Persistent microbial dysbiosis in preterm premature rupture of membranes from onset until delivery

Elizabeth Baldwin, Marina Walther-Antonio, Allison MacLean, Daryl Gohl, Kenneth Beckman, Jun Chen, Bryan White, Douglas Creedon, Nicholas Chia

Background. Preterm Premature Rupture of Membranes (PPROM) is a major leading cause of preterm births. While the cause for PPROM remains unidentified, it is anticipated to be due to subclinical infection, since a large proportion of PPROM patients display signs of chorioamnionitis. Our goal was to characterize the vaginal microbiome and amniotic fluid discharge upon PPROM, through latency antibiotic treatment, and until delivery, to detect the presence of pathogens and microbial response to treatment.

Methods. Enrolled subjects (15) underwent routine institutional antenatal care for PPROM, including the administration of latency antibiotics. Serial vaginal swabs were obtained from diagnosis of PPROM through delivery and the sequencing of the V3-V5 region of the 16S rRNA gene was performed for all collected samples.

Results. The results show that Lactobacilli species were markedly decreased when compared to vaginal swabs collected from uncomplicated pregnancy subjects with a matched gestational time. *Prevotella* and *Peptoniphilus* were the most prevalent taxa in PPROM subjects at presentation. The vaginal microbiome of the PPROM subjects varied substantially intra- and inter-subjects. Several taxa were found to be significantly reduced during and after the antibiotic treatment: *Weeksella, Lachnospira, Achromobacter,* and *Pediococcus*. In contrast, *Peptostreptococcus* and *Tissierellaceae ph2* displayed a significant increase after the antibiotic treatment. However, the relative abundance of *Lactobacillus, Prevotella*, and *Peptoniphilus* was not substantially impacted during the hospitalization of the PPROM subjects. The deficiency of *Lactobacillus*, and constancy of known pathogenic species, such as *Prevotella* and *Peptoniphilus* during and after antibiotics, highlights the persistent dysbiosis and warrants further investigation into mitigating approaches.

Discussion. PPROM is responsible for one third of all preterm births. It is thought that subclinical infection is a crucial factor in the pathophysiology of PPROM because 25-40% of patients present signs of chorioamnionitis on amniocentesis. Here we sought to directly assess the bacterial content of the vagina and leaking amniotic fluid of subjects at presentation, throughout treatment and up until delivery, in order to search for common pathogens and microbial response to latency antibiotic treatment. We have found that the

vaginal microbiome of PPROM subjects is highly variable and displays significant changes to treatment. However, the unchanging deficiency of *Lactobacillus*, and persistence of known pathogenic species, such as *Prevotella* and *Peptoniphilus* from presentation, through antibiotic treatment and up until delivery, highlights the persistent dysbiosis and warrants further investigation into mitigating approaches.

Persistent Microbial Dysbiosis in Preterm Premature Rupture of Membranes from Onset until Delivery

Elizabeth Baldwin, MD[1,+], Marina R.S. Walther-António, PhD[2,+], Allison M. MacLean, BS[3], Daryl Gohl, PhD[3], Kenneth B. Beckman, PhD[3], Jun Chen, PhD[4], Bryan A. White, PhD[5,6], Douglas J. Creedon, MD, PhD[1,*], Nicholas Chia, PhD[2,7,*]

[1]Obstetrics and Gynecology, Mayo Clinic, Rochester, MN, USA

[2]Department of Surgical Research, Mayo Clinic, Rochester, MN, USA

[3]Genomics Center, University of Minnesota, Minneapolis, MN, USA

[4]Biomedical Statistics and Informatics, Mayo Clinic, Rochester, MN, USA

[5]Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA

[6]Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, IL, USA

[7]Department of Physiology and Biomedical Engineering, Mayo College, Rochester, MN, USA;

[+]These authors contributed equally:

Baldwin.Elizabeth@mayo.edu, WaltherAntonio.Marina@mayo.edu

[*]Corresponding authors:

Douglas Creedon[1]

200 First Street SW, Rochester, MN, 55905, USA

Email address: Creedon.Douglas@mayo.edu

Nicholas Chia[2,7]

200 First Street SW, Rochester, MN, 55905, USA

Email address: Chia.Nicholas@mayo.edu

Abstract

Background. Preterm Premature Rupture of Membranes (PPROM) is a major leading cause of preterm births. While the cause for PPROM remains unidentified, it is anticipated to be due to subclinical infection, since a large proportion of PPROM patients display signs of chorioamnionitis. Our goal was to characterize the vaginal microbiome and amniotic fluid discharge upon PPROM, through latency antibiotic treatment, and until delivery, to detect the presence of pathogens and microbial response to treatment.

Methods. Enrolled subjects (15) underwent routine institutional antenatal care for PPROM, including the administration of latency antibiotics. Serial vaginal swabs were obtained from diagnosis of PPROM through delivery and the sequencing of the V3-V5 region of the 16S rRNA gene was performed for all collected samples.

Results. The results show that Lactobacilli species were markedly decreased when compared to vaginal swabs collected from uncomplicated pregnancy subjects with a matched gestational time. *Prevotella and Peptoniphilus* were the most prevalent taxa in PPROM subjects at presentation. The vaginal microbiome of the PPROM subjects varied substantially intra- and inter-subjects. Several taxa were found to be significantly reduced during and after the antibiotic treatment: *Weeksella*, *Lachnospira*, *Achromobacter,* and *Pediococcus*. In contrast, *Peptostreptococcus* and *Tissierellaceae ph2* displayed a significant increase after the antibiotic treatment. However, the relative abundance of *Lactobacillus, Prevotella,* and *Peptoniphilus* was not substantially impacted during the hospitalization of the PPROM subjects. The deficiency *of Lactobacillus,* and constancy of known pathogenic species, such as *Prevotella* and *Peptoniphilus* during and after

antibiotics, highlights the persistent dysbiosis and warrants further investigation into mitigating approaches.

Discussion: PPROM is responsible for one third of all preterm births. It is thought that subclinical infection is a crucial factor in the pathophysiology of PPROM because 25-40% of patients present signs of chorioamnionitis on amniocentesis. Here we sought to directly assess the bacterial content of the vagina and leaking amniotic fluid of subjects at presentation, throughout treatment and up until delivery, in order to search for common pathogens and microbial response to latency antibiotic treatment. We have found that the vaginal microbiome of PPROM subjects is highly variable and displays significant changes to treatment. However, the unchanging deficiency *of Lactobacillus,* and persistence of known pathogenic species, such as *Prevotella* and *Peptoniphilus* from presentation, through antibiotic treatment and up until delivery, highlights the persistent dysbiosis and warrants further investigation into mitigating approaches.

