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

Elizabeth A Baldwin, Marina Walther-Antonio, Allison M MacLean, Daryl M Gohl, Kenneth B Beckman, Jun Chen, Bryan White, Douglas J 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. Since subclinical infections can be facilitated by dysbiosis, 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, microbiota alteration, 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, microbiota changes, 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.

1   **Persistent Microbial Dysbiosis in Preterm Premature Rupture of Membranes from**

2   **Onset until Delivery**

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28   **Abstract**

29   **Background.** Preterm Premature Rupture of Membranes (PPROM) is a major leading cause of

30   preterm births. While the cause for PPROM remains unidentified, it is anticipated to be due to

31   subclinical infection, since a large proportion of PPROM patients display signs of

32   chorioamnionitis. Since subclinical infections can be facilitated by dysbiosis, our goal was to

33   characterize the vaginal microbiome and amniotic fluid discharge upon PPROM, through latency

34   antibiotic treatment, and until delivery, to detect the presence of pathogens, microbiota alteration,

35   and microbial response to treatment.

36   **Methods.** Enrolled subjects (15) underwent routine institutional antenatal care for PPROM,

37   including the administration of latency antibiotics. Serial vaginal swabs were obtained from

38   diagnosis of PPROM through delivery and the sequencing of the V3-V5 region of the 16S rRNA

39   gene was performed for all collected samples.

40   **Results.** The results show that Lactobacilli species were markedly decreased when compared to

41   vaginal swabs collected from uncomplicated pregnancy subjects with a matched gestational time.

42   *Prevotella and Peptoniphilus* were the most prevalent taxa in PPROM subjects at presentation.

43   The vaginal microbiome of the PPROM subjects varied substantially intra- and inter-subjects.

44   Several taxa were found to be significantly reduced during and after the antibiotic treatment:

45   *Weeksella, Lachnospira, Achromobacter,* and *Pediococcus*. In contrast, *Peptostreptococcus* and

46   *Tissierellaceae ph2* displayed a significant increase after the antibiotic treatment. However, the

47   relative abundance of *Lactobacillus, Prevotella,* and *Peptoniphilus* was not substantially

48   impacted during the hospitalization of the PPROM subjects. The deficiency *of Lactobacillus,* and

49   constancy of known pathogenic species, such as *Prevotella* and *Peptoniphilus* during and after

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

51   approaches.

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53   subclinical infection is a crucial factor in the pathophysiology of PPROM because 25-40% of

54   patients present signs of chorioamnionitis on amniocentesis. Here we sought to directly assess

55   the bacterial content of the vagina and leaking amniotic fluid of subjects at presentation,

56   throughout treatment and up until delivery, in order to search for common pathogens, microbiota

57   changes, and microbial response to latency antibiotic treatment. We have found that the vaginal

58   microbiome of PPROM subjects is highly variable and displays significant changes to treatment.

59   However, the unchanging deficiency *of Lactobacillus,* and persistence of known pathogenic

60   species, such as *Prevotella* and *Peptoniphilus* from presentation, through antibiotic treatment and

61   up until delivery, highlights the persistent dysbiosis and warrants further investigation into

62   mitigating approaches.

63

64   **Introduction**

65   Preterm birth is the single leading cause of neonatal morbidity and mortality in the

66   developed world, and despite efforts to identify causes, preventative strategies and treatment

67   options, the incidence of preterm birth continues to rise in the United States(Goldenberg et al.

68   2008). Preterm premature rupture of membranes (PPROM) directly causes one third of all

69   preterm births (Mercer et al. 2000). Subclinical infection likely plays a key role in the

70 pathophysiology of PPROM and subsequent onset of preterm labor, as evidenced by the fact that

71 25-40% of patients with PPROM present signs of chorioamnionitis on amniocentesis (Simhan &

72 Canavan 2005). Furthermore, specific microbes associated with bacterial vaginosis (BV) such as

73 *Gardnerella vaginalis* and *Mycoplasma hominis* have been linked to pregnancy complications,

74 including PPROM, preterm birth (Leitich et al. 2003); (McDonald et al. 1991); (Romero et al.

75 2002); (Hay et al. 1994), and intra-amniotic infections (Mendz et al. 2013). Attempts to prevent

76 preterm birth by using antibiotics prior to the onset of labor have not been very efficacious

77 (Oliver & Lamont 2013); (Hauth et al. 1995); (Brocklehurst et al. 2013). This suggests that our

78 current knowledge of the vaginal microbes associated with adverse pregnancy outcomes, mainly

79 based on clinical and traditional culture techniques remains incomplete (White et al. 2011).

