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Spatial characterisation of the reproductive tract microbiota indicates high prevalence of microbial driven aetiology in preterm prelabour rupture of the fetal membranes (PPROM)

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Article

Keywords: PPROM, preterm birth, microbiome, ascending infection, inflammation, microbiota-host immune response

Posted Date: November 1st, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11285726/v1

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Additional Declarations: Yes there is potential Competing Interest. PRB and DAM hold a patent to the use of Lactobacillus crispatus in pregnant women (International Application No. PCT/GB2022/050453; International Publication No. WO 2022/175680). DAM is a Scientific Advisory Board member of Calla Lily Clinical Care (UK) and Freya Biosciences APS (Denmark).

Abstract

A high-diversity vaginal microbiome is a risk factor for preterm prelabour rupture of the fetal membranes (PPROM) and preterm birth, major causes of infant mortality and morbidity. Associative evidence supports a mechanism for preterm birth involving ascending vaginal pathogen infection and inflammation driving early labour. However, the relationship between the vaginal microbiome and upper reproductive tract colonisation in pregnancy remains poorly described. Using spatial metataxonomic characterisation of reproductive tract microbiota during pregnancy and delivery, we found higher bacterial load ($p < 0.0001$) and higher pathogen detection in fetal membranes (FM) from PPROM patients compared to term controls (12/15,80% v 9/24,38%; $p < 0.01$, $\chi 2$ test). FM levels of proinflammatory cytokines, MMP9 and complement system proteins were increased in PPROM, particularly in pathogen-enriched samples. Temporal and between-site comparison of strain-level data indicated the vagina as a likely source of upper reproductive tract pathogen colonisation in some cases. Our findings demonstrate that pathogen colonisation of the fetal membranes can occur in the presence of a *Lactobacillus*-dominated vaginal microbiome, suggesting pathogen driven PPROM is likely more common than previously acknowledged.

Introduction

Preterm birth (PTB), defined as delivery prior to 37 weeks of gestation, accounts for 15 million deliveries worldwide[1]. It remains the leading cause of death in children under 5 years of age[2] with survivors often suffering life-long morbidities[3]. During pregnancy, the fetal membranes (FM) serve as a protective barrier between the fetoplacental and maternal compartments[4]. Preterm prelabor rupture of the fetal membranes (PPROM) precedes around 40% of all cases of spontaneous PTB[5]. Following PPROM, 80% of women deliver within 9 days[6], during which time both mother and fetus are at a heightened risk of ascending infection associated with poor pregnancy outcomes[7–12].

PPROM is associated with an increased prevalence of high-diversity vaginal microbiota communities depleted of commensal *Lactobacillus* species, often enriched for potentially pathogenic bacteria including *Prevotella*, *Sneathia* and *Streptococcus* species. These appear to become established early in pregnancy, prior to the rupture event[13–15]. High diversity, *Lactobacillus* depleted vaginal microbiota are also predictive of early PTB[16–18]. The mechanism by which suboptimal vaginal microbiota drives PTB is thought to involve activation of local immune responses in the lower reproductive tract, which triggers an inflammatory cascade characterised by an exacerbated activation of the complement cascade[19] and increased production of chemokines, cytokines, prostaglandins and matrix metalloproteinases (MMPs), which can eventuate in PPROM[20]. Rupture of the fetal membranes subsequently exposes the uterine cavity, placenta and fetus to ascending pathogen colonisation and increases risk of poor neonatal outcomes[21] .

Whilst some studies have proposed haematogenous spread of pathogenic bacteria and subsequent colonisation of the placenta as a potential route of invasion and infection in PTB[22,23], it is widely thought that the primary route of pathogenic bacterial colonisation is ascension from the vagina[24,25]. While this paradigm has been demonstrated in animal models, for example through vaginal infection of pregnant mice with *Streptococcus agalactiae*[26], *Ureaplasma parvum*[27] and *E.coli* [28], there is no direct in-human evidence that pathogens located in the fetal membranes and placenta in PPROM originated from the vagina. Whether vaginal and upper reproductive tract microbiota are independent risk factors of PPROM and PTB remains unknown.

In this study, we spatially characterised and compared the bacterial component of the reproductive tract microbiota (vagina, fetal membranes and placenta) in PPROM and healthy pregnancies delivering at term. We also examined the relationship between microbial composition and host immune and inflammatory responses in different regions of the fetal membranes and placenta to test the hypothesis that the presence of potentially pathogenic bacteria in upper gestational tissues activate local inflammatory pathways implicated in PPROM. Finally, we undertook temporal and between-site comparison of bacterial species and strains to investigate if their detection in the upper reproductive tract at the time of delivery may have originated from earlier vaginal colonisation.