Introduction

Preterm birth is the single leading cause of neonatal morbidity and mortality in the developed world, and despite efforts to identify causes, preventative strategies and treatment options, the incidence of preterm birth continues to rise in the United States(Goldenberg et al., 2008). Preterm premature rupture of membranes (PPROM) directly causes one third of all preterm births (Mercer et al., 2000). Subclinical infection likely plays a key role in the pathophysiology of PPROM and subsequent onset of preterm labor, as evidenced by the fact that 25-40% of patients with PPROM present signs of chorioamnionitis on amniocentesis (Simhan & Canavan, 2005). Furthermore, specific microbes associated with bacterial vaginosis (BV) such as

Gardnerella vaginalis and *Mycoplasma hominis* have been linked to pregnancy complications, including PPROM and preterm birth (Leitich et al., 2003; McDonald et al., 1991; Romero et al., 2002; Hay et al., 1994). Attempts to prevent preterm birth by using antibiotics prior to the onset of labor have not been very efficacious (Oliver & Lamont, 2013; Hauth et al., 1995; Brocklehurst et al., 2013). This suggests that our current knowledge of the vaginal microbes associated with adverse pregnancy outcomes, mainly based on clinical and traditional culture techniques remains incomplete (White et al., 2011).

Recently, the advent of next generation sequencing techniques such as 16S rRNA gene hypervariable tag sequencing has allowed a more complete characterization of the vaginal microbial ecology also known as the microbiome. Studies using 16S rRNA gene technology have demonstrated a broad spectrum of microbial organisms not previously identified in the vagina using traditional culture techniques (Fredricks, Fiedler, and Marrazzo, 2005; Zhou et al., 2004; Ravel et al., 2010). In studies of healthy non-pregnant women, the vaginal microbiome is characterized by five community profiles or subtypes most primarily dominated by a mixture of *Lactobacillus* species (Ravel et al., 2010). During normal pregnancy, this dominance broadens and the microbial diversity undergoes a marked decrease with convergence toward a single subtype (Aagaard et al., 2012; Romero et al., 2014). It is therefore intriguing that increased diversity in the vaginal microbiome in non-pregnant women correlates with clinical disease such as BV, which is also correlated with adverse pregnancy outcomes (Oliver & Lamont, 2013; Oakley et al., 2008; Flynn, Helwig, and Meurer, 1997; Hauth et al., 2003). Despite this, little is known about the vaginal microbiome and amniotic fluid discharge associated with pregnancy complications such as PPROM.

After diagnosis of PPROM at a gestation of 34 weeks or less, the administration of antibiotics has been shown to prolong pregnancy latency and improve short-term neonatal outcome (Kenyon, Boulvain, and Neilson, 2013; Hutzal et al., 2008). However, although outcomes are improved by antibiotic administration, the majority of women still enter spontaneous preterm labor soon after PPROM, and maternal and neonatal infectious morbidity is not eliminated (Simhan & Canavan, 2005; Epstein, Parry, and Strauss, 1998); Soraisham et al., 2009). This suggests that there are residual microbial factors involved after broad-spectrum antibiotics. In order to directly characterize the vaginal and amniotic fluid discharge at a PPROM event, we have taken a metagenomic approach and used next generation sequencing techniques. We continued the sampling throughout the subjects' hospitalization up until delivery to capture the microbiome changes throughout the treatment and delivery events.

Materials and Methods

Ethics Statement

Subjects were consented under IRB #12-001675, which was reviewed and approved by the Mayo Clinic Institutional Review Board. All subjects provided written consent.

Subjects Enrollment

Here we report the results from 15 subjects enrolled upon admission for PPROM to the Mayo Clinic Hospital – Rochester. Inclusion criteria consisted of the following: age >18 years; no known pregnancy complications prior to admission; ability to provide written informed consent; willingness to participate in mandatory translational research component of the study; weight greater than 50 kilograms; and confirmed preterm rupture of membranes based on clinical

criteria. Exclusion criteria consisted of the following: known immunodeficiency; chronic active viral infection (including HIV, HTLV and hepatitis); known autoimmune disease; solid organ or transplant recipient; and multiple gestations. Upon enrollment, participations were requested to fill out a questionnaire detailing sexual and reproductive health history and hygiene practices. Selected questionnaire data is available in Supplemental Table S1. Metadata from the questionnaires was stored using REDCap (Harris et al., 2009). Relevant medical information related to the time of labor and delivery is also included in Supplemental Table S1. All subjects were managed according to the standard institutional protocol for PPROM. This included hospitalization, administration of a course of antenatal steroids if <34 weeks gestation on admission and a standardized course of latency antibiotics (12 subjects received ampicillin/amoxicillin and azithromycin; two subjects received clindamycin and azithromycin due to penicillin allergy; and one subject received penicillin alone due to presentation in active labor). Delivery was undertaken for spontaneous preterm labor, nonreassuring maternal or fetal status, clinical concern for infection, or when a gestational age of 34 weeks was reached.

Sample Collection

Dacron swabs were collected during sterile speculum exam performed by an obstetrician from the posterior vaginal fornix and placed in a Nucleic Acid Transport collection tube. After collection the samples were stored at -80^{o}C until processing. Vaginal samples were collected at the time of admission, every three days for the first two weeks of admission and weekly thereafter until delivery. An additional vaginal sample was collected at the time of delivery and a swab from the fetal surface of the placental membranes after delivery for 6 of the 15 subjects.

Sample Processing

Samples were thawed and centrifuged for 10 minutes at 10 000*g* to collect the bacterial cells, and the supernatant was discarded. Genomic DNA extraction was performed by using the MoBio Ultraclean Soil Kit (MoBio Laboratories, Inc., Carlsbad, CA); with the MP FastPrep (MP Biomedicals, Solon, OH) for 40 seconds at 6.0m/s. Incubation period was done for a minimum of 30 minutes. After extraction the DNA content was measured using High Sensitivity Qubit (Life Technologies Corporation, Carlsbad, CA) with the results ranging from below detection to 23 ng/ul of DNA. The V3-V5 region of the 16S rRNA was then amplified using a two-step polymerase chain reaction (PCR) protocol. The primary amplification was done using the following recipe: 3 μl template DNA, 0.5 μl nuclease-free water, 1.2 μl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.18 μl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.3 μl DMSO (Fisher Scientific, Waltham, MA) , 0.12 μl ROX (25 μM) (Life Technologies, Carlsbad, CA), 0.003 μl 2000x SYBR Green, 0.12 μl KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA), 0.3 μl forward primer (10 μM), 0.3 μl reverse primer (μl). The following cycling conditions were used: 95^{O}C for 5 minutes, followed by 25 cycles of 98^{O}C for 20 seconds, 55^{O}C for 15 seconds, 72^{O}C for 1 minute. The primers for the primary amplification contained both 16S-specific primers (357F and 926R), as well as adapter tails for adding indices in a secondary amplification. The primer sequences for the primary amplification were as follows (16S-specific sequences in bold):