80   Recently, the advent of next generation sequencing techniques such as 16S rRNA gene

81 hypervariable tag sequencing has allowed a more complete characterization of the vaginal

82 microbial ecology also known as the microbiome. Studies using 16S rRNA gene technology

83 have demonstrated a broad spectrum of microbial organisms not previously identified in the

84 vagina using traditional culture techniques (Fredricks et al. 2005); (Zhou et al. 2004); (Ravel et

85 al. 2010). In studies of healthy non-pregnant women, the vaginal microbiome is characterized by

86 five community profiles or subtypes most primarily dominated by a mixture of *Lactobacillus*

87 species (Ravel et al. 2010). During normal pregnancy, this dominance broadens and the

88 microbial diversity undergoes a marked decrease with convergence toward a single subtype

89 (Aagaard et al. 2012); (Romero et al. 2014b); (Walther-António et al. 2014). Although, the

90 vaginal microbiome changes in relative abundance of taxa experienced by women that delivered

91 at term has not been shown to be significantly different from women that delivered preterm

92 (Romero et al. 2014a), higher absolute abundances of particular microorganisms such as

93   *Leptotrichia/Sneathia, Mobiluncus spp.* (Nelson et al. 2014), and *Mycoplasma* (Wen et al. 2014)

94   have been correlated to preterm deliveries. It is therefore intriguing that increased diversity in the

95   vaginal microbiome in non-pregnant women correlates with clinical disease such as BV, which is

96   also correlated with adverse pregnancy outcomes (Oliver & Lamont 2013); (Oakley et al. 2008);

97   (Flynn et al. 1997); (Hauth et al. 2003). Despite this, little is known about the vaginal

98   microbiome and amniotic fluid discharge associated with pregnancy complications such as

99   PPROM.

100   After diagnosis of PPROM at a gestation of 34 weeks or less, the administration of

101   antibiotics has been shown to prolong pregnancy latency and improve short-term neonatal

102   outcome (Kenyon et al. 2013); (Hutzal et al. 2008). However, although outcomes are improved

103   by antibiotic administration, the majority of women still enter spontaneous preterm labor soon

104   after PPROM, and maternal and neonatal infectious morbidity is not eliminated (Simhan &

105   Canavan 2005); Epstein, (Epstein et al. 1998); (Soraisham et al. 2009). This suggests that there

106   are residual microbial factors involved after broad-spectrum antibiotics. In order to directly

107   characterize the vaginal and amniotic fluid discharge at a PPROM event, we have taken a

108   metagenomic approach and used next generation sequencing techniques. We continued the

109   sampling throughout the subjects' hospitalization up until delivery to capture the microbiome

110   changes throughout the treatment and delivery events.

111

112   **Materials and Methods**

113   **Ethics Statement**

114   Subjects were consented under IRB #12-001675, which was reviewed and approved by the Mayo

115   Clinic Institutional Review Board. All subjects provided written consent.

116

Subjects Enrollment

118 Here we report the results from 15 subjects enrolled upon admission for PPROM to the Mayo

119 Clinic Hospital – Rochester. Inclusion criteria consisted of the following: age >18 years; no

120 known pregnancy complications prior to admission; ability to provide written informed consent;

121 willingness to participate in mandatory translational research component of the study; weight

122 greater than 50 kilograms; and confirmed preterm rupture of membranes based on clinical

123 criteria. Exclusion criteria consisted of the following: known immunodeficiency; chronic active

124 viral infection (including HIV, HTLV and hepatitis); known autoimmune disease; solid organ or

125 transplant recipient; and multiple gestations. Upon enrollment, participations were requested to

126 fill out a questionnaire detailing sexual and reproductive health history and hygiene practices.

127 Selected questionnaire data is available in Supplemental Table 1. Metadata from the

128 questionnaires was stored using REDCap (Harris et al. 2009). Relevant medical information

129 related to the time of labor and delivery is also included in Supplemental Table 1. All subjects

130 were managed according to the standard institutional protocol for PPROM. This included

131 hospitalization, administration of a course of antenatal steroids if <34 weeks gestation on

132 admission and a standardized course of latency antibiotics (12 subjects received

133 ampicillin/amoxicillin and azithromycin; two subjects received clindamycin and azithromycin

134 due to penicillin allergy; and one subject received penicillin alone due to presentation in active

135 labor). Delivery was undertaken for spontaneous preterm labor, nonreassuring maternal or fetal

136 status, clinical concern for infection, or when a gestational age of 34 weeks was reached.

137

138 **Sample Collection**

139   Dacron swabs were collected during sterile speculum exam performed by an obstetrician from

140   the posterior vaginal fornix and placed in a Nucleic Acid Transport collection tube. After

141   collection the samples were stored at -80℃ until processing. Vaginal samples were collected at

142   the time of admission, every three days for the first two weeks of admission and weekly

143   thereafter until delivery. An additional vaginal sample was collected at the time of delivery and

144   a swab from the fetal surface of the placental membranes after delivery for 6 of the 15 subjects.

145

146   **Sample Processing**

147   Samples were thawed and centrifuged for 10 minutes at 10 000g to collect the bacterial cells, and

148   the supernatant was discarded. Genomic DNA extraction was performed by using the MoBio

149   Ultraclean Soil Kit (MoBio Laboratories, Inc., Carlsbad, CA); with the MP FastPrep (MP

150   Biomedicals, Solon, OH) for 40 seconds at 6.0m/s. Incubation period was done for a minimum

151   of 30 minutes. After extraction the DNA content was measured using High Sensitivity Qubit

152   (Life Technologies Corporation, Carlsbad, CA) with the results ranging from below detection to

153   23 ng/ul of DNA. The V3-V5 region of the 16S rRNA was then amplified using a two-step

154   polymerase chain reaction (PCR) protocol. The primary amplification was done using the

155   following recipe: 3 μl template DNA, 0.5 μl nuclease-free water, 1.2 μl 5x KAPA HiFi buffer

156   (Kapa Biosystems, Woburn, MA), 0.18 μl 10 mM dNTPs (Kapa Biosystems, Woburn, MA), 0.3