Results

Characteristics of the study cohorts

To minimise potential contamination of FM and placenta during vaginal delivery, we limited our investigations to women who had experienced PPROM and were delivered by Caesarean section prior to the onset of labour (n = 15). Our control group consisted of women with uncomplicated pregnancies delivered by Caesarean section at term, prior to the onset of labour and with intact fetal membranes (n = 24; Fig. 1A). As expected, women with PPROM had a significantly reduced gestational age at delivery (Mann-Whitney Test, $p < 0.0001$, Table 1). There were no significant differences in maternal age (Mann-Whitney Test, $p = 0.17$), ethnicity (Fisher's Exact Test, $p = 0.52$) or BMI (Mann-Whitney Test, $p = 0.71$) between PPROM and control groups.

Table 1
Demographics of the study population. Data is presented as median (interquartile range). BMI body mass index, GA gestational age. P values: Mann Whitney U test for continuous data and Fisher's exact for categorical data.

Total number	PPROM	Term	P-value
	n = 15	n = 24	
Age*	34 (32–40)	33 (31–35)	0.17
BMI*†	25 (22–28)	25 (22–29)	0.71
Ethnicity			0.52
Black	4 (27%)	4 (17%)	
Other	4 (27%)	11 (46%)	
White	7 (47%)	9 (38%)	
GA at delivery (days)*	232 (204–244)	273 (268–274)	< 0.0001
*Median (IQR);			
†Missing value in 1 term sample			

Spatial metataxonomic profiling of reproductive tract microbiota in PPROM and term pregnancies

Metataxonomic profiling was performed on matched vaginal, fetal membranes (FM) samples at the rupture, mid- and distal- to rupture sites, and placental samples from term (n = 24) and PPROM (n = 15) pregnancies. The mean sequencing read count was significantly higher for vaginal samples (27,168 ±2746 SEM) compared to FM samples rupture site (11,913 ±1137 SEM), mid-site (10,634 ±994.0 SEM) and distal site (10,905 ±959.9 SEM) and placental (9,236 ±963.6 SEM) (Mann-Whitney Test, $p < 0.0001$) (Supplementary Fig. 1). To detect the metataxonomic signature above background contamination, negative controls for delivery theatre environment (T), sample processing environment (S), as well as laboratory environments and kit reagents (L) were used to identify potential sources of contaminant ASVs.

We next compared the relative abundance of the top 20 species detected across all samples along the reproductive tract in term (Fig. 1B) and PPROM pregnancies (Fig. 1D). Most vaginal samples from term pregnancies were dominated by *Lactobacillus spp*. (17/24, 71%), specifically *Lactobacillus crispatus* (42%), or *Lactobacillus iners* (21%), with the remaining samples being dominated by *Gardnerella vaginalis* (29%). Matching FM and placental samples from these women had similar microbiota profiles to negative controls (Fig. 1B) indicating that other than very low relative abundance of *L. crispatus* and *L. iners*, there was no upper reproductive tract microbiota signature detectable in healthy term samples. In

both PPROM and term control cohorts, total bacterial load, estimated by RT-qPCR quantification of total 16S rRNA gene copies, was significantly lower in fetal membrane and placental samples compared to matched vaginal samples (Mann-Whitney Test, $p \leq 0.0001$) (Fig. 1C, E). In contrast to women delivering at term, vaginal microbiota composition of women presenting with PPROM was frequently depleted of *Lactobacillus* species and enriched with potentially pathogenic bacteria such as *Prevotella*, *Ureaplasma*, *Sneathia* and *Streptococcus* species (Fig. 1D). Pathogenic species could also be detected in the upper reproductive tract above background levels observed in negative control. In PPROM rupture site FM samples, pathogenic bacteria were detected (> 1% relative abundance) in 12/15 (80%) of samples compared to 9/24 (38%) ($p < 0.01$, $\chi 2$ test) in term control samples.

Consistent with these results, the overall bacterial composition across sample sites significantly differed depending on pregnancy outcome. In term pregnancies, vaginal samples had a significantly different composition compared to upper reproductive tract and negative control samples ($p = 0.001$, PERMANOVA), whereas in PPROM samples, negative control samples were significantly distinct from vaginal and upper reproductive tract samples ($p = 0.001$, PERMANOVA) (Fig. 2A, B). Mean bacterial load in vaginal samples was similar between term and PPROM pregnancies (6.22 ± 0.20 SEM v 6.20 ±0.22 SEM log10 copies/ml, $p = 0.7996$, Mann-Whitney test). However, bacterial load was significantly higher in FM collected from the rupture site (MR) ($p < 0.0001$), mid-site (MM) ($p = 0.0001$), distal site (MD) ($p < 0.0001$) and the placenta (PL) ($p = 0.0073$, Mann-Whitney test) in PPROM compared to equivalent sample sites in term pregnancies (Fig. 2C).