V3F_Nextera:

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGAGGCAGCAG**

V5R_Nextera:

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CCGTCAATTCMTTTRAGT**

Next, these amplicons were diluted 1:100 in sterile, nuclease-free water, and a second PCR reaction was set up to add the Illumina flow cell adapters and indices. The secondary amplification was done using the following recipe: 5 μl template DNA, 1 μl nuclease-free water, 2 μl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.3 μl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.5 μl DMSO (Fisher Scientific, Waltham, MA), 0.2 μl KAPA HiFi Polymerase (Kapa Biosystems, Woburn, MA), 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (μl). The following cycling conditions were used: 95^{o}C for 5 minutes, followed by 10 cycles of 98^{o}C for 20 seconds, 55^{o}C for 15 seconds, 72^{o}C for 1 minute, followed by a final extension at 72^{o}C for 10 minutes. The indexing primers are as follows (X marks the positions of the 8 bp indices):

Forward indexing primer:

AATGATACGGCGACCACCGAGATCTACACXXXXXXXXTCGTCGGCAGCGTC

Reverse indexing primer:

CAAGCAGAAGACGGCATACGAGATXXXXXXXXGTCTCGTGGGCTCGG

The products of the amplification were quantified using a PicoGreen dsDNA assay (Life Technologies, Carlsbad, CA), and the samples were normalized, pooled, and approximately 1 μg of material was concentrated to 10 μl using 1.8x AMPureXP beads (Beckman Coulter, Brea, CA). The pooled sample was then size selected at 723 bp +/- 20% on a Caliper XT DNA 750 chip (Caliper Life Science, Hopkinton, MA). The size-selected material was cleaned up using AMPureXP beads, and eluted in 20 μl of EB buffer (10 mM Tris-HCl, pH 8.5). The final pooled sample was quantified using the PicoGreen dsDNA assay, and analyzed using an Agilent Bioanalyzer High Sensitivity Chip (Agilent Technologies, Santa Clara, CA). Finally, the sample pool was diluted to 2 nM based on the PicoGreen measurements, and 10 μl of the 2 nM pool was

159 denatured with 10 μl of 0.2 N NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 20%
160 phiX, heat denatured at 96^{O}C for 2 minutes, and sequenced using a MiSeq 600 cycle v3 kit
161 (Illumina, San Diego, CA).
162
163 **Processing of Controls**
164 Control samples consisted of five "empty collection" replicates. In brief, five swabs were
165 immersed in the collection buffer and DNA was extracted, amplified, and sequenced using the
166 same procedures and reagents used for the PPROM samples. In addition, three other controls
167 were performed: 1) one extraction with only the collection buffer was performed (no swab), 2) a
168 PCR negative control was sequenced, 3) a positive control of a pure isolate of *Campylobacter*
169 *jejuni*. There was no detectable amplification in the negative controls by qPCR. The positive
170 control (*Campylobacter jejuni*) was carried forward to sequencing as well to guarantee that the
171 negative controls were sequenced at comparable depth. The eight control samples were spiked
172 into a MiSeq run at a comparable per-sample concentration to that of the PPROM sample MiSeq
173 run. As expected, the extraction and water negative controls yielded much lower numbers of
174 reads (between 0.0084% and 0.0406% of the total reads in the run).
175
176 **Sequencing Processing and Outcome**

177 To remove the 5'-Adapter-primer sequence on forward reads which has a total length of 58 bps,
178 for each forward read, we took 60 bps from the 5' end and BLAST against the 5'-Adapter-primer
179 sequence allowing one base mismatch, and the matched region of the reads were removed if
180 present at the beginning of the sequence read. To remove the 5'-Adapter-barcode-primer
181 sequence on reverse reads which has a total length of 65 bps, we first generated a group of

sequences, through a combination of different barcodes and wobbly bases. There are a total of 73 samples taken from 15 subjects, each sample with a unique barcode. And there are two wobbly bases M and G in the sequence, each of which denotes an alternative base of A or C and A or G respectively. Thus the total number of sequence combinations is 77 x 2 x 2=292. We created a BLAST database from the 292 sequences. Then, for each reverse read, we took 67 bps from the 5' end and BLAST against the created database allowing one base mismatch except for the barcode region. The matched region of each read was removed if present at the beginning of the sequence read and the samples were de-multiplexed by the matched barcode on each read. Due to the low number of R2 samples passing quality control (at least 187bp per sequence read and a minimum of 2,000 sequence reads per sample), only R1 reads were used for analysis. A total of 5,575,178 R1 sequence reads (5,527 to 174,479 sequence reads per sample) passed quality control.

Sequence Analysis

Sequence reads were aligned with our own custom multiple alignment tool known as the Illinois-Mayo Taxon Operations for RNA Dataset Organization (IM-TORNADO) that merges paired end reads into a single multiple alignment and obtains taxa calls (Sipos et al., 2010). Operational taxonomic units were clustered using UPARSE (Edgar, 2013). Further processing for visualization and statistical analysis was performed using QIIME (Caporaso et al., 2010). To identify differential abundant bacteria genera, we fit a linear regression model to the square-root transformed genus proportion data. We used the t-statistic as the test statistic. To address the non-normality of the outcome variable as well as within-subject correlation, we used permutation to assess the statistical significance. Permutation was performed 1,000 times and was

constrained within the subject to retain the original correlation structure. We also fit a generalized mixed effects model to the genus presence/absence data using PQL method, assuming a random intercept for each subject. Statistical significance was assessed based on Wald test. False discovery control (B-H procedure) was used for correcting multiple testing. Sequences are publicly available at SRA, study accession: SRP061714. The taxonomic assignments are provided in Supplemental Table S2.

Results

Our cohort consisted of 15 subjects (age range: 19-37 years old; Mean ± Standard Deviation: 29 ± 4 years old). The majority were Caucasian (14), and one was East Asian. Within the cohort, there were no complications specific to pregnancy prior to or subsequent to diagnosis of PPROM including no diagnoses of gestational diabetes or hypertensive disorders of pregnancy. The cohort included 10 parous and 5 nulliparous women, with four subjects having a history of preterm delivery with a previous pregnancy. From these 15 subjects, a total of 61 vaginal samples and 6 placental samples were collected (Fig 1; selected metadata associated with each subject can be found in Table S1).

