% of preterm births. Vaginal microorganisms have been shown to reach the cervix, chorioamnion, placenta, or amniotic fluid and trigger early labor via pro-inflammatory pathways[56,61]. Four major routes have been proposed to describe microbial invasion of the uterine cavity: ascending from the vagina and cervix, hematogenous dissemination through the placenta, retrograde through the fallopian tubes, and introduction during invasive procedures; with the ascending pathway being the most common route.[24]

A review by Goldenberg, describes a mechanism by which infection can lead to PTB. In these mechanism, microbial invasion and colonization of the choriodecidual space activates maternal and fetal membranes to release exotoxins, endotoxins, and cytokines. The release of these inflammatory mediators promotes production and secretion of prostaglandins, metalloproteinases, as well as, chemotaxis and activation of neutrophils. Spontaneous preterm birth is then initiated by prostaglandin mediated uterine contraction and metalloproteases induced membrane rupture. Fetal infection could be another pathway leading to preterm birth by increasing cortisol production, which in turn stimulates release of prostaglandins.[24]

In the presence of intrauterine infection, the levels of multiple inflammatory mediators have been found to be altered in the amniotic fluid, cervix, and serum. Mothers with symptoms of preterm labor show higher concentrations of G-CSF, TNF- alpha, IL-1, IL-6, IL-8, C-reactive protein, & fetal fibronectin, while asymptomatic mothers with intrauterine infection have shown

elevated levels of G-CSF, IL-6, fetal fibronectin, IL-8, and ferritin in routine screenings prior to the onset of preterm labor.[22,24,40]

Previously, it was believed the placenta was a sterile environment in the absence of intrauterine infection.[68] However, recent studies have demonstrated that the placenta has a microbiome, even in uneventful healthy pregnancies. In a case report of a woman presenting with spontaneous preterm labor and chorioamnionitis at 28 weeks of gestation, a microbial biofilm was identified in the amniotic fluid sludge.[56] Intra-amniotic microbial infections studied using culture and 16S rRNA-based culture-independent methods have demonstrated presence of *Fusobacterium nucleatum, Leptotrichia* (*Sneathia*) spp., *Bergeyella* sp., *Peptostreptococcus* sp., *Bacteroides* spp., and a species of the order *Clostridiales*.[29]

In a study were placenta samples were collected from both vaginal and cesarean deliveries, DNA of intestinal bacteria *Bifidobacterium* spp. and *Lactobacillus rhamnosus* were identified in more than 90% of samples. However, when attempting to detect any viable cells by cultivation they were not able to detect *Bifidobacteria* or *L. rhamnosus*.[58] Gram-positive and negative intracellular bacteria have been identified in placental basal plates, with up to 54% of spontaneous preterm deliveries <28 weeks and 26% of term spontaneous deliveries (p=0.2) showing evidence of intracellular bacteria in the placental basal plates.[61]

Aagaard and colleagues demonstrated that the placenta has its own microbiome even in uneventful pregnancies and that this composition was more similar to the human oral microbiome than to that of any other body site. Further, the authors reported an association between the placental microbiome composition and APOs.[1] However, a study by Laude and colleagues, challenge these results by arguing that due to the low bacterial biomass of placenta samples, some or all of the bacterial DNA could be derived from contamination. Authors

quantified total 16sRNA gene copies using PCR and found they could not distinguish between communities from placental samples and contamination controls.

Studies have sought differences in the placental microbiomes of PTB in mothers suffering from different medical conditions or pregnancy complications such as pre-eclampsia, gestational weight gain, and intramniotic infection. In a cross-sectional study evaluating placental microbiome in PTB, subjects with chorioamnionitis showed high abundance of urogenital and oral commensal bacteria[54]. A study comparing the microbiome of placental tissues of mothers with pre-eclampsia with that normotensive mothers found that 12.7% of placental tissue from women with pre-eclampsia were PCR-positive, while all samples from control women were PCR-negative. When the PCR-positive samples were analyzed using next-generation sequencing, the authors were able to identify multiple organisms commonly associated with gastrointestinal infections, respiratory infections, and periodontitis[3]. Another study investigating PTB placental microbiome found no significant alterations in the placental microbiome in the presence of maternal obesity, however, excess maternal gestational weight gain was associated with an altered microbiome.[4]

Doyle and colleagues compared the microbiome of placental membranes of very preterm and term deliveries. Using 16S rDNA sequencing, they were able to identified one family (*Enterobacteriaceae*) and six genera (*Fusobacterium, Streptococcus, Mycoplasma, Aerococcus, Gardnerella* and *Ureaplasma*) that were present in greater relative abundances in PT samples or absent in term deliveries.[18] Zheng and colleagues, investigated whether the placental microbiome varies with low birth weight in full-term infants and found significant variations in the composition of placental microbiota between LBW and NBW infants at phylum and genus

levels. Authors concluded that in fact the placental microbiome varies in association with low birth weight in full-term infants.[70]

Role of Periodontal Disease on Pathogenic Mechanisms of Adverse Pregnancy Outcomes

Oral microorganisms have been postulated to contribute to preterm birth. [35,41,43,47,66] In fact, periodontitis, a common oral disease of bacterial etiology and potent inflammatory component is strongly associated with pre-term birth. Further, levels of periodontal pathogens, such as *Porphyromonas gingivalis* and *Tannerella forsythia* have been detected in significantly higher levels in subgingival biofilm samples of mothers who delivered preterm babies.[34] In addition, those pathogens have also been detected more commonly in the amniotic fluid and subgingival plaque samples of patients who gave birth to preterm neonates.[19]

Several animal studies have evaluated the effects of periodontal pathogens on adverse pregnancy outcomes (APOs). In an animal study by Collins and colleagues, the effects of LPS from oral microorganisms on golden hamster pregnancy outcomes were investigated. Authors found that exposure to *Escherichia coli* LPS and *Porphyromonas gingivalis* LPS induced harmful effects on the developing fetus causing significant decreases in fetal weight, therefore, demonstrating that oral bacteria can cause adverse pregnancy outcomes[16]. In a similar animal study by the same group of researchers, the effects of *P. gingivalis* infection on the production of inflammatory mediators and pregnancy outcomes in golden hamsters were evaluated. *P. gingivalis* infection was found to significantly increase the levels of PGE2 and TNF-α and it was also found to induce adverse pregnancy outcomes including decreased fetal weight, as well as, increased embryolethality and fetal resorption.[17]

Evidence of hematogenous transmission of oral bacteria to placenta was shown in an animal study were *F. nucleatum* was intravenously injected into pregnant mice which resulted in

premature delivery, stillbirths, and non-sustained live births[28] . *P. gingivalis* has also demonstrated ability to invade maternal and fetal tissues in a rodent model, resulting in chorioamnionitis and placentitis.[8]

Offenbacher and colleagues challenged pregnant mice with *Campylobacter rectus* and found that this periodontal pathogen is able to induce placental architectural changes and inflammation, increase fetal brain expression of IFN-ϒ by 2.8 fold, and pup lethality by 3.9 fold.[49] *C. rectus* infection has also been found to increase fetal resorption and fetal growth restriction in the mouse model.[69] *C. rectus* has also been shown to down-regulate placental expression of critical growth and developmental related genes leading to fetal growth restriction.[12]

Fardini and colleagues employed a pregnant murine model to identified oral microbial species that are able to translocate to the placenta, hematogenously by injecting pooled saliva and pooled subgingival plaques samples into pregnant mice. Authors found that majority of the mice developed mixed infections with two or more different bacterial species present. These mice were preferentially infected by *Neisseri*a spp. (in eight mice); *Streptococcus* spp. (in seven mice); *Aggregatibacter segnis, Leptotrichia* spp., and *Selenomonas* spp. (each in three mice); and *Fusobacterium nucleatum* and *Eikenella corrodens*, however, a total of 16 different genera/species were identified in the murine placentas[20]. This study was able to prove that oral microorganisms are able to disseminate in the blood and infect distant sites such as the placenta and fetus.

Toll-like receptors have been suggested to have a role in pregnancy maintenance, placental immune protection, and initiation of delivery[51]. In pregnant mice infected with *C. rectu*s, TLR4 mRNA expression was found to be elevated and microscopic analysis showed

evidence of increased immunofluorescence of TLR4 in trophoblasts in the placental labyrinth layer[5]. These findings suggest a possible mechanism of how periodontal pathogens are able to invade and colonize the placenta.

Several human studies have shown evidence of oral microorganisms translocation to the intrauterine cavity. *Streptococcus* spp. and *F. nucleatum* have been identified in the amniotic fluid and a significant association was found between detection of bacterial DNA and adverse pregnancy outcomes in previous pregnancies[7]. Periodontal pathogens such as *P. gingivalis*, *C. rectus, T. forsythia*, and *F. nucleatum* have been detected in the amniotic fluid of mother's with subgingival plaque samples positive for such pathogens.[19,33] *F. nucleatum* has been detected in chorionic tissues of high-risk pregnant women, but not in tissues of normal pregnant women. Authors found that *F. nucleatum* is capable of inducing elevated production of IL-6 and CRH production in chorion-derived cells.[64] In a case report of a term stillbirth, *F. nucleatum* was shown to translocate hematogenously to the placenta and fetus, in a mother whose subgingival plaque was positive for this microorganism.[27]

Cahill and colleagues, evaluated samples of fetal membrane from preterm neonates and were able to detect *Mycoplasma hominis, Pasturella multocida, Pseudomonas PH1, Fusobacterium nucleatum, E. coli.* and *Prevotella bivia. F. nucleatum* was the most common organism identified and was shown to be linked to fetal membrane rupture and preterm delivery.[14]

P. gingivalis antigens have been detected in placental tissues of mothers presenting with chorioamnionitis using immunocytochemistry.[32] Vanterpool and colleagues, compared the presence and location of P.g. in placental and umbilical cord samples from PTB (25-32 weeks of gestation) and TB using immunofluorescent histology. Authors were able to detect *P. gingivalis*

in 51% of placentas and 41% of umbilical cord samples in the PTB group. When evaluating placental tissues, *P. gingivalis* was found to be significantly associated with shorter gestational lengths and C-section delivery but not with histological presence of chorioamnionitis or preeclampsia. However, *P. gingivalis* was significantly associated with preeclampsia when found in umbilical cord samples. In this study, the term group only showed presence of *P. gingivalis* in 6% of placentas, however, it was not able to be detected in any of the umbilical term samples. Pg was only detected within villous mesenchyme of the placenta in samples of PT group, while presence of *P. gingivalis* in syncytiotrophoblasts was found in both PT and term groups. This study was able to establish an association between the presence and location of *P. gingivalis* in placental and umbilical tissues and APOs.[65] Furthermore, periodontal pathogens have been found in high numbers in plaque and placentas of mothers presenting with preeclampsia, suggesting a possible role of these microorganisms in the pathogenesis of this disease.[6,62]

Several human studies have investigated the association between periodontal disease and increased risk of PTB. In the 1990's, a case-control study by Offenbacher and colleagues showed that periodontal disease is a statistically significant risk factor for preterm low birth weight (PLBW) with odd ratios of 7.9 for all PLBW cases.[41] A 5-year prospective study in pregnant women periodontal disease incidence and progression was found to be significantly associated with low weight for gestational age, as well as, increased relative risk for PTB and very PTB. Severity of maternal periodontal disease was also shown to be a predictor of more severe APOs. [47,48] A meta-analysis by Vergnes, concluded that periodontal disease may be an independent risk factor for preterm low birth weight, with odds ratio of 2.83.[66]

Boggess and colleagues evaluated the relationship between increased risk of PTB and fetal immune and inflammatory responses to oral microorganisms. Authors found fetal IgM

response to *Campylobacter rectus, Peptostreptococcus micros, Prevotella nigrescens, Prevotella intermedia* or *Fusobacterium nucleatum* in 35% of the cases and it was associated with an elevated risk of PTB (<35 weeks). Fetuses also show evidence of an immune response to infection with oral pathogens measured by the presence of TNF-α, PGE2, IL-1β, IL-6, and 8-isoprostane. The risk for PTB was significantly increased when fetuses developed both an immune and an inflammatory response.[13]

Reduced maternal IgG antibody response to periodontal pathogens has also been shown to be associated with increased risk of PTB.[34] In a study by Offenbacher and colleagues, 2.9-fold higher prevalence of IgM seropositivity for one or more organisms of the Orange or Red complex was observed among preterm babies, as compared to term babies. The prevalence of positive fetal IgM to *C. rectus* was significantly higher for preterm as compared to full-term infants. Reduced maternal IgG antibody to periodontal pathogens of the Red complex was associated with an increased rate of PTB with odds ratio (OR) = 2.2. These findings support the theory that the lack of a protective maternal antibody response allows periodontal pathogens to travel in the blood and invade the intrauterine cavity, ultimately leading to APOs.[36]

To date, interventions studies examining the effect of periodontal therapy during pregnancy on APOs present conflicting results. Michalowicz and colleagues found that periodontal treatment in pregnant women while safe for the pregnancy, does not significantly alter rates of preterm birth, low birth weight, or fetal growth restriction.[38] A pilot study by Offenbacher and colleagues found periodontal intervention significantly reduced the odds ratio for PTB (OR 0.26; 95% CI 0.08-0.85).[48] However, a later study by the same group found that periodontal therapy during pregnancy had no statistically significant effect in reducing the incidence of PTB.[42]

A meta-analysis by Polyzos and colleagues found that periodontal therapy during pregnancy is associated with significantly lower risk of PTB (OR 0.55, 95% CI 0.35-0.86), lower LBW (OR 0.48; 95% CI 0.23-1.00),although no difference was observed for spontaneous abortion or stillbirth (OR 0.73; 95% CI 0.41-1.31).[53] In contrast, a systematic review and meta-analysis by Rosa and colleagues found that periodontal treatment during pregnancy had no significant effect on rate of PTB (RR 0.90; 95% CI 0.68-1.19) and low birth weight (RR 0.92; 95% CI 0.71-1.20)[57]. Similar results were found in a meta- and trial sequential analysis by Schwendicke and colleagues, were periodontal intervention was shown to reduce the risk of APOs only in populations with high occurrence (>20%) of PTB and LBW.[59]

Madianos and colleagues proposed a possible biological model for the association between PD and APOs. In these model, the authors emphasizes that hormonal changes during pregnancy lead to increased vascular permeability in oral tissues which facilitates periodontal pathogens to gain access to the systemic circulation, where they can travel to distant sites and reach the placenta and fetus. The presence of oral bacteria and their products in the intrauterine cavity elicits a fetal immune and inflammatory response characterized by production of IgM against periodontal pathogens and stimulates the release of inflammatory cytokines. If the immune response is able to contain the infection, no APOs should occur. [35]

However, if the infection is not controlled the increase in inflammatory mediators can induce premature rupture of membranes and uterine contraction, resulting in spontaneous abortion or PTB. This model also considers the possibility that inflammatory cytokines in gingival tissues can disseminate hematogenously and reach the uterine circulation, enhancing the inflammatory response in the intrauterine cavity. Furthermore, intrauterine infection can lead to downregulation of key genes for placental and fetal growth and development. This could cause

placental architectural changes that can impair the movement of nutrients into the placenta, ultimately leading to APOs such as low weight for gestational age and fetal mortality.[35]

However, a gap in knowledge remains regarding whether oral pathogens can consistently reach and affect fetal tissues, such as the placenta and the umbilicus and whether this exposure is determined by the level of periodontitis.