157   μl DMSO (Fisher Scientific, Waltham, MA) , 0.12 μl ROX (25 μM) (Life Technologies,

158   Carlsbad, CA), 0.003 μl 2000x SYBR Green, 0.12 μl KAPA HiFi Polymerase (Kapa

159   Biosystems, Woburn, MA), 0.3 μl forward primer (10 μM), 0.3 μl reverse primer (μl). The

160   following cycling conditions were used: 95℃ for 5 minutes, followed by 25 cycles of 98℃ for

161   20 seconds, 55℃ for 15 seconds, 72℃ for 1 minute. The primers for the primary amplification

162 contained both 16S-specific primers (357F and 926R), as well as adapter tails for adding indices

163 in a secondary amplification. The primer sequences for the primary amplification were as

164 follows (16S-specific sequences in bold):

165 V3F_Nextera:

166 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGAGGCAGCAG**

167 V5R_Nextera:

168 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CCGTCAATTCMTTTRAGT**

169 Next, these amplicons were diluted 1:100 in sterile, nuclease-free water, and a second PCR

170 reaction was set up to add the Illumina flow cell adapters and indices. The secondary

171 amplification was done using the following recipe: 5 μl template DNA, 1 μl nuclease-free water,

172 2 μl 5x KAPA HiFi buffer (Kapa Biosystems, Woburn, MA), 0.3 μl 10 mM dNTPs (Kapa

173 Biosystems, Woburn, MA), 0.5 μl DMSO (Fisher Scientific, Waltham, MA), 0.2 μl KAPA HiFi

174 Polymerase (Kapa Biosystems, Woburn, MA), 0.5 μl forward primer (10 μM), 0.5 μl reverse

175 primer (μl). The following cycling conditions were used: 95°C for 5 minutes, followed by 10

176 cycles of 98°C for 20 seconds, 55°C for 15 seconds, 72°C for 1 minute, followed by a final

177 extension at 72°C for 10 minutes. The indexing primers are as follows (X marks the positions of

178 the 8 bp indices):

179 Forward indexing primer:

180 **AATGATACGGCGACCACCGA**GATCTACACXXXXXXXXTCGTCGGCAGCGTC

181 Reverse indexing primer:

182 **CAAGCAGAAGACGGCATACGA**GATXXXXXXXXGTCTCGTGGGCTCGG

183 The products of the amplification were quantified using a PicoGreen dsDNA assay (Life

184 Technologies, Carlsbad, CA), and the samples were normalized, pooled, and approximately 1 μg

185   of material was concentrated to 10 µl using 1.8x AMPureXP beads (Beckman Coulter, Brea,

186   CA). The pooled sample was then size selected at 723 bp +/- 20% on a Caliper XT DNA 750

187   chip (Caliper Life Science, Hopkinton, MA). The size-selected material was cleaned up using

188   AMPureXP beads, and eluted in 20 µl of EB buffer (10 mM Tris-HCl, pH 8.5). The final pooled

189   sample was quantified using the PicoGreen dsDNA assay, and analyzed using an Agilent

190   Bioanalyzer High Sensitivity Chip (Agilent Technologies, Santa Clara, CA). Finally, the sample

191   pool was diluted to 2 nM based on the PicoGreen measurements, and 10 µl of the 2 nM pool was

192   denatured with 10 µl of 0.2 N NaOH, diluted to 8 pM in Illumina's HT1 buffer, spiked with 20%

193   phiX, heat denatured at 96°C for 2 minutes, and sequenced using a MiSeq 600 cycle v3 kit

194   (Illumina, San Diego, CA).

195

196   **Processing of Controls**

197   Control samples consisted of five "empty collection" replicates. In brief, five swabs were

198   immersed in the collection buffer and DNA was extracted, amplified, and sequenced using the

199   same procedures and reagents used for the PPROM samples. In addition, three other controls

200   were performed: 1) one extraction with only the collection buffer was performed (no swab), 2) a

201   PCR negative control was sequenced, 3) a positive control of a pure isolate of *Campylobacter*

202   *jejuni*. There was no detectable amplification in the negative controls by qPCR. The positive

203   control (*Campylobacter jejuni*) was carried forward to sequencing as well to guarantee that the

204   negative controls were sequenced at comparable depth. The eight control samples were spiked

205   into a MiSeq run at a comparable per-sample concentration to that of the PPROM sample MiSeq

206   run. As expected, the extraction and water negative controls yielded much lower numbers of

207   reads (between 0.0084% and 0.0406% of the total reads in the run).

208

209   **Sequencing Processing and Outcome**

210   To remove the 5'-Adapter-primer sequence on forward reads which has a total length of 58 bps,

211   for each forward read, we took 60 bps from the 5' end and BLAST against the 5'-Adapter-primer

212   sequence allowing one base mismatch, and the matched region of the reads were removed if

213   present at the beginning of the sequence read. To remove the 5'-Adapter-barcode-primer

214   sequence on reverse reads which has a total length of 65 bps, we first generated a group of

215   sequences, through a combination of different barcodes and wobbly bases. There are a total of 73

216   samples taken from 15 subjects, each sample with a unique barcode. And there are two wobbly

217   bases M and G in the sequence, each of which denotes an alternative base of A or C and A or G

218   respectively. Thus the total number of sequence combinations is 77 x 2 x 2=292. We created a

219   BLAST database from the 292 sequences. Then, for each reverse read, we took 67 bps from the

220   5' end and BLAST against the created database allowing one base mismatch except for the

221   barcode region. The matched region of each read was removed if present at the beginning of the

222   sequence read and the samples were de-multiplexed by the matched barcode on each read. Due

223   to the low number of R2 samples passing quality control (at least 187bp per sequence read and a

224   minimum of 2,000 sequence reads per sample), only R1 reads were used for analysis. A total of

225   5,575,178 R1 sequence reads (5,527 to 174,479 sequence reads per sample) passed quality

226   control.