PPROM Before Antibiotic Treatment. In order to assess the näive maternal microbiome at time of presentation with PPROM, we examined the admission samples from nine subjects that were obtained before the administration of antibiotics and compared them with those of a companion study, where 12 women experiencing an uncomplicated pregnancy were sampled longitudinally (Walther-António et al., 2014). Using gestational age-matched samples from the companion study we compared the vaginal microbiome taxonomic assignments to the PPROM subjects. The microbiome in the PPROM subjects exhibited a diversity of taxa that was in stark contrast to the

normal pregnancy subjects (Fig 2), and was marked by a marked underrepresentation of lactobacilli (Fig 3). Overall, the most common pathogens detected in the PPROM subjects before the administration of antibiotics were *Prevotella* and *Peptoniphilus* (Fig 4).

PPROM During and After Antibiotic Treatment. The administration of antibiotics did not significantly impact the relative abundance of *Lactobacillus, Prevotella,* or *Peptoniphilus* during or after the antibiotic treatment (Table 1 and Table 2). However, during the administration of the treatment, *Weeksella, Lachnospira, Achromonacter* and *Pediococcus* showed a significant decrease, while *Peptostreptococcus* and *Tissierellaceae ph2* showed a significant increase (Table 1 shows presence/absence significance most powerful for low abundance taxa, Table 2 shows proportion significance more powerful for high abundance taxa).

Placental Microbiome. To assess the placental microbiome, we compared the six placental samples with the matched maternal vaginal samples obtained at the time of delivery. The placental microbiome was statistically distinct from the vaginal microbiome (Bray Curtis distance p-value 0.08 – PERMANOVA, within subject 1,000 permutations). However, given the limited amount of samples, there was insufficient statistical power to identify differential taxa. Placental samples showed high individual variability and weak correlation with the maternal vaginal microbiome (Fig 5). The most prevalent taxa recovered were *Alicyclobacillus* and *Corynebacterium*.

Controls. The sequencing of our controls – Negative PCR control, five quality control (QC) replicates (empty swab collections) and the DNA extraction and amplification of the TE buffer used for the sample collection – showed very little overlap with the major taxa seen in the study samples (Table 3).

Discussion

Our results also show that the vaginal microbiota of pregnant subjects with PPROM is distinct from that of subjects undergoing an uncomplicated pregnancy. The findings indicate that the vaginal microbiome after PPROM is more diverse and dynamic than previously observed during normal pregnancies (Walther-António et al., 2014). Of specific interest is the difference in lactobacilli abundance in PPROM. Lactobacilli acidify their environment and limit diversity through a process of niche expansion—allowing for the dominance of lactobacilli in most vaginal microbiota observed to date. The loss of lactobacilli, along with many other factors, may place a key role in the destabilization of the PPROM-associated vaginal microbiome. This depletion may be due to the displacement either by the constant leakage of alkaline amniotic fluid or may precede the PPROM event. It is also worth emphasizing that although the swabs were physically placed in the posterior fornix for the sampling, this was likely to contain varying amounts of amniotic fluid in PPROM subjects. Once there is a rupture of the membranes, amniotic fluid continues to leak from the cervix and when a patient is in the supine position the posterior fornix is where that fluid will collect. The patients remain hospitalized until delivery, and the samples were obtained in the patient's hospital bed. So unless the patient had been ambulating just prior to sampling, there will be some amniotic fluid pooled in the posterior fornix. Once lactobacilli are displaced, opportunistic bacteria may occupy the available niche leading to an unbalanced microbial ecology (dysbiosis) with potentially negative consequences for health. The transient nature of the PPROM microbiome is consistent with the idea of an ecology that is out of equilibrium. *Prevotella* and *Peptoniphilus* emerge as taxa of particular interest in PPROM given their prevalence at presentation and persistence throughout treatment, evidence of prominent role in persistent bacterial vaginosis and preterm labor (Marrazzo et al.,

2008; Smayevsky et al., 2001; Wang et al., 2013; Mikamo et al., 1999) and display of broad-spectrum antibiotic resistance (Sherrard et al., 2013; Tanaka et al., 2006). The highly variable placental microbiome recovered and the weak correlation with the maternal microbiome raises the possibility that the uterine microbiome may be an independent driver of the placental microenvironment. Although the vaginal microbiome likely exerts a strong influence in the uterine microbiome in the initial stages of pregnancy, the formation of the cervical mucus plug as early as seven weeks of gestation provides a barrier between the two environments. The influence of the vaginal microbiome in the uterine microbial niche is therefore anticipated to be reduced for as long as the mucus plug is intact. The fact that in this study *Prevotella* was a prominent pathogen found in the vaginal, amniotic fluid discharge and placental membranes indicates a very likely role in the etiology of PPROM. The known association of *Prevotella* with bacterial vaginosis and preterm labor strengthen this possibility.

Our study is limited by the small number of patients. Another limitation is that the vaginal swabs collected from PPROM subjects likely contained both amniotic fluid and vaginal fluid. We are unable to determine the proportion of amniotic fluid in each sample, which is expected to vary with each collection. By contrast, the vaginal swabs collected from uncomplicated pregnancy subjects are not anticipated to have had a significant presence of amniotic fluid.

Despite the low amount of microbial DNA present in the samples, we were able to rule out that the relevant taxa in this study could be the result of contamination. As shown in Table 3 we were able to find several well-known contaminants (Salter et al., 2014), but not the taxa relevant to our study with one exception, *Corynebacterium*. This taxa was predominant in the placenta of our PPROM subjects, which supports previous findings in the placenta of preterm

297 deliveries (Oh et al., 2010). It is possible that its presence may be overemphasized in this study
298 due to its detection in the collection buffer, but it is undoubtedly present in the study samples as
299 well.
300
301 **Conclusions**
302 The deficiency in lactobacilli species and persistence of known pathogenic species at
303 admission, and during and after antibiotics, highlights a marked dysbiosis with high individual
304 variability. An interesting area of future research would be to assess the changes in the
305 microbiome noted in our study as a potential causative factor predating diagnosis of PPROM by
306 longitudinal assessment of the vaginal microbiome in pregnant women at high risk for preterm
307 premature rupture of membranes.
308
309
310 **Competing Interests**
311 DC and NC have licensed intellectual property with Whole Biome, Inc. All other authors declare
312 that they have no competing interests.
313
314 **Authors Contributions**
315 EB performed sample collection and drafted the manuscript. MWA performed the laboratory
316 processing, data analysis and drafted the manuscript. AMM performed the laboratory processing.
317 DG performed the laboratory processing and sequence processing. KBB performed the sequence

processing. JC performed the statistical analysis. BW conceived the study and participated in its design. DC conceived the study and design and coordinated tasks. NC participated in the design, coordination and draft of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors wish to acknowledge clinical, diagnostic and clerical staff at Mayo Clinic.