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CHAPTER 2: THE UMBILICAL AND PLACENTAL MICROBIOMES AND THEIR RELATIONSHIP WITH PRE-TERM BIRTH

INTRODUCTION

WHO defines preterm birth as babies born alive before 37 weeks of pregnancy are completed, with subcategories moderate-late preterm (32 to < 37 weeks), very preterm (28 to <32 weeks), and extremely preterm (<28 weeks). Worldwide an estimated 11.1% of all live births in 2010 were born preterm (14.9 million babies born before 37 weeks of gestation), with preterm birth rates increasing in most countries with reliable trend data. Direct complications of preterm birth account for one million deaths each year, and preterm birth is a risk factor in over 50% of all neonatal deaths.[4] The effect of preterm birth among some survivors may continue throughout life, impairing neuro-developmental functioning through increasing the risk of cerebral palsy, learning impairment and visual disorders and affecting long-term physical health with a higher risk of non-communicable disease.[32]

Preterm births can be classified into two main categories: spontaneous births or provider-initiated preterm birth.[14] Spontaneous preterm labor can be initiated by different mechanisms, which include immunologically mediated processes, infection and inflammation, uterine overdistension, uteroplacental ischaemia or hemorrhage, stress, among others. [14, 33] Multiple maternal and fetal features have been linked to preterm birth, among these are pregnancy history, current pregnancy characteristics, maternal demographic characteristics, infection, uterine contractions and cervical length, nutritional status, psychological characteristics, adverse behaviors, and biological and genetic markers.[14]

The pathogenesis of preterm birth while complex, it appears to be driven by microbial colonization and invasion.[15] Studies have suggested that intrauterine infection could be the cause of 25-40% of preterm births. Vaginal microorganisms have been shown to reach the cervix, chorioamnion, placenta, or amniotic fluid, and trigger early labor via pro-inflammatory pathways. [34,37] Four major routes have been proposed to described microbial invasion of the uterine cavity: ascending from the vagina and cervix, hematogenous dissemination through the placenta, retrograde through the fallopian tubes, and introduction during invasive procedures; with the ascending pathway being the most common route.[14] Previously, it was believed the placenta was a sterile environment in the absence of intrauterine infection.[39] However, recent studies have demonstrated that the placenta has a microbiome, even in uneventful healthy pregnancies. Aagaard and colleagues, demonstrated that the placenta has its own microbiome with a composition most similar to the human oral microbiome. Further, the authors reported an association between the placental microbiome composition and APOs.[1]

Oral microorganisms have been postulated to contribute to preterm birth.[24,25,27,38] In fact, periodontitis, a common oral disease of bacterial etiology and potent inflammatory component is strongly associated with pre-term birth. Further, levels of periodontal pathogens, such as *Porphyromonas gingivalis* and *Tannerella forsythia* have been detected in significantly higher levels in subgingival biofilm samples of mothers who delivered preterm babies.[22] In addition, those pathogens have also been detected more commonly in the amniotic fluid and subgingival plaque samples of patients who gave birth to preterm neonates.[11] However, a gap in knowledge remains regarding whether oral pathogens can consistently reach and affect fetal tissues, such as the placenta and the umbilicus and whether this exposure is determined by the level of periodontitis. Therefore, the aim of this study was to compare the microbiomes of placenta and

umbilicus samples from women presenting periodontal diseases who experienced full term (FTB, n=22) and preterm births (PTB; <35 weeks) using 16S rRNA MiSeq Illumina sequencing.

MATERIALS AND METHODS

Study Population

Samples used in this study were collected from pregnant women previously enrolled in the Maternal Oral Therapy to Reduce Obstetric Risk (MOTOR U01DE014577) Study[26] as part of the study demographic, medical and dental (periodontal) data were collected as well as maternal subgingival biofilm samples and fetal tissues (umbilicus and placenta). All participants of the MOTOR Study signed a consent that allowed the use of all the available biological samples collected and stored, for the purposes of the present investigation. Inclusion criteria: pregnant and able to complete periodontal treatment prior to 23 weeks gestation, at least 16 years old at enrollment, minimum of 20 teeth present, three (3) or more periodontal sites with $\geq$ 3mm clinical attachment loss. Exclusion criteria: multiple gestation, systemic medical conditions, rampant decay, symptomatic teeth or any other dental finding such as periodontal abscess or endodontic fistula that would preclude enrollment, chronic use of medications that cause gingival enlargement such as phenytoin, cyclosporin-A, or calcium channel antagonists, chronic use of steroids, or concomitant use of orthodontic appliances (braces).

For the present study, a case-control design with a ratio of 1:1 was employed. Case group consisted of 22 mothers who had preterm delivery (< 35 weeks gestational age), while the control group consisted of 22 mothers who had normal, term deliveries and uneventful pregnancies. Control cases were selected using a random sampling method. In the case group, a non-random sampling method was used due to the limited numbers of subjects in the MOTOR database. In this report we present the following data from each subject: maternal pre-partum and

post-partum plaque samples, as well as, placental and umbilical tissue samples collected at delivery.

Sample Collection and Processing

Placental and umbilical cord tissue samples were obtained within the first hour of delivery. Samples were collected using sterile tissue tweezers or hemostat and scissors. Each sample was stored into a cryovial numbered for that tissue specimen. The cryovials were snap frozen in liquid nitrogen. Samples were stored temporarily on dry ice, transported to the laboratory at the University of North Carolina at Chapel Hill and subsequently stored at -80°C. The first step in preparation for the microbial analysis of the umbilical and placental tissues was tissue homogenization, using a sterile mortar and pestle and liquid nitrogen until a powder consistency was achieved. Immediately proceeded with DNA extraction using the MasterPure DNA Purification kit (Epicentre, Illumina, Madison, WI) according to the manufacturer's recommendations. DNA quantification was performed using Quant-iTTM PicoGreen ® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA). Sample fluorescence was measured using a fluorescence microplate reader (excitation ~480 nm, emission ~520 nm).

Library preparation and 16S rRNA sequencing

The protocol used has been described elsewhere (Furquim et al 2016).[13] In brief, samples prepared for sequencing had a minimum DNA concentration of 10 ng/uL in at least 10 uL volume. A modified protocol was used for 16S rRNA amplicon sequencing library preparation.[7] The variable region V3-V4 of the 16S rRNA gene were PCR amplified using barcoded 341F and 806R 16S primers.[6] The amplicon size was approximately 460 bp. PCR products were purified (AMPure beads Beckman Coulter, Brea, CA) and quantified (Quant-iT™ PicoGreen ® dsDNA Reagent, Molecular Probes, Waltham, MA). Equimolar amounts of each library were pooled,

gel-purified, analyzed and quantified (2100 Bioanalyzer, DNA High Sensitivity chip;Agilent Technologies, Santa Clara, CA). The library mixture (6 picomolars spiked with 30% PhiX) were run on a MiSeq instrument (Illumina, San Diego, CA). Automated cluster generation and paired–end sequencing using 500 cycle reagent kit were performed according to the manufacturer's instructions.

Clinical Measurements

During the MOTOR Study subjects received a comprehensive periodontal examination performed by calibrated dental examiners at baseline and delivery. The clinical examination protocol of the study has been described elsewhere (Offenbacher et al 2009).[26]. For all subjects, there are baseline and post-partum data for the following clinical parameters: plaque score gingival index, probing pocket depth (PD), bleeding on probing (BOP), and clinical attachment level (CAL).

Data Analysis

After removal of chimeric sequences and sequences that failed quality control, sequencing reads were evaluated using the QIIME analysis pipeline (Quantitative Insights Into Microbial Ecology, qiime.org, version 1.8)[5] Reads were grouped into Operational Taxonomic Units (OTUs) using UCLUST [10] and the bacterial taxonomy was determined using HOMD and Greengenes. After taxonomic assignment, OTUs were combined with sample metadata. Species-level taxonomy was determined using HOMINGS. The relative abundance of the taxa present in each sample was computed at the phylum and genus levels. The demographic and clinical measurements of the study population were analyzed using Student's t-test and Chi Square test. Microbial differences between samples from PTB and FTB were sought using Kruskall-Wallis and Principal Component Analyses (PCA). Correlations between microbial taxa and periodontal

parameters were sought using the Spearman correlation coefficient. False discovery rate (FDR) was used to adjust for multiple comparisons. All statistical analysis was performed in SAS version 9.4.

RESULTS

Socio-Demographic and Clinical Characteristics of the Study Population

A total of forty-four pregnant women were included in this study, with twenty-two mothers in the term group and twenty-two mothers in preterm group. Preterm and term groups at baseline did not differ in maternal age, marital status, maternal BMI, public assistance, previous pregnancy, or smoking history. However, significant differences were observed regarding maternal race, with 62% of Caucasian mothers in the term group and 73% of African American mothers in the preterm group (Table 1). With regard to ante-partum clinical measures, both groups had similar mean gingival index (GI), plaque index (PI), bleeding on probing (BOP), extent of sites with probing depth (PD) at least 4mm, and attachment level (AL) at least 3mm. In terms of postpartum clinical measures, no differences were observed between groups (Table 1).

Overall Sequencing Data and Microbial Profile

All samples were sequenced in the same run, which generated a total of reads, and yielded, on average 2,911 reads (±3,376; median: 2,255) for placenta samples, ranging from 669 to 22,630 reads and an average of 14, 741 reads (± 24,699; median: 3,378) for umbilicus samples, ranging from 653 to 121,896 reads (Table 2). Sequencing of placenta samples revealed 8 different phyla, 84 genera, and 132 species. While, umbilicus samples revealed 8 different phyla, 100 genera, and 225 species. Comparison of sequencing results using HOMD and Greengenes Databases demonstrated similar results regarding percentage of unassigned genera

and most abundant genera detected (Table 3). However, certain differences regarding genera detection were also observed between databases (Table 4).

Impact of Sample Type and Time of Delivery

Principal component analyses of placenta and umbilicus samples showed that the microbiome of the placenta is different from the umbilicus microbiome (Fig 1). When comparing the umbilicus PTB samples with FTB samples, principal component analysis showed a tendency for samples of the different groups to cluster together, suggesting that PTB and FTB samples exhibit distinct microbial profiles (Fig 2).

Microbial Profiles in PTB/FTB

Placenta Tissues

The most abundant phylum in placental tissues were: *Firmicutes* (mean relative abundance ± standard deviation) (FTB: 44.2% ± 14.2%, PTB: 40.9% ± 15.0%; p: 0.481), followed by *Fusobacteria* (FTB: 9.4% ± 9.0%, PTB: 15.0% ± 9.7%; p: 0.069) and *Proteobacteria* (FTB: 12.7% ± 10.4%, PTB: 9.3% ± 8.3%; p = 0.222) (Fig 3). The most abundant genera in placental samples were *Veillonella* (FTB: 16.1% ± 10.3%, PTB: 18.7% ± 11.4%, p= 0.573), *Fusobacterium* (FTB: 9.4% ± 9.0%, PTB: 14.9% ± 9.8%, p= 0.076) and *Streptococcus* (FTB: 6.4% ± 6.5%, PTB: 6.4% ± 4.5%, p= 0.511). The following genera demonstrated significant differences between groups: *Slacki*a (FTB: 0.0% ± 0.0%, PTB: 0.6% ± 1.7%, p= 0.04) and *Actinomyces* (FTB: 0.1% ± 0.5%, PTB: 0.7% ± 1.4%, p= 0.03) (Fig 5).

The most abundant species were *Neisseria elongata* (FTB: 4.78% ± 4.95%, PTB: 3.84% ± 4.51%, p= 0.53), *Gemella morbillorum* (FTB: 3.21% ± 4.80%, PTB: 3.98% ± 5.09%, p= 0.45), *Streptococcus constellatus* (FTB: 3.99% ± 4.44%, PTB: 3.16% ± 3.68%, p= 0.52) and *Anaeroglobus geminatus* (FTB: 2.39% ± 3.28, PTB: 3.19% ± 4.84%, p= 0.66) (Fig 7). Several

periodontal microorganisms were detected in placental samples, including *Fretibacterium fastidiosum, Campylobacter gracilis, Parvimonas micra, Capnocytophaga gingivalis, Prevotella nigrescens, Eikenella corrodens* and *Treponema denticola.* Key periodontal pathogens such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis* and *Tanerella forsythia*, were not detected.

Umbilicus Tissues

Most abundant phylum in umbilical tissues were: *Firmicutes* (mean relative abundance ± standard deviation) (FTB: 32.0% ± 17.0%, PTB: 36.6% ± 20.3%; p= 0.851), followed by *Proteobacteria* (FTB: 15.1% ± 20.5%, PTB: 8.1% ± 7.0%; p= 0.467) and *Synergistetes* (FTB: 7.2% ± 12.7%, PTB: 7.7% ± 14.7%; p= 0.823) (Fig 4). The most abundant genera were *Veillonella* (FTB : 9.7% ± 8.5%, FTB: 18.9% ± 18.2%, p = 0.064), *Pyramidobacter* (FTB: 6.2% ± 12.2%, PTB: 6.5% ± 14.7%, p = 0.807), and *Fusobacterium* (FTB: 6.4% ± 65.5%, PTG: 6.4% ± 6.4%, p = 0.814). The following genera demonstrated significant differences between groups: *Bifidobacterium* (FTB: 2.2% ± 3.1, PTB: 0.6% ± 1.0%, p= 0.04), *Megasphaera* (FTB: 2.3% ± 2.8%, PTB: 0.7% ± 1.0%, p= 0.02), and *Lactobacillus* (FTB: 3.7% ± 8.3%, PTB: 1.4% ± 4.5%, p= 0.01) (Fig 6).

The most abundant species were: *P. piscolens* (FTB: 6.1% ± 11.8%, PTB: 6.3% ± 14.5%, p= 0.64), *G. morbillorum* (FTB: 1.5% ± 1.7%, PTB: 1.8% ± 2.5%, p= 0.98), *Streptococcus constellatus* (FTB: 1.8% ± 2.0%, PTB: 1.7% ± 2.2%, p=0.79), and *Neisseria elongata* (FTB: 1.5% ± 2.3%, PTB: 1.6 % ± 3.0%, p= 0.93). The following species were found to be significantly different between groups: *Granulicatella adiacens* (FTB: 0.2% ± 0.6%, PTB: 1.2% ± 1.5%, p=0.01), *Anaeroglobus geminatus* (FTB: 2.1% ± 2.7%, PTB: 0.5% ± 0.9%, p=0.03), *Lactobacillus iners* (FTB: 2.3% ± 6.7%, PTB: 0.5% ± 2.2%, p=0.02), *Peptostreptococcaceae*

[XI][G-7] HOT 081 (FTB: 0.0 ± 0.1, PTB: 0.4 ± 1.1; p=0.02), and *Eubacterium nodatum* (FTB: 0.0 ± 0.1, PTB: 0.4 ± 0.7; p= 0.04) (Fig 8). Several periodontal microorganisms were detected in umbilical samples, including *Campylobacter gracilis, Fretibacterium fastidiosum, Fusobacterium HOT 204, Parvimonas micra, P. gingivalis, Prevotella intermedia, Prevotella nigrescens* and *Treponema denticola*. Key periodontal pathogens such as *Aggregatibacter actinomycetemcomitans* and *Tanerella forsythia*, were not detected.

Unique Taxa Detected

The only genus detected in both placental and umbilical FTB samples was *Ruminococcaceae*. *Mycoplasma*, *Peptoniphilaceae*, and *Shuttleworthia* genera were detected in both placental and umbilical PTB samples (Table 5). Some species were unique to FTB samples such as *Gardenella vaginalis, Peptosreptococcacea [XI][G7]*, and *Mobiluncus mulieris* (Table 6). While, certain species were unique to PTB samples such as *Bacteroides heparinolyticus, Enterococcus faecalis*, and *Pseudoramibacter alactolyticus* (Table 7).