227

228   **Sequence Analysis**

229   Sequence reads were aligned with our own custom multiple alignment tool known as the Illinois-

230   Mayo Taxon Operations for RNA Dataset Organization (IM-TORNADO) that merges paired end

231   reads into a single multiple alignment and obtains taxa calls (Sipos et al. 2010). Operational

232   taxonomic units were clustered using UPARSE (Edgar 2013). Further processing for

233   visualization and statistical analysis was performed using QIIME (Caporaso et al. 2010). To

234   identify differential abundant bacteria genera, we fit a linear regression model to the square-root

235   transformed genus proportion data. We used the t-statistic as the test statistic. To address the

236   non-normality of the outcome variable as well as within-subject correlation, we used permutation

237   to assess the statistical significance. Permutation was performed 1,000 times and was

238   constrained within the subject to retain the original correlation structure. We also fit a

239   generalized mixed effects model to the genus presence/absence data using PQL method,

240   assuming a random intercept for each subject. Statistical significance was assessed based on

241   Wald test. False discovery control (B-H procedure) was used for correcting multiple testing.

242   Sequences are publicly available at SRA, study accession: SRP061714. The taxonomic

243   assignments are provided in Supplemental Table 2.

244

245   **Results**

246   Our cohort consisted of 15 subjects (Table 1; age range: 19-37 years old; Mean ±

247   Standard Deviation: 29 ± 4 years old). The majority were Caucasian (14), and one was East

248   Asian. Within the cohort, there were no complications specific to pregnancy prior to or

249   subsequent to diagnosis of PPROM including no diagnoses of gestational diabetes or

250   hypertensive disorders of pregnancy.   The cohort included 10 parous and 5 nulliparous women,

251   with four subjects having a history of preterm delivery with a previous pregnancy. From these 15

252   subjects, a total of 61 vaginal samples and 6 placental samples were collected (Fig 1; selected

253   metadata associated with each subject can be found in Table S1).

254   PPROM Before Antibiotic Treatment. In order to assess the näive maternal microbiome at time

255   of presentation with PPROM, we examined the admission samples from nine subjects that were

256   obtained before the administration of antibiotics and compared them with those of a companion

257   study, where 12 women experiencing an uncomplicated pregnancy were sampled longitudinally

258   (Walther-António et al. 2014). Using gestational age-matched samples from the companion

259   study we compared the vaginal microbiome taxonomic assignments to the PPROM subjects. The

260   microbiome in the PPROM subjects exhibited a diversity of taxa that was in stark contrast to the

261   normal pregnancy subjects (Fig 2), and was marked by an underrepresentation of lactobacilli

262   (Fig 3). Overall, the most common pathogens detected in the PPROM subjects before the

263   administration of antibiotics were *Prevotella* and *Peptoniphilus* (Fig 4).

264   PPROM During and After Antibiotic Treatment. The administration of antibiotics did not

265   significantly impact the relative abundance of *Lactobacillus, Prevotella,* or *Peptoniphilus* during

266   or after the antibiotic treatment (Table 2 and Table 3). However, during the administration of the

267   treatment, *Weeksella, Lachnospira, Achromonacter* and *Pediococcus* showed a significant

268   decrease, while *Peptostreptococcus* and *Tissierellaceae ph2* showed a significant increase (Table

269   2 shows presence/absence significance most powerful for low abundance taxa, Table 3 shows

270   proportion significance more powerful for high abundance taxa). We also compared the Bray-

271   Curtis (BC) and UniFrac distances (with similar results) to address relevant questions. One of

272   them was whether there was a statistical difference in the vaginal microbiome of subjects that

273   underwent a cesarian-section (C-section) when compared to subjects that had a normal

274   spontaneous vaginal delivery (NSVD). We have 5 subjects that underwent a C-section and 10

275   with a NSVD. PERMANOVA based on BC distance produced a p-value of 0.23. Therefore, we

276   do not have enough supporting evidence that the delivery mode affects the microbiome. We

277   further enquired whether parity was a determining factor for the vaginal microbiome. There are

278   10 parous subjects (parity > 0) and 5 nulliparous subjects (parity=0). PERMANOVA based on

279   BC distance produced a p-value of 0.20. Therefore, we do not have enough supporting evidence

280   that parity influences the vaginal microbiome.

281   Placental Microbiome. To assess the placental microbiome, we compared the six placental

282   samples with the matched maternal vaginal samples obtained at the time of delivery. The

283   placental microbiome was statistically distinct from the vaginal microbiome (Bray Curtis

284   distance p-value 0.08 – PERMANOVA, within subject 1,000 permutations). However, given the

285   limited amount of samples, there was insufficient statistical power to identify differential taxa.