References

Aagaard K, Riehle K, Ma J, Segata N, Mistretta T-A, Coarfa C, Raza S, Rosenbaum S, Van den Veyver I, and Milosavljevic A. 2012. A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. *PLoS One* 7:e36466.

Brocklehurst P, Gordon A, Heatley E, and Milan SJ. 2013. Antibiotics for treating bacterial vaginosis in pregnancy. *The Cochrane Library*.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, and Gordon JI. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature methods* 7:335-336.

Edgar RC. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature methods* 10:996-998.

Epstein FH, Parry S, and Strauss JF. 1998. Premature rupture of the fetal membranes. *New England Journal of Medicine* 338:663-670.

Flynn CA, Helwig AL, and Meurer LN. 1997. Bacterial vaginosis in pregnancy and the risk of prematurity: a meta-analysis. Medical College of Wisconsin.

Fredricks DN, Fiedler TL, and Marrazzo JM. 2005. Molecular identification of bacteria associated with bacterial vaginosis. *New England Journal of Medicine* 353:1899-1911.

Goldenberg RL, Culhane JF, Iams JD, and Romero R. 2008. Epidemiology and causes of preterm birth. *The Lancet* 371:75-84.

Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, and Conde JG. 2009. Research electronic data capture (REDCap)—a metadata-driven methodology and workflow process for providing translational research informatics support. *Journal of biomedical informatics* 42:377-381.

Hauth JC, Goldenberg RL, Andrews WW, DuBard MB, and Copper RL. 1995. Reduced incidence of preterm delivery with metronidazole and erythromycin in women with bacterial vaginosis. *New England Journal of Medicine* 333:1732-1736.

Hauth JC, MacPherson C, Carey JC, Klebanoff MA, Hillier SL, Ernest J, Leveno KJ, Wapner R, Varner M, and Trout W. 2003. Early pregnancy threshold vaginal pH and Gram stain scores predictive of subsequent preterm birth in asymptomatic women. *American journal of obstetrics and gynecology* 188:831-835.

Hay PE, Lamont RF, Taylor-Robinson D, Morgan DJ, Ison C, and Pearson J. 1994. Abnormal bacterial colonisation of the genital tract and subsequent preterm delivery and late miscarriage. *Bmj* 308:295-298.

Hutzal CE, Boyle EM, Kenyon SL, Nash JV, Winsor S, Taylor DJ, and Kirpalani H. 2008. Use of antibiotics for the treatment of preterm parturition and prevention of neonatal morbidity: a metaanalysis. *American journal of obstetrics and gynecology* 199:620. e621-620. e628.

Kenyon S, Boulvain M, and Neilson JP. 2013. Antibiotics for preterm rupture of membranes. *The Cochrane Library*.

Leitich H, Bodner-Adler B, Brunbauer M, Kaider A, Egarter C, and Husslein P. 2003. Bacterial vaginosis as a risk factor for preterm delivery: a meta-analysis. *American journal of obstetrics and gynecology* 189:139-147.

Marrazzo JM, Thomas KK, Fiedler TL, Ringwood K, and Fredricks DN. 2008. Relationship of

specific vaginal bacteria and bacterial vaginosis treatment failure in women who have sex with women. *Annals of internal medicine* 149:20-28.

McDonald H, O'LOUGHLIN J, Jolley P, Vigneswaran R, and McDonald P. 1991. Vaginal infection and preterm labour. *BJOG: An International Journal of Obstetrics & Gynaecology* 98:427-435.

Mercer BM, Goldenberg RL, Meis PJ, Moawad AH, Shellhaas C, Das A, Menard MK, Caritis SN, Thurnau GR, and Dombrowski MP. 2000. The preterm prediction study: prediction of preterm premature rupture of membranes through clinical findings and ancillary testing. *American journal of obstetrics and gynecology* 183:738-745.

Mikamo H, Kawazoe K, Sato Y, and Tamaya T. 1999. Elastase activity of anaerobes isolated from amniotic fluid with preterm premature rupture of membranes. *American journal of obstetrics and gynecology* 180:378-380.

Oakley BB, Fiedler TL, Marrazzo JM, and Fredricks DN. 2008. Diversity of human vaginal bacterial communities and associations with clinically defined bacterial vaginosis. *Applied and Environmental Microbiology* 74:4898-4909.

Oh KJ, Lee KA, Sohn Y-K, Park C-W, Hong J-S, Romero R, and Yoon BH. 2010. Intraamniotic infection with genital mycoplasmas exhibits a more intense inflammatory response than intraamniotic infection with other microorganisms in patients with preterm premature rupture of membranes. *American journal of obstetrics and gynecology* 203:211. e211-211. e218.

Oliver R, and Lamont R. 2013. Infection and antibiotics in the aetiology, prediction and prevention of preterm birth. *Journal of Obstetrics & Gynaecology* 33:768-775.

Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, Karlebach S, Gorle R, Russell J, and Tacket CO. 2010. Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences*:201002611.

Romero R, Espinoza J, Chaiworapongsa T, and Kalache K. 2002. Infection and prematurity and the role of preventive strategies. Seminars in Neonatology: Elsevier. p 259-274.

Romero R, Hassan SS, Gajer P, Tarca AL, Fadrosh DW, Nikita L, Galuppi M, Lamont RF, Chaemsaithong P, and Miranda J. 2014. The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome* 2:4.

Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, Turner P, Parkhill J, Loman NJ, and Walker AW. 2014. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC biology* 12:87.

Sherrard LJ, Graham KA, McGrath SJ, McIlreavey L, Hatch J, Muhlebach MS, Wolfgang MC, Gilpin DF, Elborn JS, and Schneiders T. 2013. Antibiotic resistance in Prevotella species isolated from patients with cystic fibrosis. *Journal of Antimicrobial Chemotherapy*:dkt191.

Simhan HN, and Canavan TP. 2005. Preterm premature rupture of membranes: diagnosis, evaluation and management strategies. *BJOG: An International Journal of Obstetrics & Gynaecology* 112:32-37.

Sipos M, Jeraldo P, Chia N, Qu A, Dhillon AS, Konkel ME, Nelson KE, White BA, and Goldenfeld N. 2010. Robust computational analysis of rRNA hypervariable tag datasets. *PLoS One* 5:e15220.

Smayevsky J, Canigia LF, Lanza A, and Bianchini H. 2001. Vaginal microflora associated with bacterial vaginosis in nonpregnant women: reliability of sialidase detection. *Infectious*

diseases in obstetrics and gynecology 9:17-22.

Soraisham AS, Singhal N, McMillan DD, Sauve RS, Lee SK, and Network CN. 2009. A multicenter study on the clinical outcome of chorioamnionitis in preterm infants. *American journal of obstetrics and gynecology* 200:372. e371-372. e376.