Correlations between Placenta Samples Microbial Profile and Clinical Measurements

Positive correlations were observed between presence of *C. tuscaniense* in placenta samples and extent of AL >3mm (CC: 0.495, p = 0.001), extent of PD >4mm (CC: 0.302 , p= 0.046), GI (CC: 0.405, p=0.006), and PI (CC: 0.422, p=0.0064. *Leptotrichia* HOT 223 showed positive correlations for extent of AL >3mm (CC: 0.495, p = 0.001), extent of PD >4mm (CC: 0.302 , p= 0.046), GI (CC: 0.405, p=0.006), and PI (CC: 0.422, p=0.0064. While, *Fusobacterium* HOT 204 showed positive correlations for extent of AL >3mm (CC: 0.389, p = 0.009), GI (CC: 0.344, p=0.022), and PI (CC: 0.386, p=0.010).

Correlations between Umbilicus Samples Microbial Profile and Clinical Measurements

Positive correlations were found between presence of *P. gingivalis* in umbilical tissues and extent of AL >3mm (CC: 0.482, p = 0.001), GI (CC: 0.365, p=0.015), and PI (CC: 0.408, p=0.006). The presence of TM7 HOT 347 in umbilical tissues showed positive correlations with extent of AL >3mm (CC: 0.439, p=0.003), extent of PD >4mm (CC: 0.388, p=0.009), GI (CC: 0.398, p=0.008), and GI (CC:0.378, p= 0.011). Presence of *Prevotella loescheii* in umbilical tissues showed a positive correlation with GI (CC: 0.383, p=0.01) and PI (CC: 0.42, p= 0.005) (Table 8).

DISCUSSION

The intrauterine cavity was previously thought to be a sterile environment in the absence of intrauterine infection.[39] Recent studies, have demonstrated the presence of a placental microbiome even in uneventful, healthy pregnancies. Fetal membranes from PTB and TB have shown presence of bacteria, although, a more diverse microbial profile was found in PTB.[19] Gram-positive and negative intracellular bacteria have been identified in placental basal plates of both preterm and term pregnancies.[37] Aagaard and colleagues, demonstrated the presence of a placental microbiome in healthy pregnancies, with a microbial composition most similar to the human oral microbiome.[1]

Our study demonstrated the presence of a low-abundance placental microbiome in both term and preterm births. Furthermore, we were able to describe the microbial profile of the umbilicus for the first time. Some of the most common phylum detected in our samples *Firmicutes, Fusobacteria, and Proteobacteria* have been previously described in the literature as part of the placental microbiome.[1] Among the genera detected in our study were *Fusobacterium, Streptococcus, and Lactobacillus*; these were described by Payne and colleagues as some of the

most commonly detected microorganisms in the placenta and amniotic fluid.[30] Similar findings were demonstrated by Satokari and colleagues, who were able to detect *Bifidobacterium* and and *Lactobacillus* in placenta samples from both vaginal and cesarean deliveries.[36]

Consistent with previous studies, a PCA analysis of our samples demonstrated differences in the microbial profile of the PTB and FTB samples. A study by Doyle and colleagues, compared the microbiome of placental membranes of very preterm and term deliveries; using 16S rDNA sequencing they were able to identified 1 family (*Enterobacteriaceae*) and 6 genera (*Fusobacterium, Streptococcus, Mycoplasma, Aerococcus, Gardnerella,* and *Ureaplasma*) that were present in greater relative abundances in PT samples or absent in term deliveries.[9] Zheng and colleagues, investigated whether the placental microbiome varies with low birth weight in full-term infants and found significant variations in the composition of placenta microbiota between LBW and NBW infants at phylum and genus levels. Authors concluded that in fact the placental microbiome varies in association with low birth weight in full-term infants.[41] Similarly, differences in placental microbiome have been associated with the development of Preeclampsia and excess maternal gestational weight gain.[2,3,20]

The pathogenesis of PTB seems to be complex and multifactorial, however, infection of the intrauterine cavity seems to be one of the leading causes.[15,23,30] In recent years, mounting evidence supporting the establishment of the placental microbiome via hematogenous dissemination has been reported. The placental microbiome has been shown to be more similar to the oral microbiome than any other microbiome in the human body.[1] These findings, suggest that oral microorganisms have the ability to invade tissues and disseminate in the blood to reach the intrauterine cavity.[23] Hematogenous translocation of bacteria into the placenta was demonstrated in pregnant murine model, in which intravenous injection of *F. nucleatum* resulted

in premature delivery, nonsustained live births, and stillbirths.[17] *F. nucleatum* has been shown to have the ability to adhere and invade epithelial and endothelial cells via novel FadA adhesion; in mixed infections, *F. nucleatum* could facilitate the hematogenous spread of other microorganisms.[12] *Bergeyella* was detected in a case of PTB with intrauterine infection, the same strain of *Bergeyella* was detected in the subgingival plaque of the mother but in the vaginal tract.[16] In our study, we were able to identify several genera common to the oral cavity such as *Veillonella, Fusobaterium, Streptococcus, Pyramidobacter,* and *Prevotella*. Interestingly, *Pyramidobacter* genus has only been described in the oral cavity.[8] When comparing the results from HOMD and Greengenes databases, once again the majority of the genera detected were common to the oral cavity. Several studies have demonstrated a strong association between PTB and periodontal disease.[24,25,27,38] Our samples demonstrated presence of multiple periodontal microorganisms that have been associated to APOs were detected in our samples: *E. corrodens, P. intermedia, P. gingivalis, P. micra, P. nigrescens*, and *T. denticola*.[18,31]

There are limitations in the present study. The first one is its sample size. This was a pilot study, therefore, we were not able to performed power calculations. Also, we were limited by the amount of available samples from subjects that met all the criteria for our study. A second limitation of the present study is its cross sectional design, which precludes any inference on a causal role of the microbiome in PTB. A third limitation is the low biomass of our samples. Previously, it has been suggested that the low biomass of placenta samples preclude from differentiating between placental DNA and contamination (i.e. dust and commercial reagents) introduced during DNA purification.[21] The collection and processing of our samples were performed following a sterile protocol. To rule out contamination, we obtained swabs from our sterile mortar and pestles before use and performed DNA extraction of those control samples.

Negative numbers were obtained when performing DNA quantification using Quant-iTTM PicoGreen ® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA), therefore, samples did not contain enough DNA to allow 16S rRNA sequencing to be performed. Furthermore, we were not able to detect common environmental contaminants such as *Propionibacterium* species in our samples.[35,40]

CONCLUSIONS

We demonstrated that the umbilicus has a microbiome, that it is the case even in uneventful deliveries and it is different from that of the placenta. It may contribute to PTB via the presence of high levels of *Veillonella, Fusobacterium* and *Peptostreptococcacea* species. The presence of *P. gingivalis* in the umbilical microbiome was positively correlated with periodontal parameters.

Table 1: Demographics and Periodontal Parameters of Study Population

	Term	*Preterm*	*p-value*
Maternal Race			
African American	4 (27%)	11 (73%)	
Caucasian	18 (62%)	11 (38%)	0.03
Mean (SD)			
Maternal Age	25.2 (3.6)	25.7 (6.7)	0.74
Mean (SD) Pre Pregnancy			
BMI	26.5 (4.5)	27.9 (5.3)	0.36
Married	11 (55%)	9 (45%)	
Not Married	11 (46%)	13 (54%)	0.54
WIC	18 (51%)	17 (49%)	
No	4 (44%)	5 (56%)	0.71
Nulliparous	4 (36%)	7 (64%)	
No	18 (55%)	15 (46%)	0.3
Smoking During Pregnancy	3 (60%)	2 (40%)	
No	19 (49%)	20 (51%)	0.63
Ante Partum Clinical Measures			
Mean (SD)			
Extent AL >=3mm	18.5 (9.6)	19.0 (12.3)	0.9
Mean (SD)			
Extent PD >=4mm	26.3 (14.7)	24.3 (19.1)	0.69
Mean(SD) BOP	47.7 (29.4)	44.0 (31.7)	0.66
Mean (SD) GI	1.3 (0.4)	1.2 (0.5)	0.26
Mean (SD) PI	2.9 (0.5)	2.8 (0.6)	0.56
Post Partum Clinical Measures			
Mean (SD)			
Extent AL >=3mm	12.9 (14.4)	10.7 (13.0)	0.61
Mean (SD)			
Extent PD >=4mm	27.6 (19.1)	22.1 (19.8)	0.37
Mean (SD) BOP	42.7 (28.1)	26.1 (25.4)	0.06
Mean (SD) GI	1.1 (0.4)	0.9 (0.4)	0.13
Mean (SD) PI	3.0 (0.5)	2.8 (0.6)	0.36

Table 2: Descriptive Statistics of Sequencing Results

Sample	Available Samples	Mean reads ± SD	Median	Range	Samples <1000 reads	Samples <5000 reads
Placenta	44	2,911 ± 3, 376	2,255	669-22,630	5	36
Umbilicus	44	14, 741 ± 24, 699	3,378	653-121,896	3	23

Table 3: Comparison of Databases: HOMD versus Greengenes

#OTU ID GREENGENES	*Mean*	*SD*	*#OTU ID HOMD*	*Average*	*SD*
Unassigned	22.80%	20.90%	*Unassigned*	25.30%	21.90%
Veillonella	15.90%	13.00%	*Veillonella*	15.80%	13.00%
Fusobacterium	9.30%	8.50%	*Fusobacterium*	9.30%	8.50%
Streptococcus	5.60%	4.80%	*Streptococcus*	5.70%	5.00%
Pyramidobacter	4.20%	10.00%	*Pyramidobacter*	4.20%	10.00%
Prevotella	3.00%	4.00%	*Prevotella*	3.00%	4.00%
f__Neisseriaceae	3.00%	4.30%	*Kingella*	3.00%	4.10%
f__Enterobacteriaceae	2.80%	9.90%	*f__Enterobacteriaceae*	1.60%	6.60%
f__Gemellaceae	2.70%	3.70%	*Gemella*	2.70%	3.70%
Megasphaera	2.40%	3.60%	*Megasphaera*	2.40%	3.60%
Campylobacter	2.30%	3.50%	*Campylobacter*	2.30%	3.50%
Lactobacillus	2.10%	5.10%	*Lactobacillus*	2.10%	5.10%
Parvimonas	1.90%	3.20%	*Parvimonas*	1.90%	3.20%
Dethiosulfovibrionaceae;g__TG5	1.60%	3.70%	*Fretibacterium*	1.60%	3.70%
Corynebacterium	1.40%	2.00%	*Corynebacterium*	1.40%	2.00%

Table 4: Distinct Taxonomy Between Databases

#OTU ID GREENGENES	Mean	SD	#OTU ID HOMD	Average	SD
Ureaplasma	*1.10%*	*9.00%*	*Kluyvera*	*1.20%*	*7.60%*
f_[Mogibacteriaceae]	*0.40%*	*1.10%*	*Afipia*	*0.10%*	*0.20%*
[Ruminococcus]	*0.10%*	*0.40%*	*Arsenicicoccus*	*0.10%*	*0.70%*
f_Bifidobacteriaceae	*0.20%*	*1.30%*	*Atopobium*	*0.10%*	*0.30%*
Brachybacterium	*0.10%*	*0.70%*	*Bacteroidetes_[G-6]*	*1.00%*	*1.90%*
Bradyrhizobium	*0.10%*	*0.20%*	*Bergeyella*	*0.20%*	*1.40%*
Brevibacterium	*0.30%*	*1.30%*	*Clostridiales_[F-3][G-1]*	*1.00%*	*4.70%*
Cloacibacterium	*0.10%*	*1.40%*	*Lachnoanaerobaculum*	*0.20%*	*1.00%*
Clostridium	*0.90%*	*4.60%*	*Lachnospiraceae_[G-2]*	*0.10%*	*0.40%*
Collinsella	*0.10%*	*0.50%*	*Lachnospiraceae_[G-8]*	*0.20%*	*0.60%*
Coprococcus	*0.10%*	*0.30%*	*Leptotrichia*	*0.10%*	*0.30%*
f_Coriobacteriaceae	*0.10%*	*0.40%*	*Peptostreptococcaceae_[XI][G-1]*	*0.20%*	*0.60%*
Faecalibacterium	*0.10%*	*0.80%*	*Peptostreptococcaceae_[XI][G-6]*	*0.10%*	*0.40%*
Gemella	*0.10%*	*0.50%*	*Peptostreptococcaceae_[XI][G-7]*	*0.70%*	*1.80%*
Lactococcus	*0.10%*	*0.80%*	*Ralstonia*	*0.10%*	*0.20%*
Methylobacterium	*0.20%*	*0.90%*			
Odoribacter	*1.00%*	*1.90%*			
Thermus	*0.10%*	*0.30%*			

Table 5: Unique Genera to FTB or PTB

PTB		***FTB***	
Placenta	***Umbilicus***	***Placenta***	***Umbilicus***
Shuttleworthia	***Peptoniphilaceae***	*Lachnospiraceae [G2]*	***Ruminococcaceae***
Eggerthella	*Bacteroides [G2]*	***Ruminococcaceae***	*Scardovia*
Anaeroglobus	***Shuttleworthia***	*Pseudomonas*	
Propionibacterium	*Microbacterium*	*Finegoldia*	
Capnocytophaga	*Tannerella*	*Peptoniphilus*	
Leptotrichia	*Stomatobaculum*	*Enterococcus*	
Mycobacterium	***Mycoplasma***	*Arsenicicoccus*	
Mycoplasma		*Bergeyella*	
Sneathia		*Clostridiales [F3][G1]*	
Peptoniphilaceae			
Peptostreptococcaceae [G9]			
Slackia			
Haemophilus			

Table 6: Species Unique to Full-Term Birth

PLACENTA	UMBILICUS
Agrobacterium tumefaciens	*Actinomyces HOT 175*
Alloprevotella HOT 308	*Actinomyces timonensis*
Alloprevotella rava	*Bacteroides pyogenes*
Bacteroides zoogleoformans	*Bacteroidetes [G-3] HOT365*
Campylobacter gracilis	*Bulleidia_extructa*
Catonella morbi	*Butyrivibrio HOT 455*
Delfia acidovorans	*Capnocytophaga HOT 335*
Enterococcus faecalis	*Capnocytophaga HOT 412*
Finegoldia magna	*Capnocytophaga HOT 901*
Gardnerella vaginalis	*Capnocytophaga sputigena*
Gemella morbillorum	*Chlamydophila pneumoniae*
Lactococcus lactis	*Corynebacterium mucifaciens*
Megasphaera HOT 841	*Haemophilus parainfluenzae*
Neisseria oralis	*Lachnospiraceae[G-9] HOT 924*
Oribacterium asaccharolyticum	*Leptotrichia HOT 217*
Oribacterium sinus	*Mitsuokella HOT 131*
Peptoniphilus lacrimalis	***Mobiluncus mulieris***
Peptostreptococcaceae [XI][G7]	*Neisseria HOT 523*
Prevotella denticola	*Olsenella profusa*
Prevotella HOT 376, HOT 300	*Parvimonas micra*
Prevotella loescheii	*Pedobacter HOT 933*
Prevotella saccharolytica	*Peptinophilaceae[G-2] HOT 790*
Rothia aeria	*Peptostreptococcaceae[XI][G-1] sulci*
	Peptostreptococcaceae[XI][G-7]
	Porphyromonas pasteri
	Porphyromonas HOT 930
	Prevotella fusca
	Prevotella intermedia
	Prevotella multisaccharivorax
	Prevotella oulorum
	Prevotella HOT 376
	Propionibacterium avidum
	Proteus mirabilis
	Selenomonas HOT 501
	Streptococcus agalactiae
	Treponema HOT 260
	Turicella otitidis
	Veillonella atypica
	Veillonella rogosae
	Veillonellaceae[G-1] HOT 155