286   Placental samples showed high individual variability and weak correlation with the maternal

287   vaginal microbiome (Fig 5). The most prevalent taxa recovered were *Alicyclobacillus* and

288   *Corynebacterium*. In order to determine if the detected placental microbiome could be the result

289   of contamination from the vaginal microbiome we performed a permutation test and compared

290   the BC and Unifrac distances. There are six subjects that have matched placental and vaginal

291   samples. Two subjects underwent a C-section and four subjects had a NVSD. We do not see that

292   the placental swabs from the vaginal deliveries are significantly closer to the vaginal swabs than

293   the placental swabs from C-sections (p=0.53). However, one placental swab from a NVSD

294   subject (PPROM_7) has potential contamination indicated by a very small distance (0.48) when

295   compared to the range among the remaining NSVD distances (0.97-0.99) and C-sections (0.88-

296   0.91). We also looked into whether the delivery vaginal microbiome was the most similar to the

297   placental swab or if the placental swab was rather closer to a previous vaginal swab from the

298   same subject. There are four subjects with multiple vaginal swabs and a placental swab. By

299   comparing the distance from the placental swab to the delivery swab, to the distance from the

300   placental swab to other vaginal swabs within the same subject, we see a trend that the

301   placental swab is more similar to the last delivery sample (p=0.078, permutation test, shuffling

302   the vaginal swabs within the same subject).

303   Controls. The sequencing of our controls – Negative PCR control, five quality control (QC)

304   replicates (empty swab collections) and the DNA extraction and amplification of the TE buffer

305   used for the sample collection – showed very little overlap with the major taxa seen in the study

306   samples (Supplemental Table 3).

307

308   **Discussion**

309

310   **Principal findings of the study.** The analysis of the vaginal microbiome of 15 PPROM subjects

311   from presentation, throughout their hospital stay and treatment, and up until delivery, shows that

312   the microbiome is dysbiotic and highly variable between subjects since presentation. The

313   antibiotic treatment administered for the condition did not eliminate the presence of pathogenic

314   species such as *Prevotella* and *Peptoniphilus*, which remained until the time of delivery, as did

315   the deficiency in lactobacilli species.

316   Our results also show that the vaginal microbiota of pregnant subjects with PPROM is

317   distinct from that of subjects undergoing an uncomplicated pregnancy. The findings indicate that

318   the vaginal microbiome after PPROM is more diverse and dynamic than previously observed

319   during normal pregnancies (Walther-António et al. 2014). Of specific interest is the difference in

320   lactobacilli abundance in PPROM. Lactobacilli acidify their environment and limit diversity

321 through a process of niche expansion—allowing for the dominance of lactobacilli in most

322 vaginal microbiota observed to date. The loss of lactobacilli, along with many other factors, may

323 place a key role in the destabilization of the PPROM-associated vaginal microbiome. This

324 depletion may be due to the displacement either by the constant leakage of alkaline amniotic

325 fluid or may precede the PPROM event. It is also worth emphasizing that although the swabs

326 were physically placed in the posterior fornix for the sampling, this was likely to contain varying

327 amounts of amniotic fluid in PPROM subjects. Once there is a rupture of the membranes,

328 amniotic fluid continues to leak from the cervix and when a patient is in the supine position the

329 posterior fornix is where that fluid will collect. The patients remain hospitalized until delivery,

330 and the samples were obtained in the patient's hospital bed. So unless the patient had been

331 ambulating just prior to sampling, there will be some amniotic fluid pooled in the posterior

332 fornix. Once lactobacilli are displaced, opportunistic bacteria may occupy the available niche

333 leading to an unbalanced microbial ecology (dysbiosis) with potentially negative consequences

334 for health. The transient nature of the PPROM microbiome is consistent with the idea of an

335 ecology that is out of equilibrium. *Prevotella* and *Peptoniphilus* emerge as taxa of particular

336 interest in PPROM given their prevalence at presentation and persistence throughout treatment,

337 evidence of prominent role in persistent bacterial vaginosis and preterm labor (Marrazzo et al.

338 2008); (Smayevsky et al. 2001); (Wang et al. 2013); (Mikamo et al. 1999) and display of broad-

339 spectrum antibiotic resistance (Sherrard et al. 2013); (Tanaka et al. 2006). The highly variable

340 placental microbiome recovered and the weak correlation with the maternal microbiome raises

341 the possibility that the uterine microbiome may be an independent driver of the placental

342 microenvironment. The formation of the cervical mucus plug as early as seven weeks of

343 gestation provides a mechanical and chemical barrier between the vaginal and uterine

344   environments (Hein et al. 2002). The influence of the vaginal microbiome in the uterine

345   microbial niche is therefore anticipated to be reduced for as long as the mucus plug is intact. The

346   fact that in this study *Prevotella* was a prominent pathogen found in the vaginal fluid discharge

347   and placental membranes indicates a very likely role in the etiology of PPROM. The known

348   association of *Prevotella* with bacterial vaginosis (Hillier et al. 1993) and preterm labor (Holst et

349   al. 1994) strengthen this possibility. It is however, important to note that preterm labor and

350   PPROM are distinct conditions. While PPROM often leads to preterm labor, preterm labor can

351   be caused by a multitude of conditions unrelated to PPROM. It is therefore not surprising that

352   preterm labor studies (Romero et al. 2014a); (Mendz et al. 2013) may or may not align with our

353   findings.