Tanaka K, Mikamo H, Nakao Ki, and Watanabe K. 2006. In vitro antianaerobic activity of DX-619, a new des-fluoro (6) quinolone. *Antimicrobial agents and chemotherapy* 50:3908-3913.

Walther-António MR, Jeraldo P, Miller MEB, Yeoman CJ, Nelson KE, Wilson BA, White BA, Chia N, and Creedon DJ. 2014. Pregnancy's Stronghold on the Vaginal Microbiome. *PLoS One* 9:e98514.

Wang X, Buhimschi CS, Temoin S, Bhandari V, Han YW, and Buhimschi IA. 2013. Comparative microbial analysis of paired amniotic fluid and cord blood from pregnancies complicated by preterm birth and early-onset neonatal sepsis. *PLoS One* 8:e56131.

White BA, Creedon DJ, Nelson KE, and Wilson BA. 2011. The vaginal microbiome in health and disease. *Trends in Endocrinology & Metabolism* 22:389-393.

Zhou X, Bent SJ, Schneider MG, Davis CC, Islam MR, and Forney LJ. 2004. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. *Microbiology* 150:2565-2573.

Table 1. Taxa displaying significant changes before, during, and after antibiotic treatment*.

	Before vs During Antibiotic Treatment			During vs After Antibiotic Treatment			Before vs After Antibiotic Treatment		
Taxa (Phylum, Classe, Order, Family, Genus)	value	p-value[1]	qvalue[2]	value	p-value[1]	qvalue[2]	value	p-value[1]	qvalue[2]
Bacteroidetes, Flavobacteriia, Flavobacteriales, Weeksellaceae, **Weeksella**	-28.3	0.9998	1.0000	-2.5	0.0054	0.1482	25.8	0.9999	1.0000
Firmicutes, Clostridia, Clostridiales, Lachnospiraceae, **Lachnospira**	-2.0	0.0743	0.6956	-2.3	0.0048	0.1482	-0.3	0.7712	1.0000
Proteobacteria, Betaproteobacteria, Burkholderiales, Alcaligenaceae, **Achromobacter**	-1.2	0.2095	0.7810	-2.7	0.0033	0.1482	-1.5	0.1894	0.7564
Bacteroidetes, Bacteroidia, Bacteroidales, Prevotellaceae, **Prevotella**	28.1	0.9999	1.0000	28.3	0.9999	1.0000	0.2	1.0000	1.0000
Firmicutes, Clostridia, Clostridiales, Tissierellaceae, **Peptoniphilus**	0.7	0.5395	0.8650	0.2	0.7926	0.9266	-0.6	0.6469	0.9301

* Results achieved using a generalized mixed effects model to the present/absent taxa using the Penalized Quasi-likelihood (PQL) method, assuming a random intercept for each subject.

[1]Statistical significance was assessed based on Wald test.

[2]False discovery control Benjamini-Hochberg (B-H procedure) was used for correcting multiple testing (qvalue<0.2 (false discovery rate) considered significant).

Table 2. Significant taxa shifts before, during, and after antibiotic treatment*.

	Before	During	After	Before vs During	During vs After	Before vs After	Before vs During	During vs After	Before vs After	Signal
Taxa (Phylum, Classe, Order, Family, Genus)	Mean	Mean	Mean	p-value[1]	p-value[1]	p-value[1]	qvalue[2]	qvalue[2]	qvalue[2]	
Firmicutes, Bacilli, Lactobacillales, Lactobacillaceae, **Pediococcus**	1.9E-03	4.1E-03	1.6E-05	0.299	0.004	0.002	0.84	0.084	0.084	Decrease
Proteobacteria, Betaproteobacteria, Burkholderiales, Alcaligenaceae, **Achromobacter**	4.3E-04	1.8E-02	3.4E-06	0.267	0.003	0.018	0.84	0.084	0.308	Decrease
Firmicutes, Clostridia, Clostridiales, Peptostreptococcaceae, **Peptostreptococcus**	2.1E-03	2.7E-04	1.9E-02	0.247	0.001	0.001	0.84	0.042	0.084	Increase
Firmicutes, Clostridia, Clostridiales, Tissierellaceae, **ph2**	1.5E-05	0.0E+00	1.3E-04	0.69	0.001	0.019	0.92	0.042	0.308	Increase
Firmicutes, Clostridia, Clostridiales, Tissierellaceae, **Peptoniphilus**	2.9E-02	1.0E-02	4.2E-03	0.052	0.691	0.4	0.546	0.921	0.781	-
Firmicutes, Bacilli, Lactobacillales, Lactobacillaceae, **Lactobacillus**	2.1E-01	1.5E-01	1.4E-01	0.021	0.853	0.618	0.546	0.940	0.881	-
Bacteroidetes, Bacteroidia, Bacteroidales, Prevotellaceae, **Prevotella**	7.3E-02	1.3E-01	1.6E-01	0.457	0.782	0.661	0.868	0.940	0.881	-

* A Linear regression model was fit to the square-root transformed genus proportion data. To address the non-normality of the outcome variable as well as within-subject correlation, permutation (1,000 times) was used to assess the statistical significance. Permutation was constrained within each subject to retain the original correlation structure.

[1]Statistical significance was assessed by permutation

[2]False discovery control Benjamini-Hochberg (B-H procedure) was used for correcting multiple testing (qvalue<0.2 (false discovery rate) considered significant).