Table 7: Species Unique to Preterm Birth

PLACENTA	UMBILICUS
Bacteroides heparinolyticus	*Actinomyces gerencseriae*
Capnocytophag gingivalis	*Actinomyces oris*
Corynebacterium tuscaniense	*Atopobium parvulum*
Fusobacterium HOT 205	***Bacteroides heparinolyticus***
Leptotrichia HOT 223	*Bergeyella HOT 907*
Mycoplasma hominis	*Cardiobacterium hominis*
Peptoniphilaceae [G1] HOT 113	*Corynebacterium durum*
Peptostreptococcus anaerobius	*Eggerthella lenta*
Prevotella baroniae	***Enterococcus faecalis***
Prevotella bivia	*Haemophilus parainfluenzae*
Prevotella maculosa	*Haemophilus pittmaniae*
Prevotella melaninogenica	*Lachnospiraceae [G2] HOT 096*
Prevotella nigrescens	*Lachnospiraceae [G7] HOT 163*
Propionibacterium acnes	*Lactobacillus jensenii*
Pseudoramibacter alactolyticus	*Leptotrichia HOT 417*
Sneathia sanguinegens	*Leptotrichia HOT 463*
Stenotrophomonas maltophilia	*Neisseria flava*
Streptococcus agalactiae	*Neisseria flavescens*
Streptococcus sobrinus	*Neisseria weaveri*
Veillonella HOT 780	*Pedobacter HOT 321*
	Peptoniphilaceae [G3] HOT 929
	Peptostreptococcaceae [XI][G5], [XI][G4], [XI][G7]
	Porphyromonas HOT 275
	Prevotella loescheii
	Prevotella scopos
	Pseudomonas HOT 032
	Pseudomonas otitidis
	Pseudoramibacter alactolyticus
	Rothia aeria
	Selenomonas HOT 478
	Stomatobaculum HOT 373
	Treponema HOT 373

Table 8: Correlations between Baseline Clinical Parameters and Umbilicus Microbiome

Oral Species	Extent AL >= 3mm (nal_3/npdsites)		Extent PD >= 4mm (npd_4/npdsites)		Extent BOP (nbl/nblsites)		Average Gingival Index		Average Plaque Score	
	Coefficient	p value	Coefficient	p value	Coefficient	p value	Coefficient	p value	Coefficient	p value
Actinomyces_sp_HOT_175	0.362	0.016								
Atopobium_parvulum			0.307	0.042						
Bacteroidales[G-2]_sp_HOT_274							-0.313	0.038		
Bifidobacterium_longum							0.314	0.038		
Gemella_morbillorum			0.310	0.041						
Gemella_morbillorum										
Gemella_morbillorum	0.306	0.043								
Lactococcus_lactis									0.456	0.002
Mitsuokella_multacida							0.431	0.004		
Peptoniphilus_lacrimalis	0.484	0.001	0.331	0.028			0.419	0.005	0.410	0.006
Porphyromonas_gingivalis	0.482	0.001					0.365	0.015	0.408	0.006
Prevotella_loescheii	0.519	0.000	0.320	0.034			0.383	0.010	0.420	0.005
Prevotella_nigrescens					-0.377	0.012				
Prevotella_oralis							-0.370	0.014		
Ruminococcaceae[G-3]_sp_HOT_381							0.431	0.004		
Selenomonas_noxia									0.459	0.002
Stenotrophomonas_maltophilia							0.408	0.006		
Streptococcus_sobrinus									-0.327	0.030
TM7[G-1]_sp_HOT_347	0.439	0.003	0.388	0.009			0.398	0.008	0.378	0.011

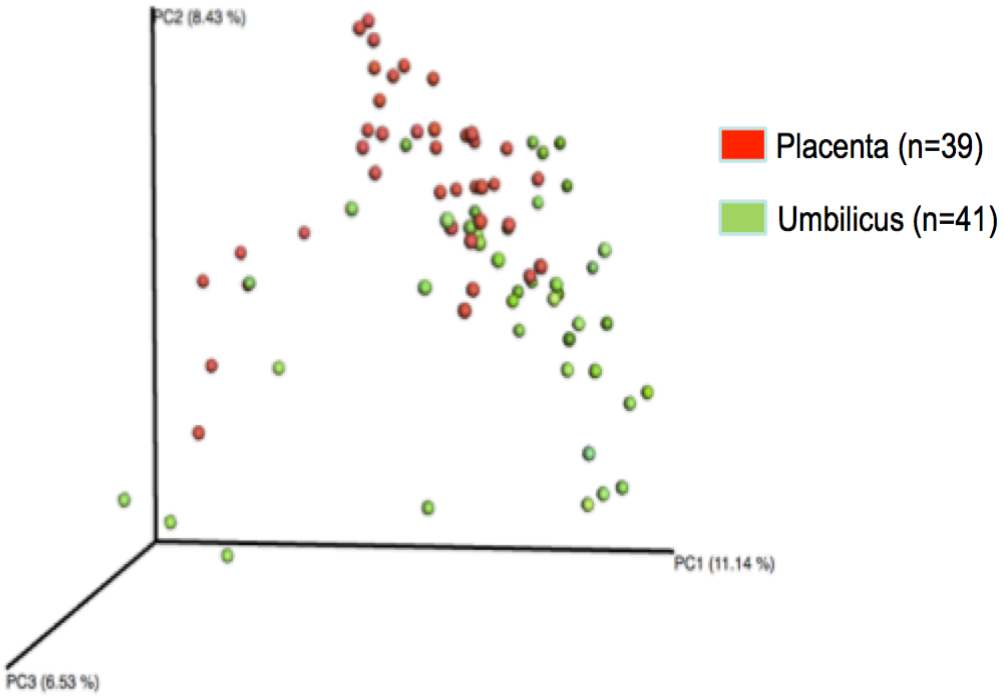

Figure 1: Principal Component Analyses of placenta and umbilicus samples. (Placenta: red, Umbilicus: green).

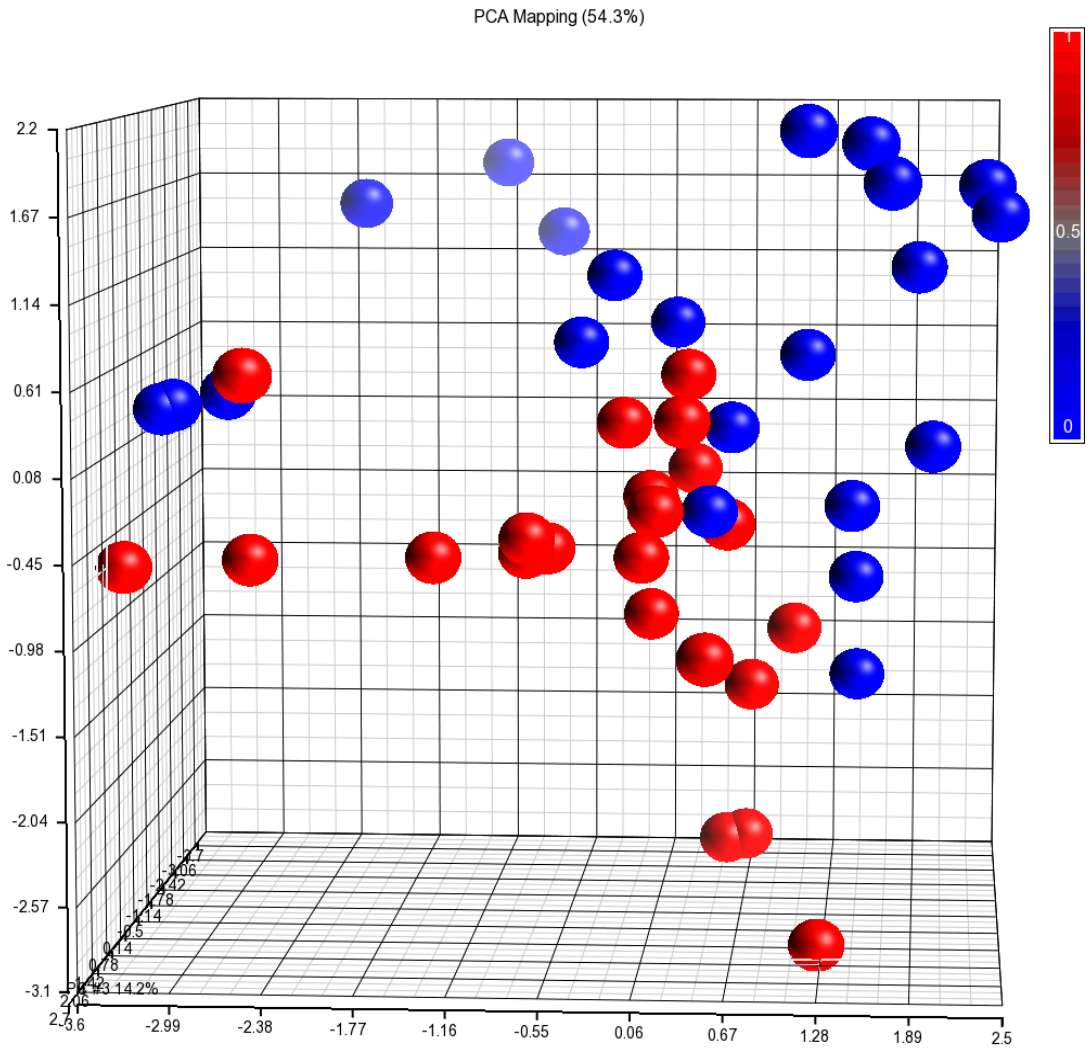

Figure 2: Principal Component Analyses of Umbilicus PTB and FTB samples. (FTB: blue, PTB: red).

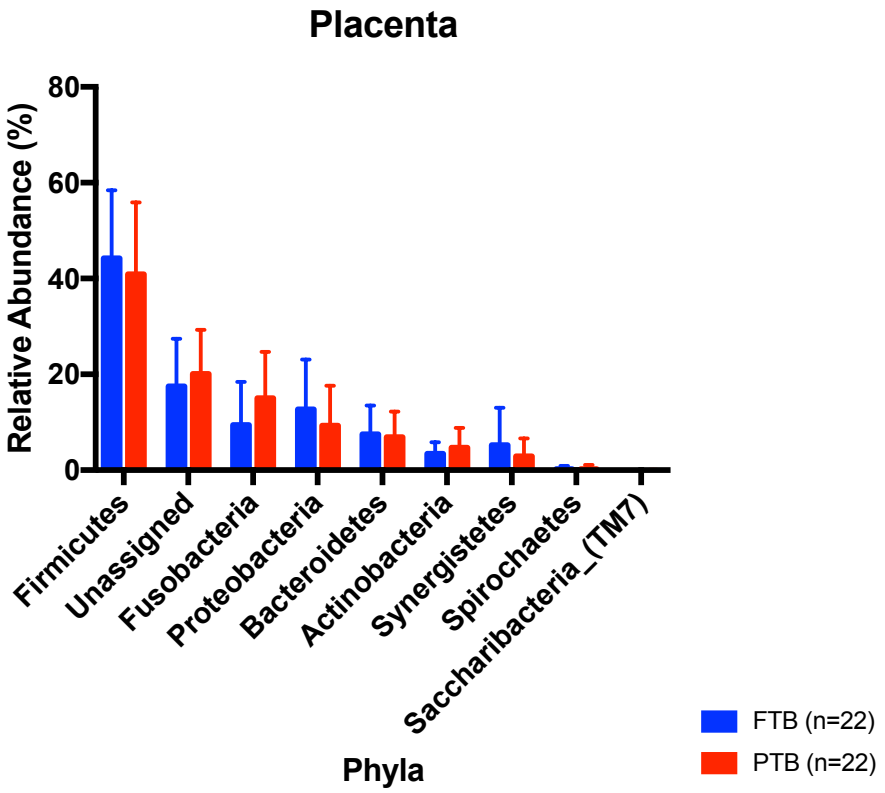

Figure 3: Mean relative abundance (% ± SD) of the most prevalent phyla detected in placenta samples (FTB: blue, PTB: red).

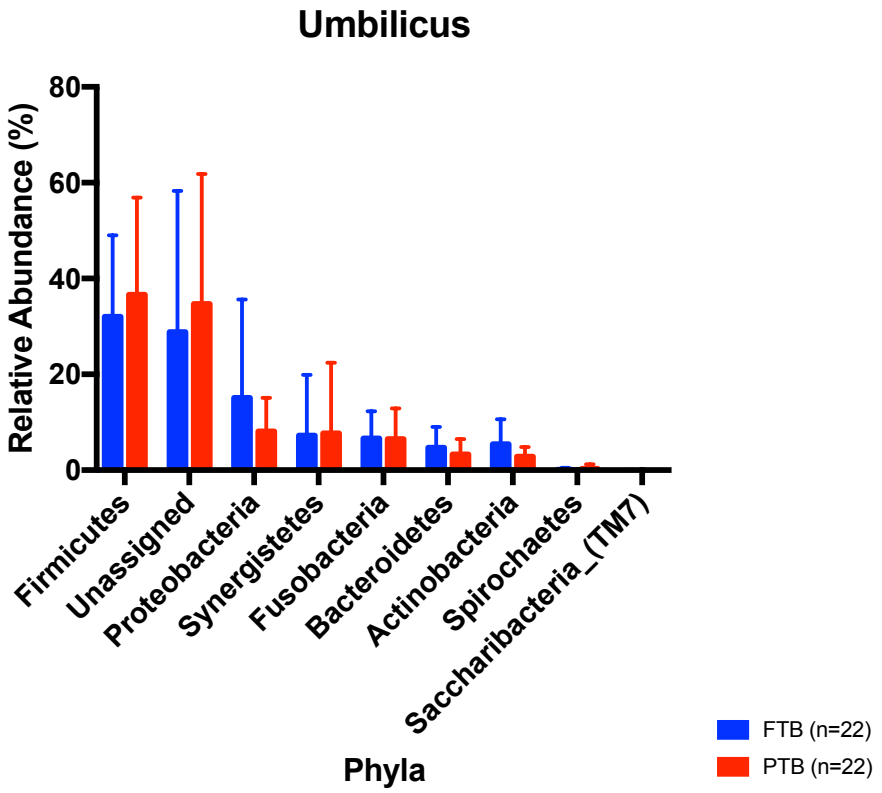

Figure 4: Mean relative abundance (% ± SD) of the most prevalent phyla detected in umbilicus samples (FTB: blue, PTB: red).