354   Our study is limited by the small number of patients. Another limitation is that the

355   vaginal swabs collected from PPROM subjects likely contained both amniotic fluid and vaginal

356   fluid. We are unable to determine the proportion of amniotic fluid in each sample, which is

357   expected to vary with each collection. By contrast, the vaginal swabs collected from

358   uncomplicated pregnancy subjects are not anticipated to have had a significant presence of

359   amniotic fluid.

360   Despite the low amount of microbial DNA present in the samples, we were able to rule

361   out that the relevant taxa in this study could be the result of contamination. As shown in Table 3

362   we were able to find several well-known contaminants (Salter et al. 2014), but not the taxa

363   relevant to our study with one exception, *Corynebacterium*. This taxa was predominant in the

364   placenta of our PPROM subjects, which supports previous findings in the placenta of preterm

365   deliveries (Oh et al. 2010). It is possible that its presence may be overemphasized in this study

366   due to its detection in the collection buffer, but it is undoubtedly present in the study samples as

367   well.

368

369   **Conclusions**

370   The deficiency in lactobacilli species and persistence of known pathogenic species at

371   admission, and during and after antibiotics, highlights a marked dysbiosis with high individual

372   variability. An interesting area of future research would be to assess the changes in the

373   microbiome noted in our study as a potential causative factor predating diagnosis of PPROM by

374   longitudinal assessment of the vaginal microbiome in pregnant women at high risk for preterm

375   premature rupture of membranes.

376

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505   vaginal microbial communities in adult healthy women using cultivation-independent
506   methods. *Microbiology* 150:2565-2573.

507

508 **Table Legends**

509

510 **Table 1.** Demographics and clinical data for all subjects enrolled. SD - Standard Deviation;
511 Gravid Status - Number of prior pregnancies; Parity - Number of prior deliveries; NSVD -
512 Normal Spontaneous Vaginal Delivery.

513

	Range	Mean (SD)
Age (years)	19 - 37 years	29 years (± 4)
Gestational age (weeks)		
On admission	23 $^1/_7$ to 34 $^5/_7$ weeks	30 $^5/_7$ weeks (± 3.6)
At delivery	28 $^1/_7$ to 34 $^6/_7$ weeks	32 $^5/_7$ weeks (± 1.9)
Latency period (days)	0 – 58 days	15 days (± 17.4)
Vaginal samples collected (no.)	1 - 11	4 (± 3.2)
Gravid status	1 - 4	2 (± 1)
Parity	0 - 3	1 (± 1) - 33% Nulliparous
Racial/ethnic background (subjects)		
White or Caucasian – 14 (93%)		
Near East Asian – 1 (17%)		
Type of delivery		
NSVD – 10 (67%)		
C-section – 5 (33%)		
Evidence of chorioamnionitis		
4 (27%)		

514 **Table 2.** Taxa displaying significant changes before, during, and after antibiotic treatment*.

Taxa (Phylum, Classe, Order, Family, Genus)	Before vs During Antibiotic Treatment			During vs After Antibiotic Treatment			Before vs After Antibiotic Treatment		
	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

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

517 [1]Statistical significance was assessed based on Wald test.

518 [2]False discovery control Benjamini-Hochberg (B-H procedure) was used for correcting multiple testing (qvalue<0.2 (false discovery rate)
519 considered significant).

520 **Table 3.** Significant taxa shifts before, during, and after antibiotic treatment.