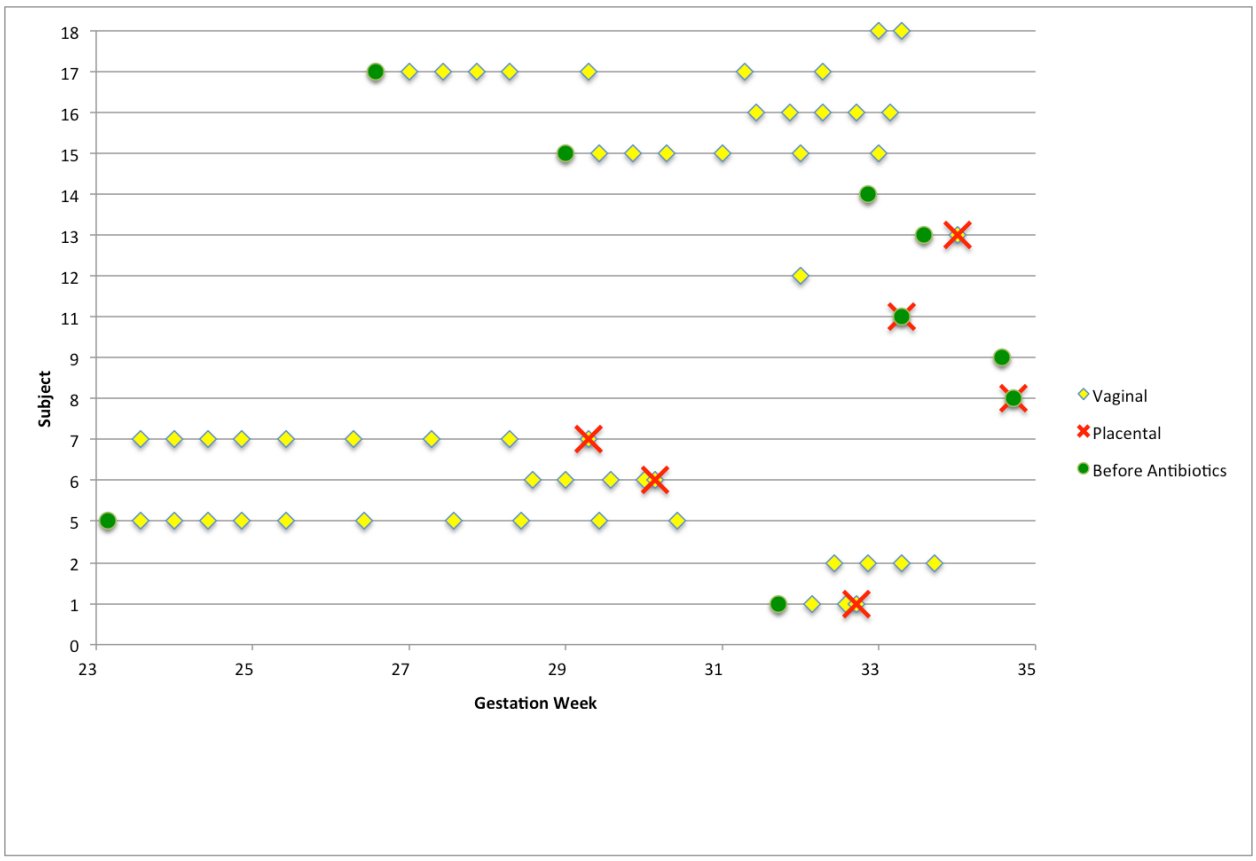

Figure 1. Samples collected and analyzed in the course of the study.

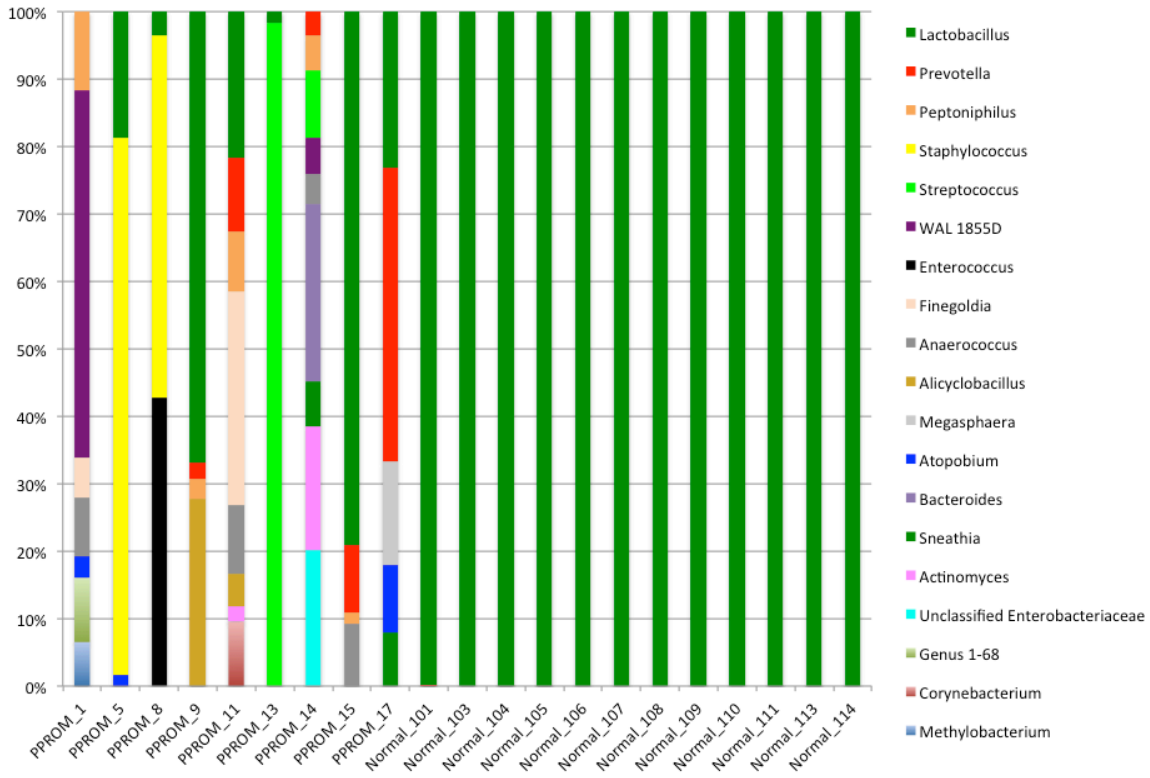

Figure 2. Comparison of relative abundance of taxa at the genus level between vaginal swabs of subjects at the time of presentation at the emergency room with PPROM before the administration of antibiotic treatment (9 subjects) and vaginal swabs from subjects that underwent an uncomplicated pregnancy at approximately 29 weeks of gestation (12 subjects). The lack of lactobacilli dominance in PPROM subjects is apparent, as is the inter-individual variation. Only taxa at >1% relative abundance in the PPROM subjects are shown for graphical clarity.

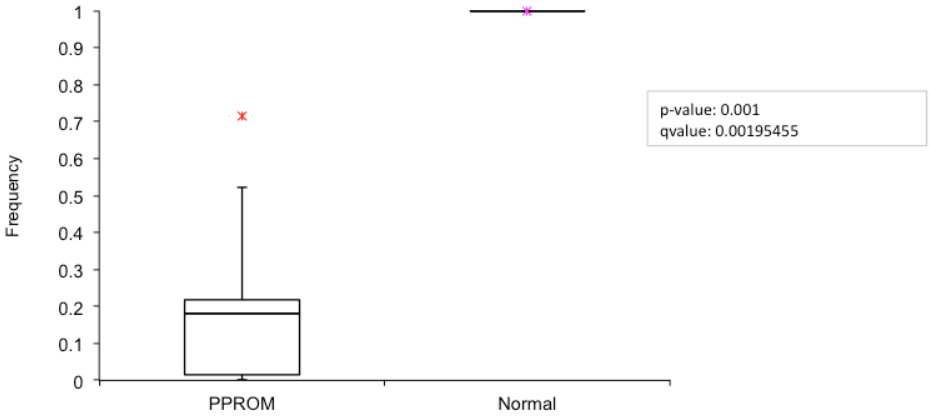

Figure 3. Boxplot representing the *Lactobacillus* frequency in the vaginal swabs of 9 PPROM subjects at presentation before the administration of antibiotic treatment. Normal is represented by the vaginal swabs of 12 subjects with uncomplicated pregnancies at approximately 29 weeks of gestation. Statistical significance found through linear regression with permutation.