Within-patient comparison of microbiota across the reproductive tract of women who experienced PPROM showed compositional disparities between the different anatomical regions sampled (Fig. 3A). In patients with vaginal *L. crispatus* dominance (Patient 1–6 in Fig. 3A), low levels of *L. crispatus* were also often observed in the matching FM and less so in placental samples. Despite *L. iners* and *G. vaginalis* dominating the vaginal microbiota of most remaining patients, these species were rarely detected in matched FM or placental samples (Fig. 3B). For example, while *G. vaginalis* was detected at > 2% total read counts in 47% of all vaginal samples, it was only observed in 7% of membranes samples at rupture site and was not detected in any placental sample. Overall, FM and placental samples showed an increased prevalence of low read, rare taxa identified as contaminants in negative controls. Despite this, potentially pathogenic species previously associated with adverse reproductive tract health including *S. agalactiae, S. anginosus*, *S. sanguinegens* and *Escherichia-Shigella* were detected as the dominant taxa in the FM and/or placental samples of 5/15 patients. Patients with high prevalence of *S. anginosus*, *S. sanguinegens* and *Escherichia-Shigella* detected in their FM and placental samples, were also histologically diagnosed with chorioamnionitis.

Assessment of upper reproductive tract microbiota-host response in PPROM

Host immune and inflammatory response to microbiota detected in the upper reproductive tract were first investigated by assessing levels of complement proteins in available matched rupture and mid-site FM and PL samples from PPROM (n = 12 women) vs term deliveries (n = 11 women) (Fig. 4). Levels of the pattern recognition molecule C1q were significantly lower ($p \leq 0.001$, Mann Whitney test) in all FM and placental samples collected from PPROM patients compared to term (Fig. 4A). A similar trend decrease in mannose binding lectin (MBL) levels in these samples was also observed. No significant differences were seen in levels of complement proteins C2, C4, C4b and C5 in the upper reproductive tract sites between PPROM and term controls (Fig. 4A). Similar levels of C3 and C3b/iC3b levels in MR and MM were observed between PPROM and term patients, however these proteins were significantly reduced ($p \leq 0.05$) in the placenta of PPROM patients compared to term controls. Both rupture and mid-site FM samples of PPROM patients had significantly higher concentrations of C5a compared to term controls ($p = 0.03$ and $p = 0.0057$ respectfully).

To investigate if complement activation is associated with pathogen composition in PPROM, levels of complement proteins C3 and C5a were assessed in MR samples collected from PPROM patients with either an *L. crispatus*-dominated vaginal microbiota, or with a pathogenic bacterial species as the dominant taxa (Fig. 4B). These proteins were specifically chosen as activation of the classical, lectin and alternative complement pathways converge at the level of C3 and proceed along a common terminal pathway leading eventually to the release of the chemoattractant complement fragment C5a. In samples dominated by pathogens, C3 was significantly lower ($p = 0.005$, Mann-Whitney test), whereas C5a was significantly increased ($p = 0.005$) (Fig. 4C, D). Consistent with enhanced downstream complement activity, the ratio of C3:C5a was significantly lower in MR samples dominated by pathogenic bacteria ($p < 0.005$) (Fig. 4E, F). These samples were also found to have significantly higher bacterial load than MR samples dominated by *L. crispatus* ($p < 0.05$) (Fig. 4G, H).

To determine if the observed complement system activation in PPROM was associated with activation of pro-inflammatory pathways which could contribute to premature rupture of the FM, we assessed levels of TNF-α, MMP9, IL-6, IL-1 β, IL-10, G-CSF, CXCL10 and IL-18 in MR and MM samples (Fig. 5). A significant increase was observed in the levels of IL-1β, IL-10, G-CSF, CXCL10, IL-8, TNF-α and MMP9 ($p < 0.05$, Mann-Whitney test) in both FM sites of PPROM compared to term controls. In the placenta, significantly increased levels of IL-6 and MMP9 ($p < 0.05$) were observed from samples collected from patients with PPROM (Fig. 5A). Consistent with microbial driven inflammation, levels of IL-1β, IL-8, IL-6, G-CSF and MMP9 ($p \leq 0.05$) were significantly higher in MR samples that contained high relative abundance of pathogenic bacteria compared to *L. crispatus* dominant samples (Fig. 5B).