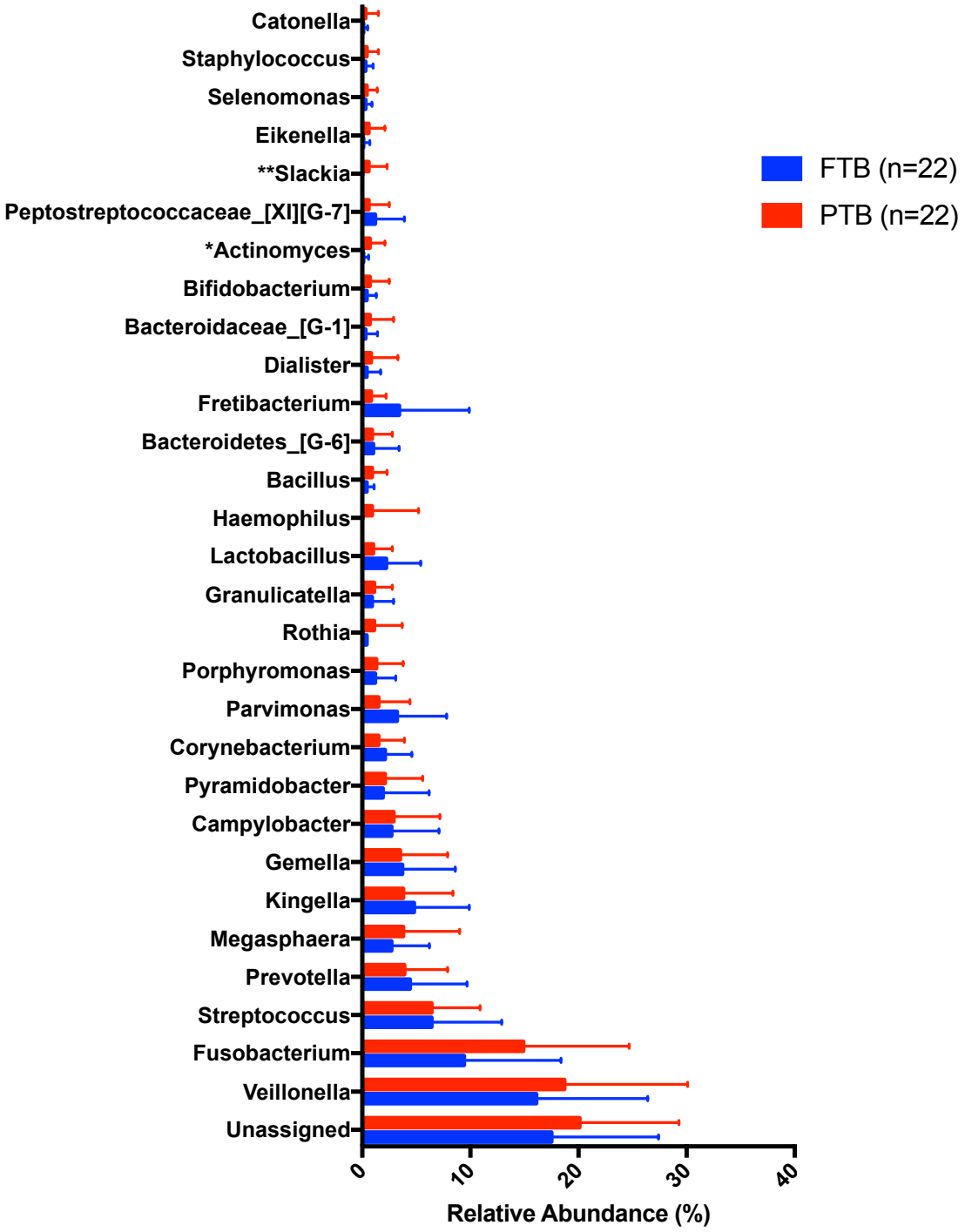

Figure 5: Mean relative abundance (% ± SD) of the most prevalent genera detected in placenta samples (FTB: blue, PTB: red). ^p=0.01, ^^p=0.02, *p=0.03, **p=0.04 (Differences did not reach FDR-adjusted statistical significance).

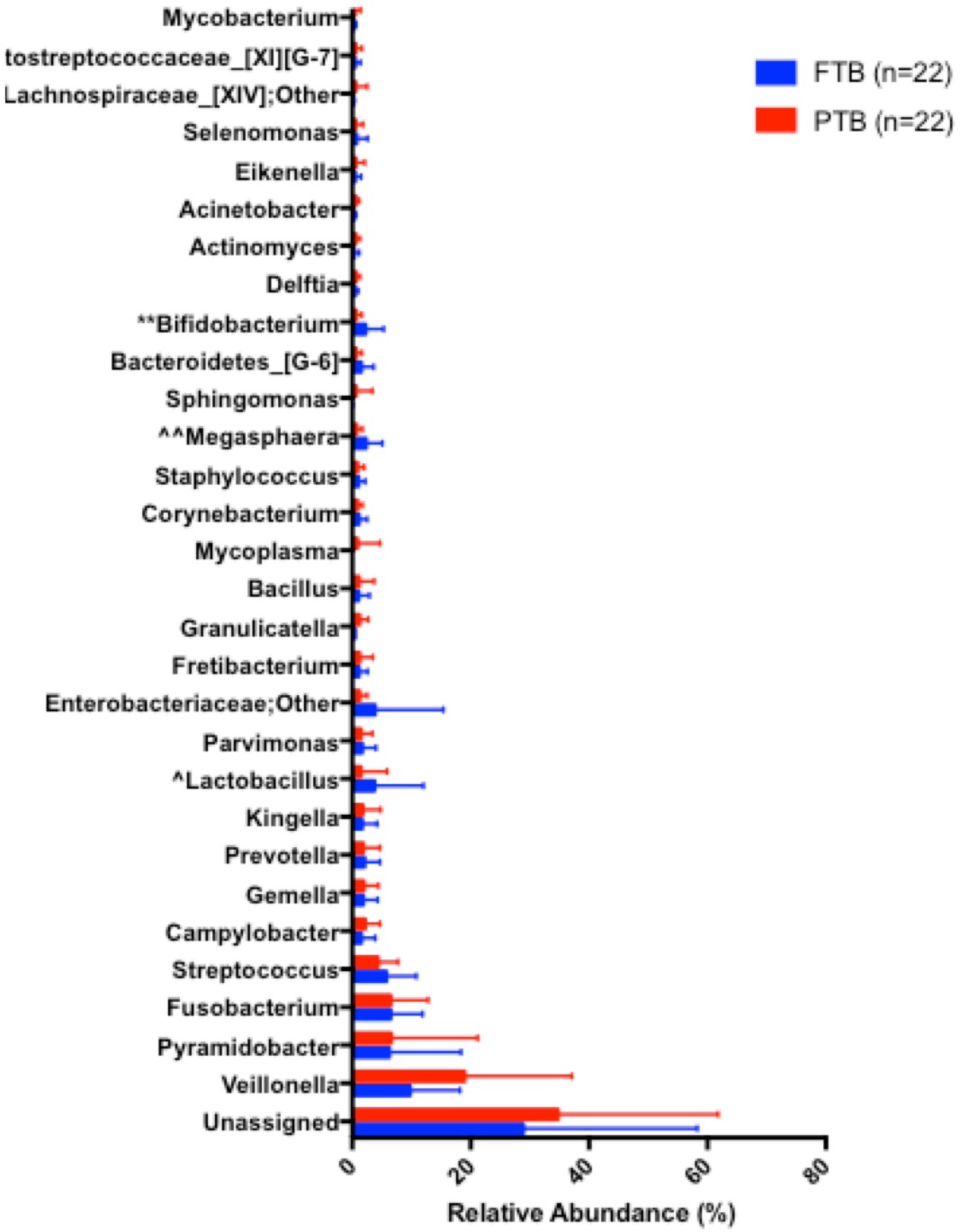

Figure 6: Mean relative abundance (% ± SD) of the most prevalent genera detected in umbilicus samples (FTB: blue, PTB: red). ^p=0.01, ^^p=0.02, *p=0.03, **p=0.04 (Differences did not reach FDR-adjusted statistical significance).

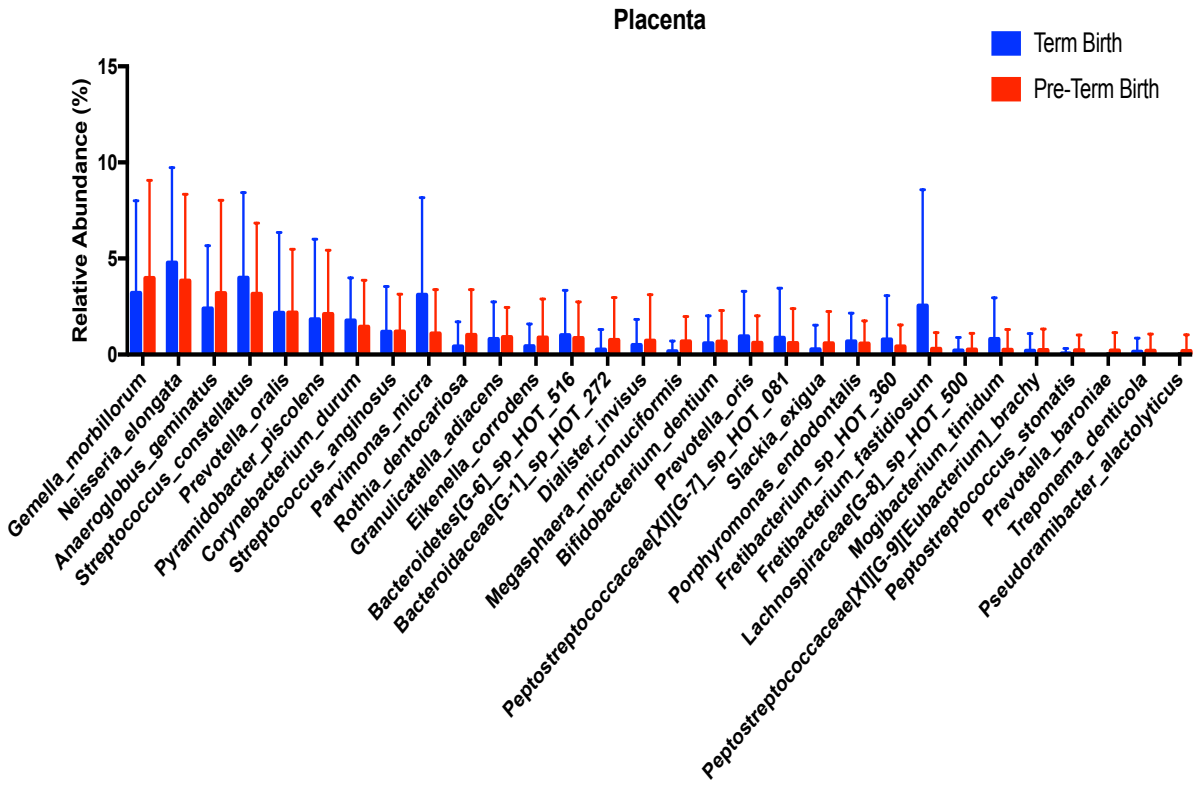

Figure 7: Mean relative abundance (% ± SD) of the most prevalent species detected in placenta samples (FTB: blue, PTB: red). ^p=0.01, ^^p=0.02, *p=0.03, **p=0.04 (Differences did not reach FDR-adjusted statistical significance).

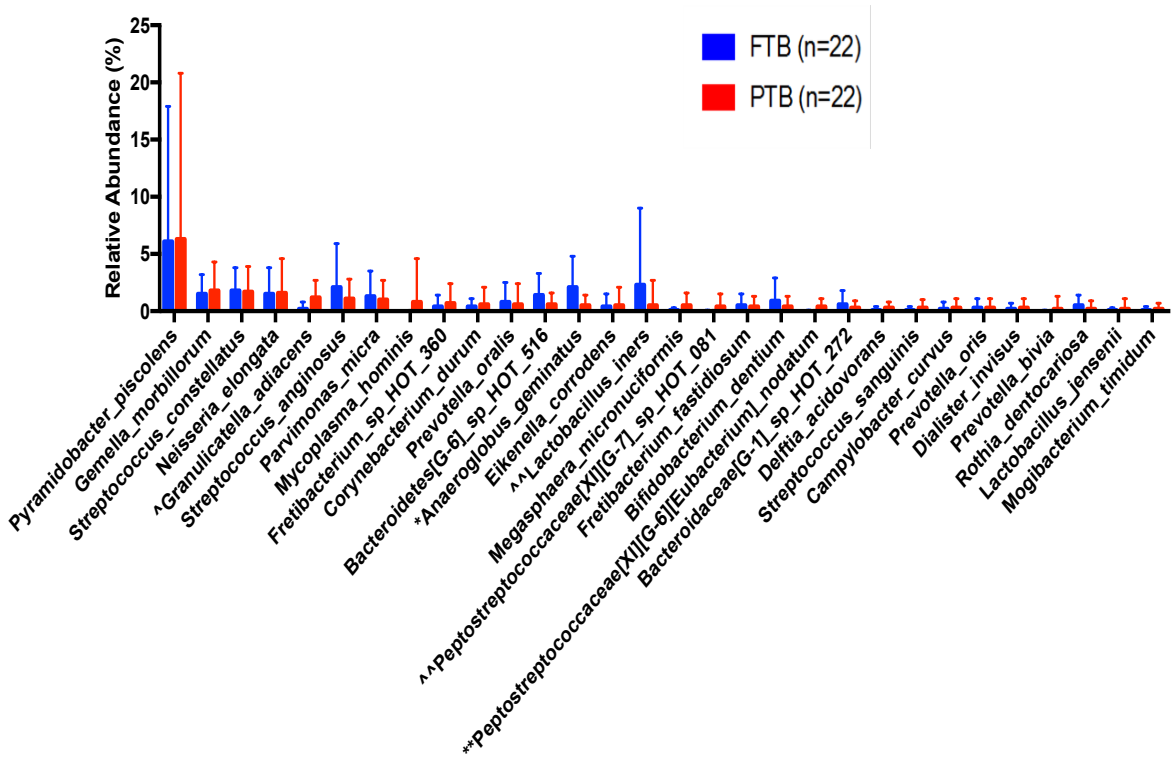

Figure 8: Mean relative abundance (% ± SD) of the most prevalent species detected in umbilicus samples (FTB: blue, PTB: red). ^p=0.01, ^^p=0.02, *p=0.03, **p=0.04 (Differences did not reach FDR-adjusted statistical significance).