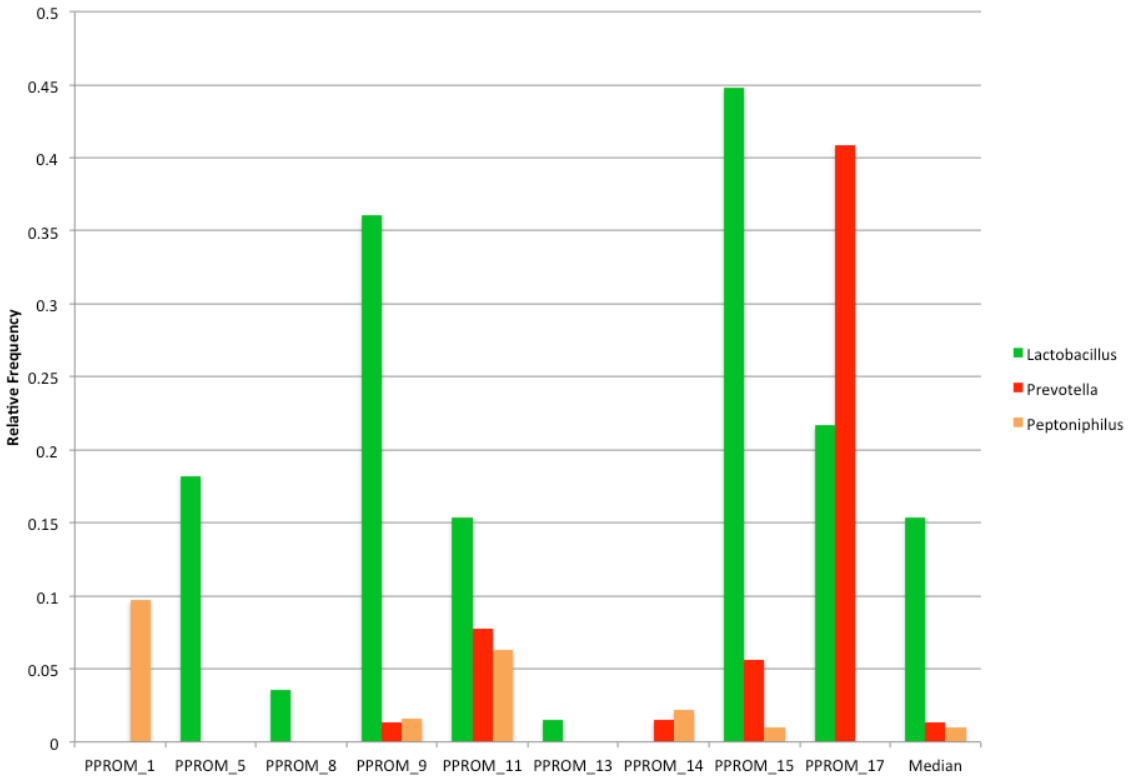

Figure 4. Summary (Median >1% relative abundance) of taxa at the genus level from all PPROM subjects at presentation before the administration of antibiotic treatment (9 subjects).

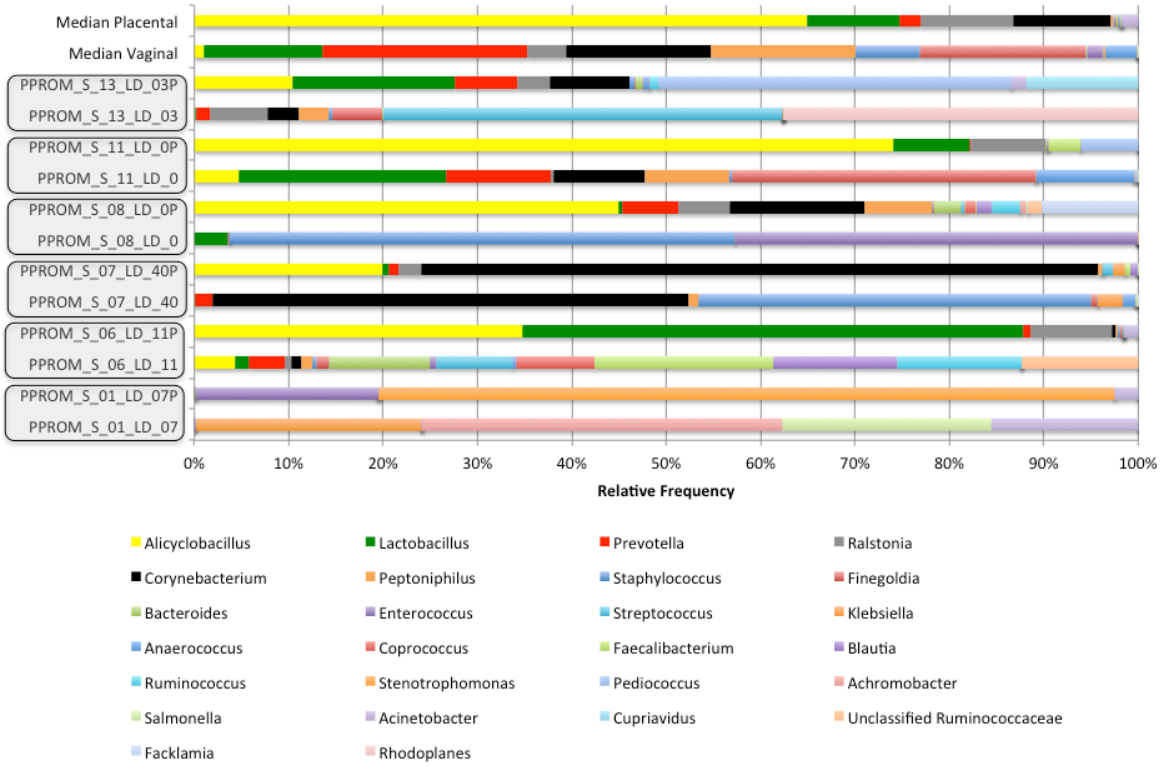

Figure 5. Placental and maternal microbiome taxa variation. S – Subject; LD – Latency Day; P – Placental sample.