521

	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	-

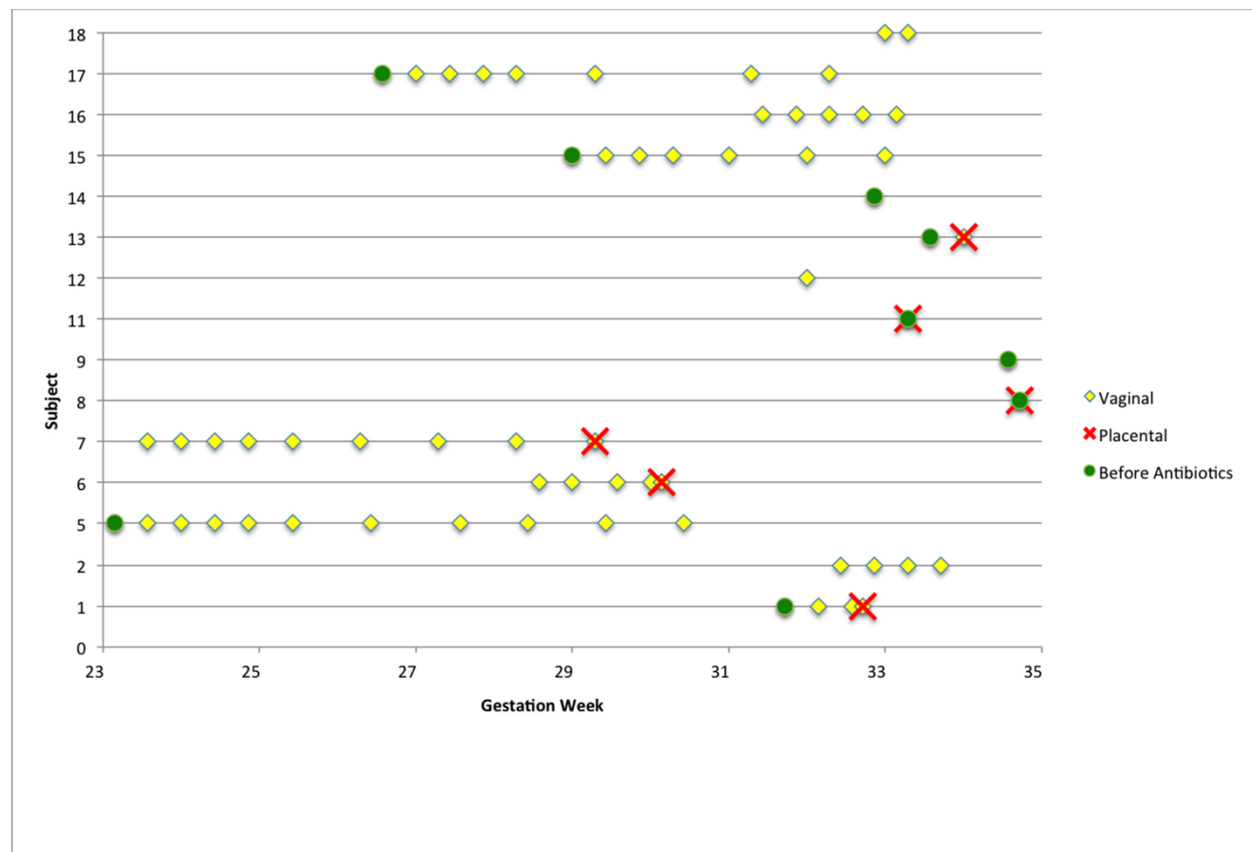
522 [*] A Linear regression model was fit to the square-root transformed genus proportion data. To address the non-normality of the outcome
523 variable as well as within-subject correlation, permutation (1,000 times) was used to assess the statistical significance. Permutation was
524 constrained within each subject to retain the original correlation structure.
525
526 [1]Statistical significance was assessed by permutation

527 [2]False discovery control Benjamini-Hochberg (B-H procedure) was used for correcting multiple testing (qvalue<0.2 (false discovery rate)
528 considered significant).

529

530 **Figure Legends**

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

532

533 **Figure 2.** Comparison of relative abundance of taxa at the genus level between vaginal swabs of

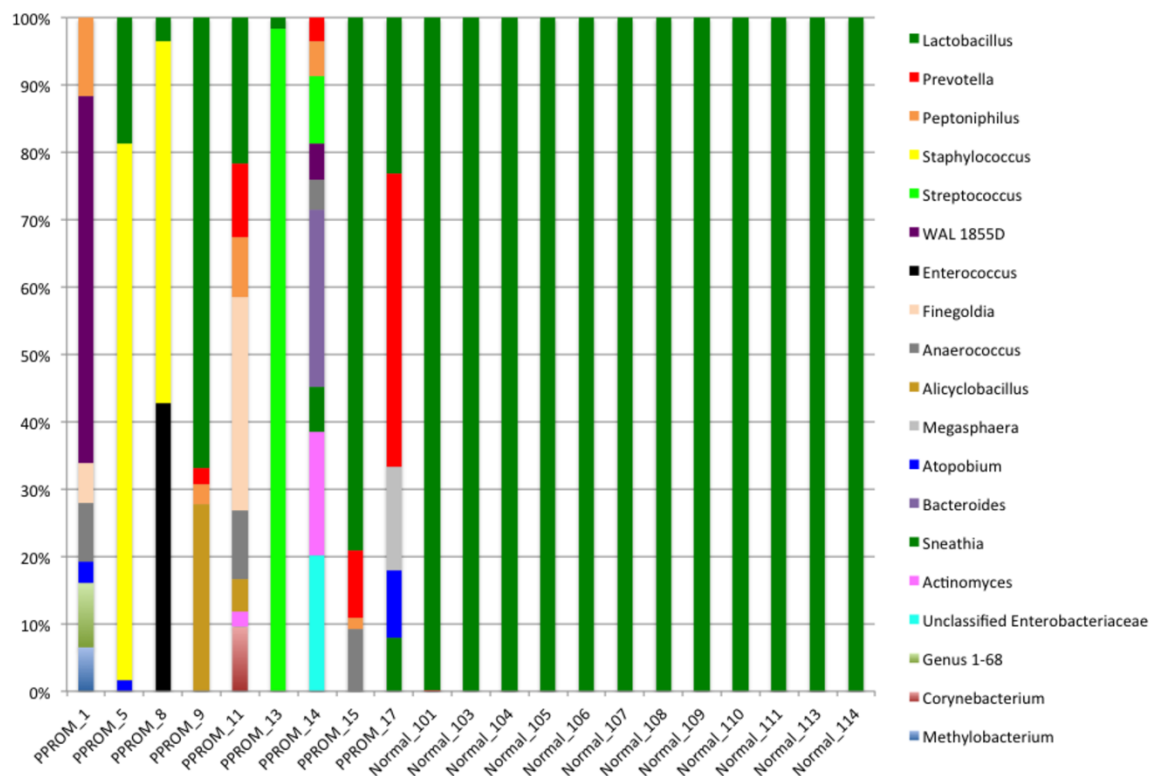
534 subjects at the time of presentation at the emergency room with PPROM before the

535 administration of antibiotic treatment (9 subjects) and vaginal swabs from subjects that

536 underwent an uncomplicated pregnancy at approximately 29 weeks of gestation (12 subjects).