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CHAPTER 3: PERIODONTAL DISEASES & PRE-TERM BIRTH: INSIGHTS FROM THE PLACENTAL, UMBILICAL AND ORAL MICROBIOMES

INTRODUCTION

Since the early 1990's periodontal disease has been suggested to have a role in the pathogenesis of preterm births (< 37 weeks gestational age). Each year, preterm birth affects almost 500,000 infants born in the U.S. In an average week, over 8000 infants are born prematurely at less than 37 weeks' gestation. Blencowe and colleagues ranked the U.S. as the sixth country with the greatest number of preterm births in the year 2010.[1,] The effect of preterm birth among some survivors may continue throughout life, impairing neuro-developmental functioning through increasing the risk of cerebral palsy, learning impairment and visual disorders and affecting long-term physical health with a higher risk of non-communicable disease. [33]

The pathogenesis of preterm birth while complex, it appears to be driven by microbial colonization and invasion.[12] Oral microorganisms have been postulated to contribute to preterm birth. [21,23,25, 27,28,40] In fact, periodontitis, a common oral disease of bacterial etiology and potent inflammatory component is strongly associated with pre-term birth. Furthermore, levels of periodontal pathogens, such as *Porphyromonas gingivalis* and *Tannerella forsythia* have been detected in significantly higher levels in subgingival biofilm samples of mothers who delivered preterm babies.[19] In addition, those pathogens have also been detected more commonly in the amniotic fluid and subgingival plaque samples of patients who gave birth to preterm neonates.[8]

Human studies have shown evidence of oral microorganisms translocation to the intrauterine cavity. Periodontal pathogens such as *P. gingivalis, Campylobacter rectus, T. forsythia*, and *Fusobacterium nucleatum* have been detected in the amniotic fluid of mother's with subgingival plaque samples positive for such pathogens.[8,18] *F. nucleatum* has been detected in chorionic tissues of high-risk pregnant women, but not in tissues of normal pregnant women. Authors found that *F. nucleatum* is capable of inducing elevated production of IL-6 and CRH production in chorion-derived cells. [39] In a case report of a term stillbirth, *F. nucleatum* was shown to translocate hematogenously to the placenta and fetus, in a mother whom subgingival plaque was positive for this microorganism.[13] Early association studies demonstrated that periodontal disease is a statistically significant risk factor for preterm low birth weight (PLBW) with odd ratios of 7.9 for all PLBW cases.[28] A meta-analysis by Vergnes, concluded that periodontal disease may be an independent risk factor for preterm low birth weight, with odds ratio of 2.83.[40]

Fetal IgM response to *Campylobacter rectus, Peptostreptococcus micros, Prevotella nigrescens, Prevotella intermedia,* or *Fusobacterium nucleatum* has been associated with an elevated risk of PTB (<35 weeks).[2] Reduced maternal IgG antibody response to periodontal pathogens has also been shown to be associated with increased risk of PTB.[19] In a study by Madianos and colleagues [21], found a 2.9-fold higher prevalence of IgM seropositivity for one or more organisms of the Orange or Red complex among preterm babies, as compared to term babies. The prevalence of positive fetal IgM to *C. rectus* was significantly higher for preterm as compared to full-term infants. A reduced maternal IgG antibody to periodontal pathogens of the Red complex was associated with an increased rate of PTB with odds ratio (OR) = 2.2. The findings of this study provide support to the theory that the lack of a protective maternal antibody

response allows periodontal pathogens to travel in the blood and invade the intrauterine cavity, ultimately leading to APOs.[21]

Interventions studies to date, examining the effect of periodontal therapy during pregnancy on APOs present conflicting results, while some studies show a positive effect of periodontal treatment on prevention of APOs, others show no benefit.[22,24,30,32,36] However, a gap in knowledge remains regarding whether oral pathogens can consistently reach and affect fetal tissues, such as the placenta and the umbilicus and whether this exposure is determined by the level of periodontitis. The aim of the present study was to compare the microbiomes of placenta and umbilicus samples from 44 women presenting periodontal diseases who experienced full term (FTB, n=22) and preterm births (PTB; <35 weeks, n=22) in relation to the levels of maternal subgingival periodontal species in each group pre and post-partum

MATERIALS AND METHODS

Study Population

Samples used in this study were collected from pregnant women previously enrolled in the Maternal Oral Therapy to Reduce Obstetric Risk (MOTOR U01DE014577) Study[24]; as part of the study demographic, medical and dental (periodontal) data were collected as well as maternal subgingival biofilm samples and fetal tissues (umbilicus and placenta). All participants of the MOTOR Study signed a consent that allowed the use of all the available biological samples collected and stored, for the purposes of the present investigation. Inclusion criteria: pregnant and able to complete periodontal treatment prior to 23 weeks gestation, at least 16 years old at enrollment, minimum of 20 teeth present, three (3) or more periodontal sites with ≥ 3mm clinical attachment loss. Exclusion criteria: multiple gestation, systemic medical conditions, rampant decay, symptomatic teeth or any other dental finding such as periodontal abscess or

endodontic fistula that would preclude enrollment, chronic use of medications that cause gingival enlargement such as phenytoin, cyclosporin-A, or calcium channel antagonists, chronic use of steroids, or concomitant use of orthodontic appliances (braces).

For the present study, a case-control design with a ratio of 1:1 was employed. Case group consisted of 22 mothers who had preterm delivery (< 35 weeks gestational age), while the control group consisted of 22 mothers who had normal, term deliveries and uneventful pregnancies. Control cases were selected using a random sampling method. In the case group, a non-random sampling method was used due to the limited numbers of subjects in the MOTOR database. In this report we present the following data from each subject: maternal pre-partum and post-partum plaque samples, as well as, placental and umbilical tissue samples collected at delivery

Sample Collection and Processing

Plaque samples were collected during the periodontal exam visit. Plaque were sampled from the deepest probed site in each quadrant. The site was isolated with a cotton roll and gross supragingival plaque or debris was removed. Samples were taken by placing a sterile curette at the base of each sulcus. Samples were stored separately at -20°C in a labeled vial containing TE and sodium hydroxide, added after collection. Since samples were stored in sodium hydroxide DNA extraction could not be performed, therefore, all plaques samples were processed using the checkerboard DNA-DNA hybridization method described by Socransky and colleagues. [37,38] DNA probes from 41 bacterial species were used.

Placental and umbilical cord tissue samples were obtained within the first hour of delivery. Samples were collected using sterile tissue tweezers or hemostat and scissors. Each sample was stored into a cryovial numbered for that tissue specimen. The cryovials were snap

frozen in liquid nitrogen. Samples were stored temporarily on dry ice, transported to the laboratory at the University of North Carolina at Chapel Hill and subsequently stored at -80°C. The first step in preparation for the microbial analysis of the umbilical and placental tissues was tissue homogenization, using a sterile mortar and pestle and liquid nitrogen until a powder consistency was achieved. Immediately proceeded with DNA extraction using the MasterPure DNA Purification kit (Epicentre, Illumina, Madison, WI) according to the manufacturer's recommendations. DNA quantification was performed using Quant-iTTM PicoGreen ® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA). Sample fluorescence was measured using a fluorescence microplate reader (excitation ~480 nm, emission ~520 nm).

Library preparation and 16S rRNA sequencing

The protocol used has been described elsewhere (Furquim et al 2016)[11]. In brief, samples prepared for sequencing had a minimum DNA concentration of 10 ng/uL in at least 10 uL volume. A modified protocol was used for 16S rRNA amplicon sequencing library preparation.[5] The variable region V3-V4 of the 16S rRNA gene were PCR amplified using barcoded 341F and 806R 16S primers.[4] The amplicon size was approximately 460 bp. PCR products were purified (AMPure beads Beckman Coulter, Brea, CA) and quantified (Quant-iT™ PicoGreen ® dsDNA Reagent, Molecular Probes, Waltham, MA). Equimolar amounts of each library were pooled, gel-purified, analyzed and quantified (2100 Bioanalyzer, DNA High Sensitivity chip;Agilent Technologies, Santa Clara, CA). The library mixture (6 picomolars spiked with 30% PhiX) were run on a MiSeq instrument (Illumina, San Diego, CA). Automated cluster generation and paired–end sequencing using 500 cycle reagent kit were performed according to the manufacturer's instructions. The entire protocol is described in supplemental material.

Clinical Measurements

During the MOTOR Study subjects received a comprehensive periodontal examination performed by calibrated dental examiners at baseline and delivery. The clinical examination protocol of the study has been described elsewhere (Offenbacher et al 2009).[24] For all subjects, there are baseline and post-partum data for the following clinical parameters: plaque score gingival index, probing pocket depth (PD), bleeding on probing (BOP), and clinical attachment level (CAL).

Data Analysis

After removal of chimeric sequences and sequences that failed quality control, sequencing reads were evaluated using the QIIME analysis pipeline (Quantitative Insights Into Microbial Ecology, qiime.org, version 1.8) [3](Caporaso et al. 2010). Reads were grouped into Operational Taxonomic Units (OTUs) using UCLUST [7](Edgar 2010) and the bacterial taxonomy was determined using HOMD and Greengenes. After taxonomic assignment, OTUs were combined with sample metadata. Species-level taxonomy was determined using HOMINGS. The relative abundance of the taxa present in each sample was computed at the phylum and genus levels. The demographic and clinical measurements of the study population were analyzed using Student's t-test and Chi Square test. Microbial differences between samples from PTB and FTB were sought using Kruskall-Wallis and Principal Component Analyses (PCA). Correlations between microbial taxa and periodontal parameters were sought using the Spearman correlation coefficient. False discovery rate (FDR) was used to adjust for multiple comparisons. All statistical analysis was performed in SAS version 9.4.

RESULTS

Socio-Demographic and Clinical Characteristics of the Study Population

A total of forty-four pregnant women were included in this study, with twenty-two mothers in the term group and twenty-two mothers in preterm group. Preterm and term groups at baseline did not differ in maternal age, marital status, maternal BMI, public assistance, previous pregnancy, or smoking history. However, significant differences were observed regarding maternal race, with 62% of Caucasian mothers in the term group and 73% of African American mothers in the preterm group (Table 1). With regard to ante-partum clinical measures, both groups had similar mean gingival index (GI), plaque index (PI), bleeding on probing (BOP), extent of sites with probing depth (PD) at least 4mm, and attachment level (AL) at least 3mm. In terms of postpartum clinical measures, no differences were observed between groups (Table 1).

Maternal Subgingival Plaque Samples Microbial Profile

Pre-partum subgingival plaque samples show high abundance of oral commensal species, such as *Actinomyces gerencseriae* (FTB: 8.3% ± 6.4%, PTB: 6.2% ± 5.4%, p= 0.24), *Actinomyces israelli* (FTB: 6.5% ± 3.9%, PTB: 5.8% ± 5.3%, p= 0.30), *Actinomyces oris* (FTB: 6.2% ± 5.6%, PTB: 6.1% ± 4.8%, p= 0.90), and *Actinomyces naeslundii* (FTB: 6.1% ± 5.3%, PTB: 4.6% ± 3.3%, p=0.39). Statistically significant differences between FTB and PTB samples were observed for *Aggregatibacter actinomycetemcomitans* (FTB: 1.0% ± 0.7%, PTB: 1.6 ± 0.9%, p= 0.02), *Campylobacter gracilis* (FTB: 1.8% ± 1.1%, PTB: 2.4 ± 1.0, p= 0.01), *Fusobacterium nucleatum ss vicentii* (FTB: 2.3% ± 1.5%, PTB: 3.4% ± 1.8%, p= 0.05), *Veillonella parvula* (FTB: 1.9% ± 3.4%, PTB: 3.4% ± 3.3%, p= 0.00), and *Treponema socranskii* (FTB: 2.3% ± 1.5 %, PTB: 3.7% ± 1.8%, p= 0.01). Periodontal pathogens of the red complex, (*P. gingivalis, T. forsythia, and T. denticola*) were also detected (Fig. 1).

When comparing pre-partum and post-partum changes in mean percentages of DNA count of subgingival plaque samples, increased in commensal organisms such as *A. oris, A. naeslundii, A. israelii*, and *A. gerencseriae* were observed in samples from PTB. However, increases in periodontal pathogens *T. denticola, P. gingivalis, and T. forsythia* were observed in subgingival plaque samples from PTB (Fig. 2).

Correlation between Ante-partum Subgingival Plaque Profile and Clinical measurements

Positive correlations were observed between presence of *P. gingivalis* in ante-partum subgingival plaque samples and the following clinical measurements: extent of AL ≥ 3mm (CC: 0.366, p=0.05), BOP (CC: 0.375, p= 0.04), and GI (CC:0.68, p= 0.00). *F. nucleatum ss nucleatum* showed positive correlations for GI (CC: 0.368, p= 0.05) only. Commensal oral microorganisms such as *Streptococcus oralis* showed negative correlations for extent of AL ≥ 3mm (CC: -0.414, p= 0.02) and GI (CC: -0.359, p= 0.05). While, *V. parvula* (CC: -0.376, p= 0.04) and *Streptococcus sanguinis* (CC: -0.359, p= 0.05) showed negative correlations for GI (Table 3).

Microbial Profile of FTB/PTB placenta samples

Sequencing of placenta samples revealed 8 different phyla, 84 genera, and 132 species. While, umbilicus samples revealed 8 different phyla, 100 genera, and 225 species. Most abundant species in placenta samples were: *Neisseria elongata* (FTB: 4.78% ± 4.95%, PTG: 3.84% ± 4.51%, p= 0.53), *Gemella morbillorum* (FTB: 3.21% ± 4.80%, PTB: 3.98% ± 5.09%, p= 0.45), *Streptococcus constellatus* (FTB: 3.99% ± 4.44%, PTB: 3.16% ± 3.68%, p= 0.52), and *Anaeroglobus geminatus* (FTB: 2.39% ± 3.28, PTB: 3.19% ± 4.84%, p: 0.66) (Fig. 3). Several periodontal microorganisms were detected in placental samples: *Fretibacterium fastidiosum, , Campylobacter gracilis, Parvimonas micra, Capnocytophaga gingivalis, Prevotella nigrescens, Eikenella corrodens,* and *Treponema denticola,* However, no statistically significant differences

were observed between groups. Key periodontal pathogens such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*, and *Tanerella forsythia* were not detected.

Microbial Profile of FTB/PTB umbilicus samples

The most abundant species in umbilicus samples were: *Pyramidobacter_piscolens* (FTB: 6.1% ± 11.8%, PTB: 6.3% ± 14.5%, p= 0.64), *Gemella morbillorum* (FTB: 1.5% ± 1.7%, PTB: 1.8% ± 2.5%, p= 0.98), *Streptococcus constellatus* (FTB: 1.8% ± 2.0%, PTB: 1.7% ± 2.2%, p=0.79), and *Neisseria elongata* (FTB: 1.5% ± 2.3%, PTB: 1.6 % ± 3.0%, p= 0.93) (Fig 4). The following species were found to be statistically significant different between groups: *Granulicatella adiacens* (FTB: 0.2% ± 0.6%, PTB: 1.2% ± 1.5%, p= 0.01), *Anaeroglobus geminatus* (FTB: 2.1% ± 2.7%, PTB: 0.5% ± 0.9%, p= 0.03), *Lactobacillus iners* (FTB: 2.3% ± 6.7%, PTB: 0.5% ± 2.2%, p= 0.02), *Peptostreptococcaceae* [XI] [G-7] HOT 081 (FTB: 0.0 ± 0.1, PTB: 0.4 ± 1.1; p: 0.02), and *Eubacterium nodatum* (FTB: 0.0 ± 0.1, PTB: 0.4 ± 0.7; p= 0.04). Several periodontal microorganisms were detected in umbilical samples: *Campylobacter gracilis, Fretibacterium fastidiosum, Fusobacterium* HOT 204*, Parvimonas micra, Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, and Treponema denticola.* Key periodontal pathogens such as *A. actinomycetemcomitans* and *T. forsythia* were not detected.

Correlation between Placenta Samples Microbial Profile and Clinical Measurements

Positive correlations were observed between presence of C. tuscaniense in placenta samples and extent of AL ≥3mm (CC: 0.495, p = 0.001), extent of PD ≥4mm (CC: 0.302 , p= 0.046), GI (CC: 0.405, p=0.006), and PI (CC: 0.422, p=0.0064. *Leptotrichia* HOT 223 showed positive correlations for extent of AL ≥3mm (CC: 0.495, p = 0.001), extent of PD ≥4mm (CC: 0.302 , p= 0.046), GI (CC: 0.405, p=0.006), and PI (CC: 0.422, p=0.0064. While, *Fusobacterium*

HOT 204 showed positive correlations for extent of AL ≥ 3mm (CC: 0.389, p = 0.009), GI (CC: 0.344, p=0.022), and PI (CC: 0.386, p=0.010).

Correlation between Umbilicus Samples Microbial Profile and Clinical Measurements

Positive correlations were found between presence of *P.gingivalis* in umbilical tissues and extent of AL ≥ 3mm (CC: 0.482, p = 0.001), GI (CC: 0.365, p=0.015), and PI (CC: 0.408, p=0.006). While, presence of TM7 [G1] HOT 347 in umbilical tissues showed positive correlations with extent of AL ≥ 3mm (CC: 0.439, p=0.003), extent of PD ≥ 4mm (CC: 0.388, p=0.009), GI (CC: 0.398, p=0.008), and GI (CC:0.378, p= 0.011). Presence of *P. loescheii* in umbilical tissues showed a positive correlation with GI (CC: 0.383, p=0.01) and PI (CC: 0.42, p= 0.005) (Table 3).

Correlation between Ante-partum Subgingival Plaque and Microbiome of Placental and Umbilical Tissues

Presence of the following periodontal pathogens in subgingival plaque showed positive correlations to periodontal microorganisms found in placenta samples. *P.gingivalis* showed a positive correlation to presence of *C. tuscaniense* (CC 0.648, p < 0001), *Fusobacterium HOT 204* (CC 0.581, p <0.001), and *Leptotrichia HOT 223* (CC 0.648, p .0001). *F. nucleatum ss vicentii* with *Mycoplasma hominis* (CC 0.672, p<0.0001), *Peptostreptococcus stomatis* (CC 0.691, p<0.0001), and *Pseudoramibacter alactolyticus* (CC 0.679, p<0.0001). *T. socranskii* with *Dialister invisus* (CC 0.594, p<0.0001). *T. denticola* with *C. gracilis* (CC 0.609, p <0.0001) and *Rothia aeria* (CC 0.590, p<0.0001). *P. nigrescens* with *Bifidobacterium dentium* (CC 0.737, p <0.0001), and *Campylobacter curvus* (CC 0.714, p <0.0001).

Presence of the following periodontal pathogens in subgingival plaque showed positive correlations to periodontal microorganisms found in umbilicus samples. *P.gingivalis* in subgingival plaque samples showed a positive correlation to presence of *Campylobacter*

tuscaniense (Correlation Coefficient [CC], p value) (0.62505, p <.0001), *Peptoniphilus lacrimalis* (CC 0.682, p <.0001), & *Prevotella loeschii* (CC 0.062733, p <.0001). *F. nucleatum ss vicentii* showed a positive correlation to presence of *Abiotrophia defectiva* (CC 0.679, p <0), *Actinomyces johnsonii* (CC 0.642, p <.0001), *Capnocytophaga leadbetteri* (CC 0.654, p <.0001), *Kingella oralis* (CC 0.679, p <.0001), *Leptotrichia HOT 392* (CC 0.679, p<.0001), and *Mycoplasma hominis* (CC 0.679, p<.0001) . *Parvimonas micra* with *S. Sanguinis* (0.603, p<.0001). *T. denticola* with *Lactococcus lactis* (CC 0.602, p <.0001) and *Selenomonas noxia* (CC 0.613, p <.0001). *F. nucleatum ss nucleatum* with *Corynebacterium Matruchotii* (CC 0.575, p <.0001) and *Peptoniphilus lacrimalis* (CC 0.590, p<.0001). *P. intermedia* with *Bifidobacterium breve* (CC 0.627, p<.0001), *Leptotrichia HOT 221* (CC 0.742, p <.0001), and *Peptostreptococcaceae [XI] [G7] [Eubacterium] _yurii_subsp_yurii_&_margaretiae* (CC 0.717, p <.0001) (Fig. 5).

DISCUSSION

The pathogenesis of PTB seems to be complex and multifactorial, however, infection of the intrauterine cavity seems to be one of the leading causes.[12,31,18] The effect of preterm birth amongs some survivors may continue throughout life, impairing neuro-developmental functioning through increasing the risk of cerebral palsy, learning impairment and visual disorders and affecting long-term physical health with a higher risk of non-communicable disease.[33]

Several studies have demonstrated an association between periodontal disease and PTB, however, the actual mechanisms have yet to be determined.[21,23,25,27,29,40] In a case-control study by Offenbacher and colleagues periodontal disease was shown to be a statistically significant risk factor for preterm low birth weight (PLBW) with odd ratios of 7.9 for all PLBW cases.[28] A 5-

year prospective study in pregnant women, found periodontal disease incidence and progression to be significantly associated with low weight for gestational age, as well as, increased relative risk for PTB and very PTB. Severity of maternal periodontal disease was also shown to be a predictor of more severe APOs.[26,29] A meta-analysis by Vergnes and colleagues, concluded that periodontal disease may be an independent risk factor for preterm low birth weight, with odds ratio of 2.83.[40] Higher prevalence of IgM seropositivity for one or more organisms of the Orange or Red complex was shown among preterm babies Reduced maternal IgG antibody to periodontal pathogens of the Red complex has been associated with an increased rate of PTB with odds ratio (OR) = 2.2.[21]

Oral microorganisms have been shown to have the ability to translocate into the intrauterine cavity via hematogenous dissemination. In a pregnant murine model, intravenous injection of *F. nucleatum* resulted in premature delivery, nonsustained live births, and stillbirths.[15] *F. nucleatum* has been shown to have the ability to adhere and invade epithelial and endothelial cells via novel FadA adhesion, in mixed infections, *F. nucleatum* could facilitate the hematogenous spread of other microorganisms.[10] *Bergeyella* has been detected cases of PTB with intrauterine infection, were the same strain of *Bergeyella* was detected in the subgingival plaque of the mother but not in the vaginal tract.[14]

We were able to demonstrate the presence of multiple oral microorganisms in both placental and umbilical tissues. Our samples showed presence of several genera found in the oral cavity, which have been shown to have the ability to translocate to the placenta in a murine model, such as, *Veillonella, Streptococcus, Porphyromonas, Parvimonas, Prevotella, Neisseria, Leptotrichia*, and *Bacteroidetes*.[9] Furthermore, we were able to detect genera of microorganisms

associated with PTB: *Leptotrichia, Neisseria, Peptostreptococcus, Streptococcus, Veillonella.*[6,16,35]

Our samples demonstrated presence of oral species previously implicated in extra-oral infections, inflammation, and APOs: *E. corrodens, P. gingivalis, P. nigrescens,* and *T. denticola.*[17] Detected species such as *G. morbillorum, E. corrodens*, and *Porphyromonas endodontalis* have been shown to have the ability to hematogenously disseminate and colonize the placenta in an animal model.[9] In our study, we were able to detect several oral species in both fetal tissues and maternal subgingival plaque, such as, *E. nodatum, E. corrodens, F. fastidiosum, G. morbillorum, P. gingivalis, P. micra, S. constellatus, S. anginosus,* and *T. denticola.* The co-occurrence of these species could explain the origin of these bacteria found in the fetal tissues.

Our subgingival plaque samples from FTB and PTB showed similar levels of periodontal pathogens of the red complex: *P. gingivalis, T. denticola*, and *T. forsythia*. However, PTB subgingival plaque samples harbored higher levels of *A. actinomycetemcomitans, T. socranskii*, and pathogens of the orange complex, such as, *F. nucleatum ss nucleatum* and *C. gracilis.* Interestingly, plaques samples of PTB subjects showed increased levels of *F. nucleatum, P. intermedia, P. gingivalis, T. denticola*, and *T.forsythia* immediately after delivery. Subgingival plaque presence of *P. gingivalis* and *F. nucleatum ss nucleatum* was positively correlated with clinical parameters, while presence of commensal organisms, *S. oralis, S. sanguinis*, and *F. nucleatum ss vicentii* were negatively correlated.

In the umbilicus microbiome *E. nodatum,* a periodontal pathogen of the orange complex showed statistically higher levels in PTB samples, while the vaginal commensal organism L. iners was statistically higher in FTB. The presence of *P. gingivalis* in umbilical tissues was

associated with mothers presenting more severe periodontal disease. The presence of *P. gingivalis* in subgingival plaque samples was not associated with the presence of *P. gingivalis* in umbilical tissues, this negative correlation could be due to the different methods used to analyze each sample type. Furthermore, taxa not detected in our samples does not necessarily indicate those microorganisms are not able to translocate to the fetal tissues, perhaps our methods for sample collection and analyses did not allow for their detection. *C. rectus* is an orange complex periodontal pathogen, which has been previously proposed as a primary fetal infectious agent that could have the ability to elicit PTB.[21] We were not able to detect *C. rectus* in our samples due to its probe not being included as part of the HOMINGS sequencing database. Future directions could include oligotyping and OTU level analysis of the microbiomes to improve taxa identification.

There are limitations in the present study. The first one is its sample size. This was a pilot study, therefore, we were not able to performed power calculations. Also, we were limited by the amount of available samples from subjects that met all the criteria for our study. A second limitation of the present study is its cross sectional design, which precludes any inference on a causal role of the microbiome in PTB. A third limitation, was that the plaque samples available for analysis did not allow for DNA extraction and 16S rRNA sequencing due to being stored in sodium hydroxide. This limited the possibility of detecting a larger number of species, than the 41 species included in checkerboard DNA-DNA hybridization method.

CONCLUSIONS

Mothers who experienced PTB harbored greater levels of pathogenic taxa in their subgingival biofilms at baseline and after delivery. Common oral bacteria were detected in placental and umbilical tissues, as well as, maternal subgingival plaque, suggesting the source of

these bacteria may be the oral cavity. The presence of *P. gingivalis* in the umbilical microbiome was positively correlated with periodontal parameters.

Table 1: Demographics and Periodontal Parameters of Study Population

	Term	*Preterm*	*p-value*
Maternal Race			
African American	4 (27%)	11 (73%)	
Caucasian	18 (62%)	11 (38%)	0.03
Mean (SD)			
Maternal Age	25.2 (3.6)	25.7 (6.7)	0.74
Mean (SD) Pre Pregnancy			
BMI	26.5 (4.5)	27.9 (5.3)	0.36
Married	11 (55%)	9 (45%)	
Not Married	11 (46%)	13 (54%)	0.54
WIC	18 (51%)	17 (49%)	
No	4 (44%)	5 (56%)	0.71
Nulliparous	4 (36%)	7 (64%)	
No	18 (55%)	15 (46%)	0.3
Smoking During Pregnancy	3 (60%)	2 (40%)	
No	19 (49%)	20 (51%)	0.63
Ante Partum Clinical Measures			
Mean (SD)			
Extent AL >=3mm	18.5 (9.6)	19.0 (12.3)	0.9
Mean (SD)			
Extent PD >=4mm	26.3 (14.7)	24.3 (19.1)	0.69
Mean(SD) BOP	47.7 (29.4)	44.0 (31.7)	0.66
Mean (SD) GI	1.3 (0.4)	1.2 (0.5)	0.26
Mean (SD) PI	2.9 (0.5)	2.8 (0.6)	0.56
Post Partum Clinical Measures			
Mean (SD)			
Extent AL >=3mm	12.9 (14.4)	10.7 (13.0)	0.61
Mean (SD)			
Extent PD >=4mm	27.6 (19.1)	22.1 (19.8)	0.37
Mean (SD) BOP	42.7 (28.1)	26.1 (25.4)	0.06
Mean (SD) GI	1.1 (0.4)	0.9 (0.4)	0.13
Mean (SD) PI	3.0 (0.5)	2.8 (0.6)	0.36

Table 2: Correlations between Baseline Clinical Parameters and Oral Microbiome

Oral Species	Extent AL >= 3mm (nal_3/npdsites)		Extent PD >= 4mm (npd_4/npdsites)		Extent BOP (nbl/nblsites)		Average Gingival Index	
	Coefficient	p value	Coefficient	p value	Coefficient	p value	Coefficient	p value
P. gingivalis	0.366	0.05			0.375	0.04	0.638	0
F. nucleatum ss vincentii			-0.425	0.02	-0.426	0.02		
V. parvula					-0.416	0.02	-0.376	0.04
S. sanguinis							-0.404	0.03
S. oralis	-0.414	0.02					-0.359	0.05
F. nucleatum ss nucleatum							0.368	0.05
P. melaninogenica							-0.391	0.03

Table 3: Correlations between Baseline Clinical Parameters and Umbilicus Microbiome

Oral Species	Extent AL >= 3mm (nal_3/npdsites)		Extent PD >= 4mm (npd_4/npdsites)		Extent BOP (nbl/nblsites)		Average Gingival Index		Average Plaque Score	
	Coefficient	p value	Coefficient	p value	Coefficient	p value	Coefficient	p value	Coefficient	p value
Actinomyces_sp_HOT_175	0.362	0.016								
Atopobium_parvulum			0.307	0.042						
Bacteroidales[G-2]_sp_HOT_274							-0.313	0.038		
Bifidobacterium_longum							0.314	0.038		
Gemella_morbillorum			0.310	0.041						
Gemella_morbillorum										
Gemella_morbillorum	0.306	0.043								
Lactococcus_lactis									0.456	0.002
Mitsuokella_multacida							0.431	0.004		
Peptoniphilus_lacrimalis	0.484	0.001	0.331	0.028			0.419	0.005	0.410	0.006
Porphyromonas_gingivalis	0.482	0.001					0.365	0.015	0.408	0.006
Prevotella_loescheii	0.519	0.000	0.320	0.034			0.383	0.010	0.420	0.005
Prevotella_nigrescens					-0.377	0.012				
Prevotella_oralis							-0.370	0.014		
Ruminococcaceae[G-3]_sp_HOT_381							0.431	0.004		
Selenomonas_noxia									0.459	0.002
Stenotrophomonas_maltophilia							0.408	0.006		
Streptococcus_sobrinus									-0.327	0.030
TM7[G-1]_sp_HOT_347	0.439	0.003	0.388	0.009			0.398	0.008	0.378	0.011

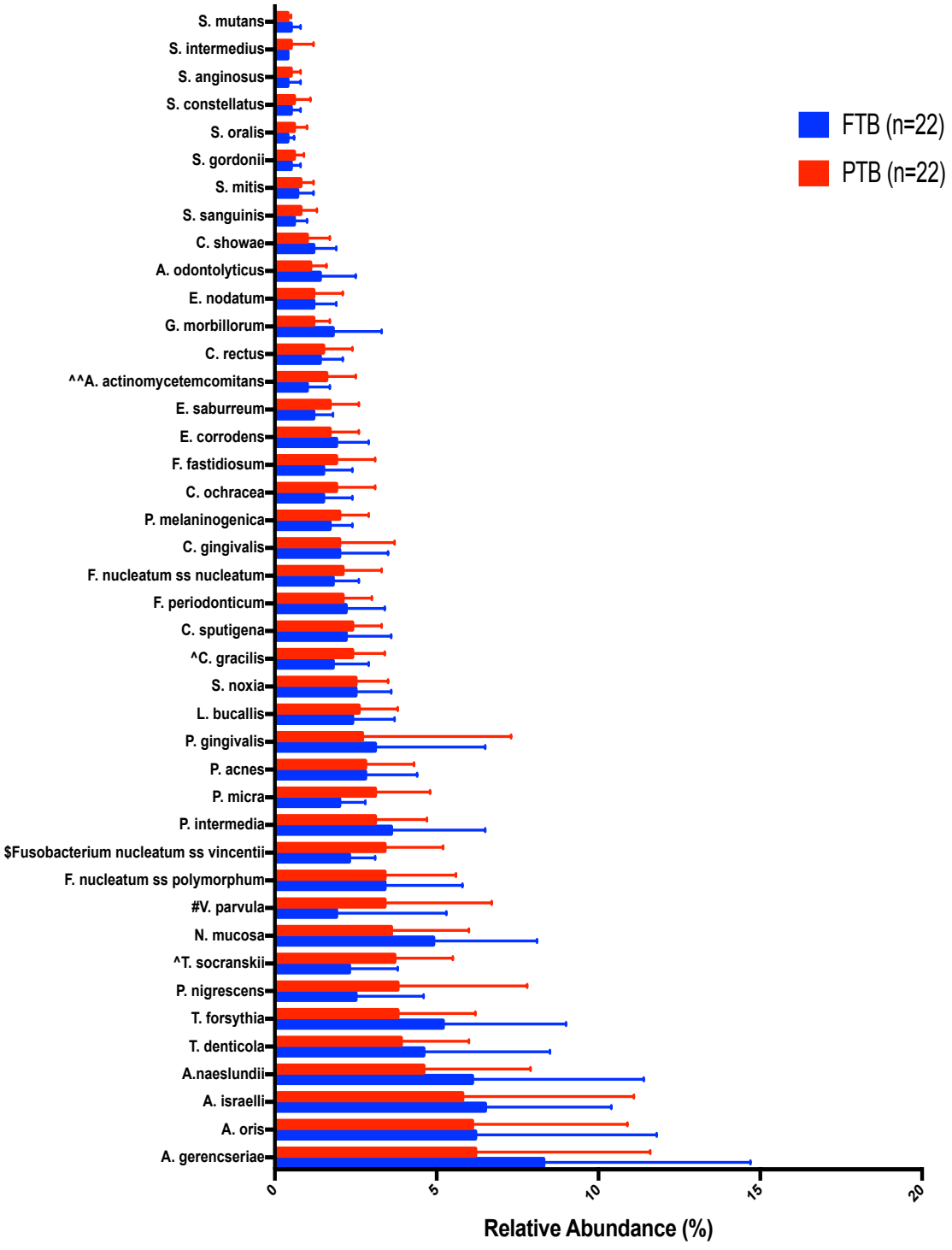

Figure 1: Pre-partum Mean (%) DNA Probe Count (± SD) of Subgingival Biofilm Samples (FTB: blue, PTB: red). ^p=0.01, ^^p=0.02, *p=0.03, **p=0.04 (Differences did not reach FDR-adjusted statistical significance).