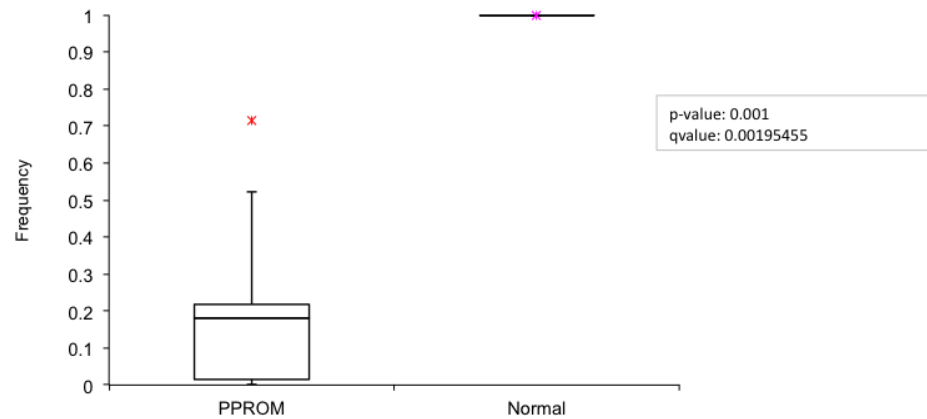
537 The lack of lactobacilli dominance in PPROM subjects is apparent, as is the inter-individual

538 variation. Only taxa at >1% relative abundance in the PPROM subjects are shown for graphical

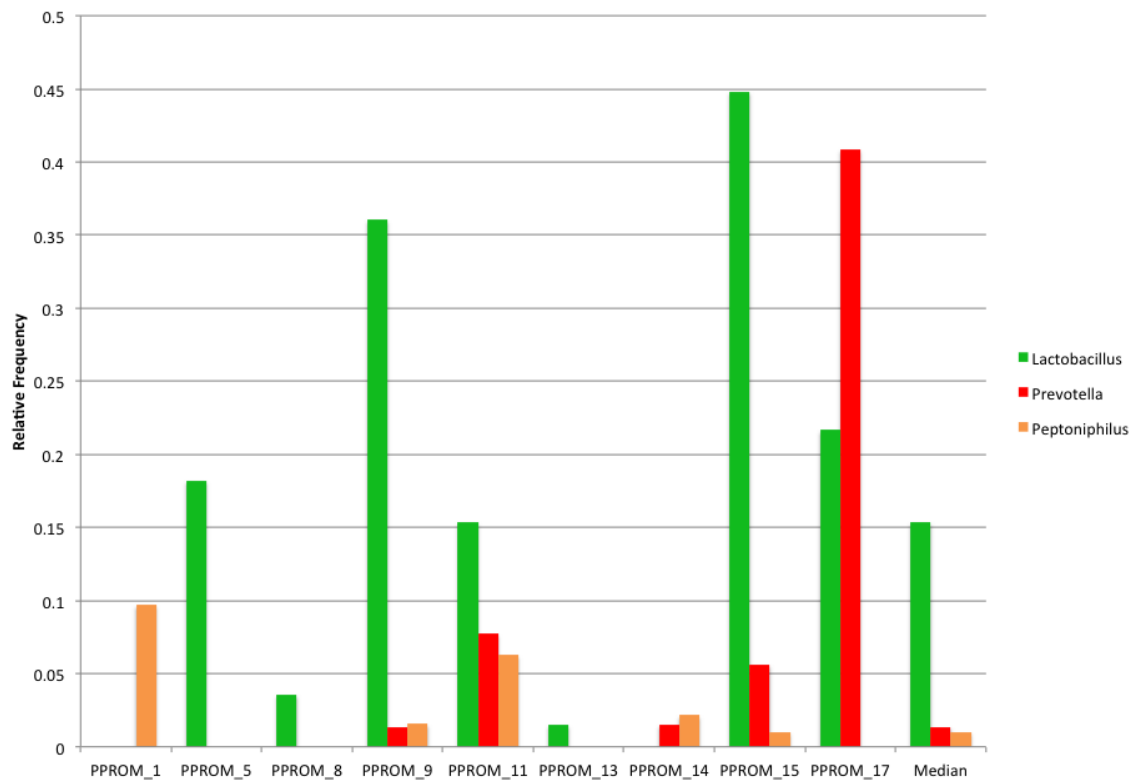
539 clarity.

540
541 **Figure 3.** Boxplot representing the *Lactobacillus* frequency in the vaginal swabs of 9 PPROM

542 subjects at presentation before the administration of antibiotic treatment. Normal is represented

543 by the vaginal swabs of 12 subjects with uncomplicated pregnancies at approximately 29 weeks

544 of gestation. Statistical significance found through linear regression with permutation.

545
546 **Figure 4.** Summary (Median >1% relative abundance) of taxa at the genus level from all

547 PPROM subjects at presentation before the administration of antibiotic treatment (9 subjects).

548

549
550   **Figure 5.** Placental and maternal microbiome taxa variation. S – Subject; LD – Latency Day; P –

551   Placental sample.

552

553

554