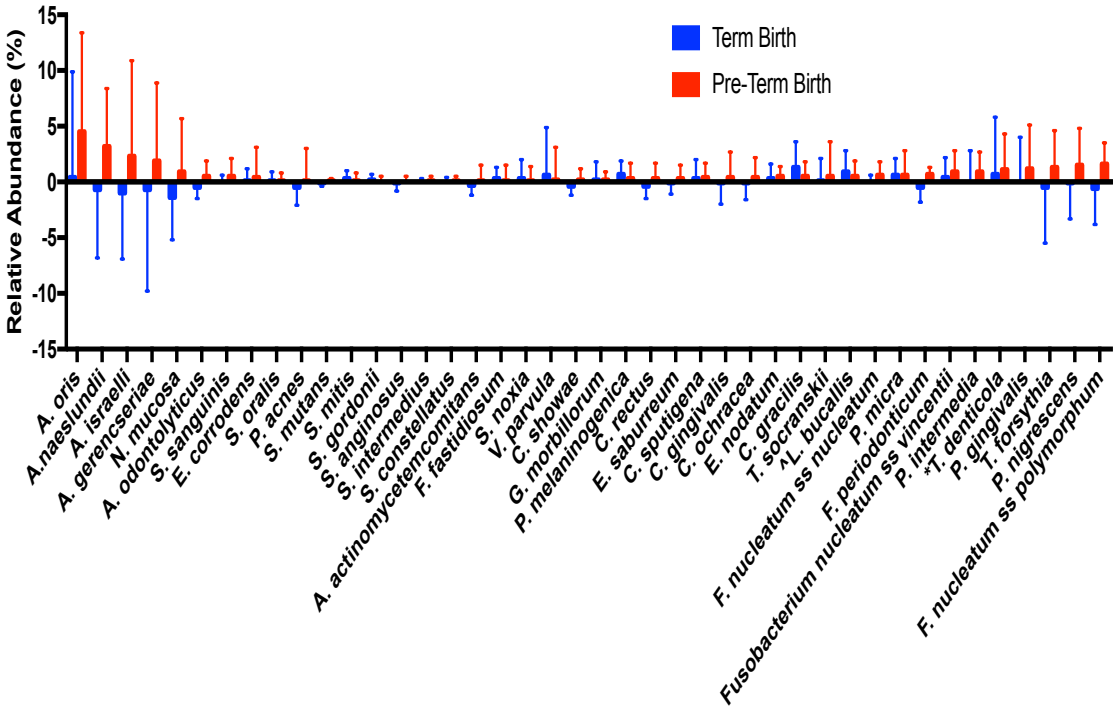

Figure 2: Pre-partum and Post-partum Changes in Mean (%) DNA Probe Count (± SD) of Subgingival Biofilm Samples (FTB: blue, PTB: red). ^p=0.01, ^^p=0.02, *p=0.03, **p=0.04 (Differences did not reach FDR-adjusted statistical significance).

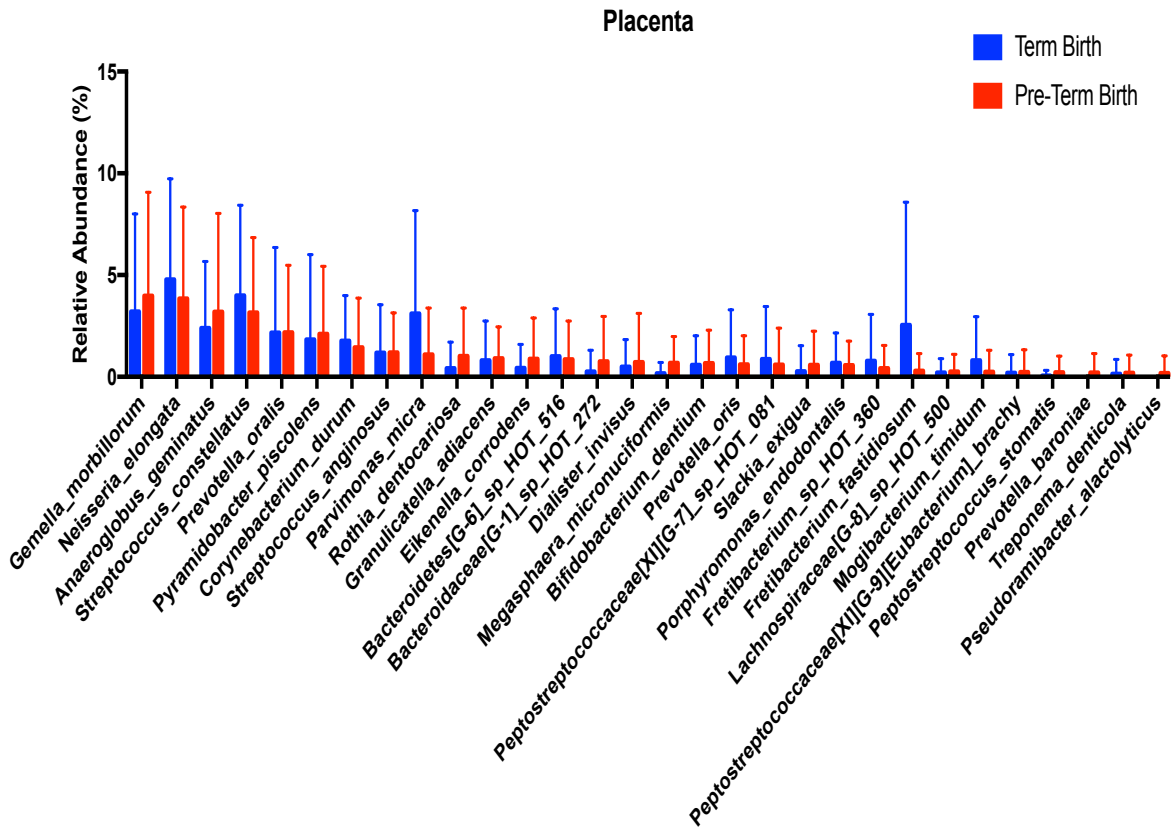

Figure 3: Mean relative abundance (% ± SD) of the most prevalent species detected in placenta samples (FTB: blue, PTB: red). ^p=0.01, ^^p=0.02, *p=0.03, **p=0.04 (Differences did not reach FDR-adjusted statistical significance).

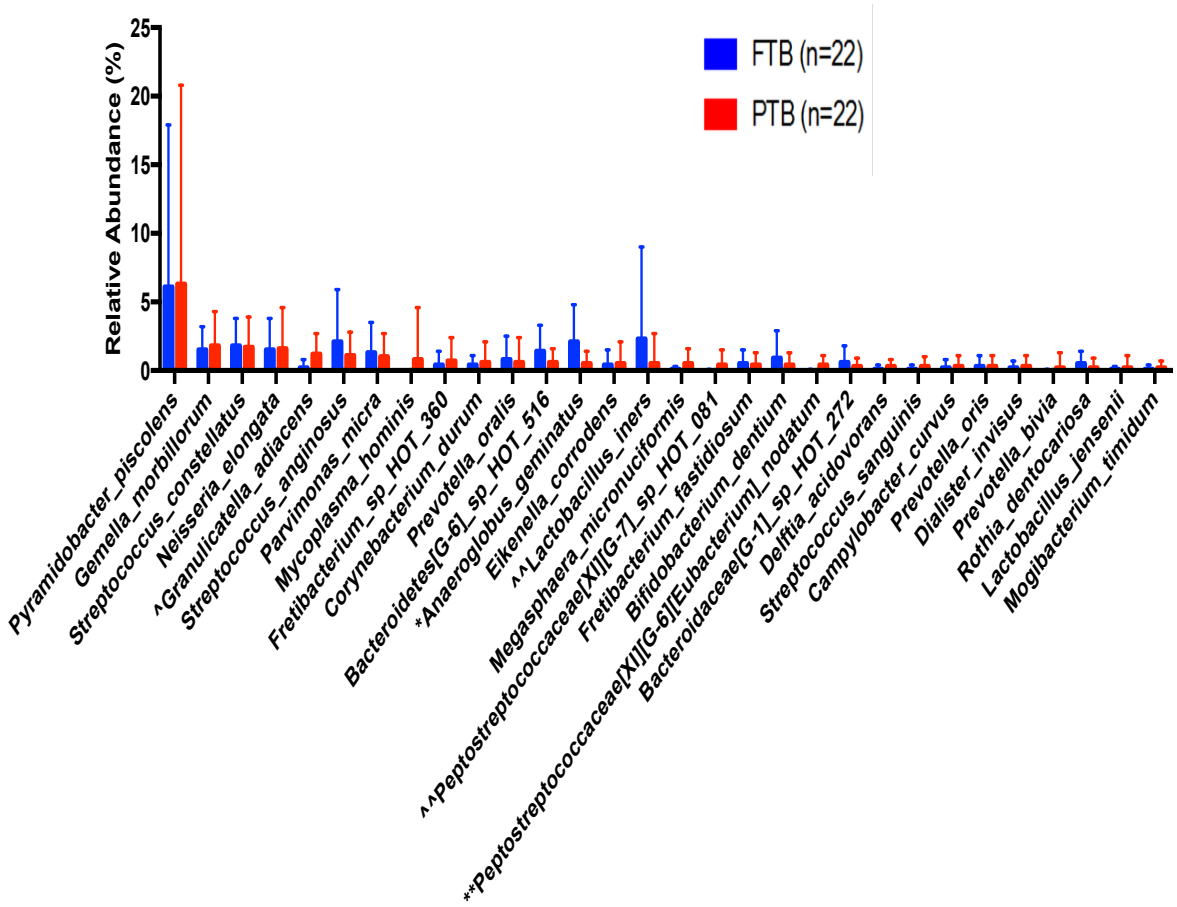

Figure 4: Mean relative abundance (% ± SD) of the most prevalent species detected in umbilicus samples (FTB: blue, PTB: red). ^p=0.01, ^^p=0.02, *p=0.03, **p=0.04 (Differences did not reach FDR-adjusted statistical significance).

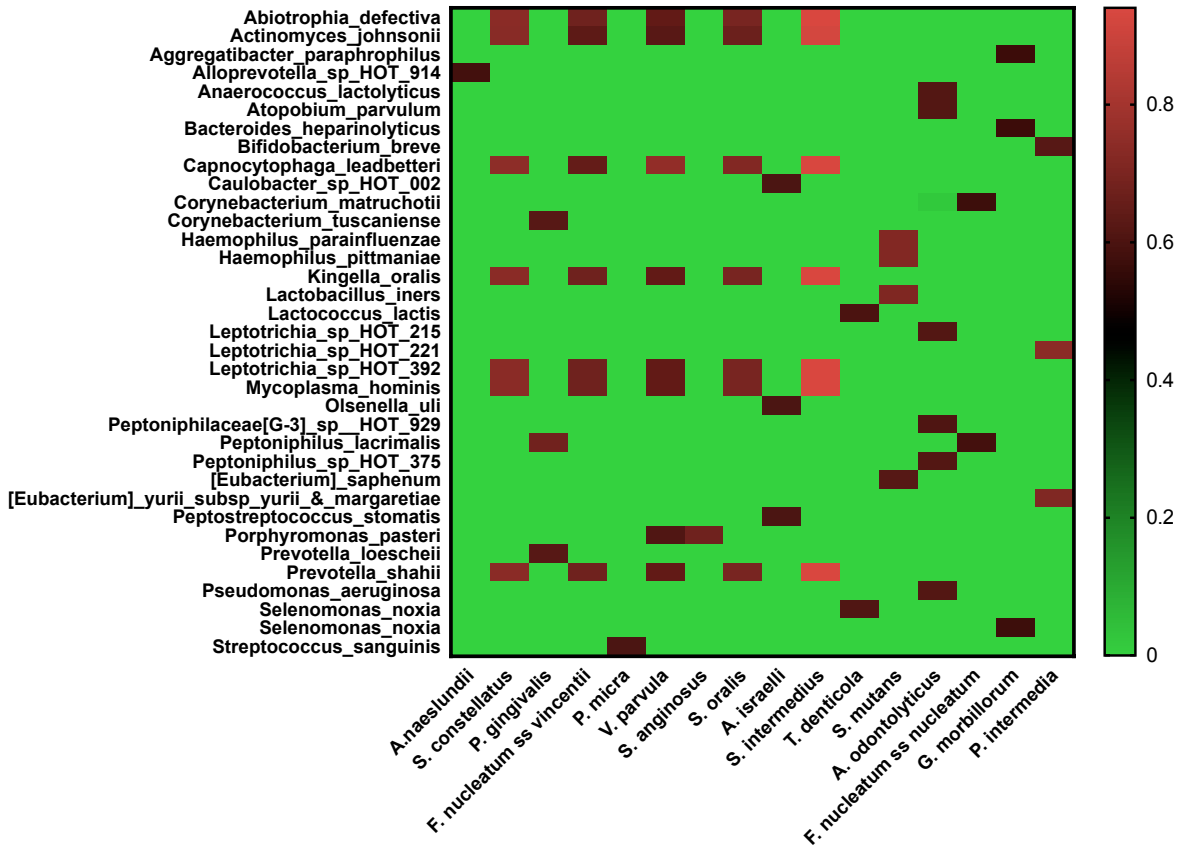

Figure 5: Correlation between Pre-term Oral and Umbilical Microbiomes ($p<0.0001$)

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