Temporal and between sample strain comparison in PPROM

Longitudinal vaginal samples collected during pregnancy prior to PPROM were available for four PPROM cases (patients 5, 6, 9 and 11) (Fig. 6A). Metataxonomic profiling of these vaginal samples indicated instability of the vaginal microbiota composition in the leadup to the membranes rupture event (Fig. 6B-E). In patient 5 (Fig. 6B), *L. crispatus* dominance at the beginning of the second trimester (13 + 4 weeks

gestation) was lost 4 weeks later (17 + 4 weeks gestation) before returning to *L. crispatus* dominance just prior to membrane rupture at 21 + 4 weeks gestation. *Lactobacillus crispatus* was also detected in the upper reproductive tract of this patient. In the case of patient 9 (Fig. 6C), *L. iners* dominance throughout most of early pregnancy was disrupted by an increase in relative abundance of potentially pathogenic bacteria at 21 + 5 weeks gestation. *Lactobacillus iners* dominance in the vagina was not reflected in the fetal membranes and placental samples of this patient. Patient 11 (Fig. 6D) had a vaginal microbiota dominated by *L. gasseri* at 11 + 1 weeks gestation but by the time of membrane rupture, this had shifted to a *G. vaginalis* dominated microbiota. However, the upper reproductive tract samples were dominated by *Escherichia shigella*. In the case of patient 6 (Fig. 6E), *L. crispatus* dominated the vaginal samples prior to and at membrane rupture. *S. anginosus* which dominated the FM and placental samples collected at delivery, could also be detected in the vagina at 22 + 0 weeks gestation, almost 4 weeks prior to the rupture event. Whole-genome shotgun metagenomic sequencing was performed on these samples to infer strain identity between samples and hypothesise routes of transmission (Fig. 6F & Supplementary Fig. 3). In patients 5 and 9, we found that a specific strain of *L crispatus* could be detected in the vagina in earlier pregnancy greater than 4 weeks before membranes rupture, then later detected in FM samples post rupture (Supplementary Fig. 3). However, certain pathogenic strains of *Gardnerella vaginalis* in patient 9 and *Escherichia coli* in patient 11 (Supplementary Fig. 3) were detected in the FM but were not detected earlier on in pregnancy in the vagina. Furthermore, these 2 patients were dominated by *L iners* and *G vaginalis* respectively and lacked the presence of *L crispatus* in the vagina. In patient 6, metagenomic sequencing confirmed the presence of *S. anginosus* in all three sampled sites of the FM and in the vagina prior and after the membrane rupture, but not in the placenta (Fig. 6F). After applying strain-level analysis with StrainPhlAn using an expanded set of marker genes and only positions covered by two or more reads, the phylogenetic tree revealed the same strain of *S. anginosus* was detected in the samples of patient 6, which was distinct to the strain from patient 11(Fig. 6G). The directionality of colonisation could not be inferred from the data as the accumulation of single nucleotide variants in such short time is not biologically plausible.

Discussion

PPROM is a major contributor to preterm birth and is associated with increased risks for both the mother and the newborn. Despite this, the underlying mechanisms driving PPROM remain unclear[21]. Weakening of the membranes can be caused by increased oxidative stress or intraamniotic pressure[31], however microbial induced inflammation driving protease activation and collagen degradation within the fetal membranes is thought to be a major causal pathway in around 40% of all cases[29]. This microbiological aetiology is widely considered to be driven by bacteria ascending from the vagina. This is supported by several lines of associative evidence including studies which have described similarities between bacteria species found in the FM and amniotic fluid to those found in the vagina of preterm birth cases[24,30]; and that neutrophil invasion of the FM and microbial invasion of the amniotic cavity are most frequently observed at the site of membranes rupture, close to the cervix[31] and in the presenting first born twin[32,33], respectively. Ascending vaginal infection leading to preterm birth has also been

demonstrated in several animal models[26,34–38]. However direct evidence of microbial ascension from the vagina to the upper reproductive in humans remains elusive.

To investigate this, we undertook spatial comparison of microbiota composition throughout the lower and upper reproductive tract in women with PPROM and controls who experienced uncomplicated delivery at term. Consistent with previous reports, we observed that the vaginal microbiome of women delivering at term were typically low in diversity and dominated by *Lactobacillus* species, particularly *L. crispatus*, a commensal strongly linked to optimal reproductive health[39] with immunoregulatory properties[40]. We also observed that FM and placental samples derived from term pregnancies had a very low bacterial load and compositional structure indistinguishable from negative controls. These findings provide further evidence that in uncomplicated term pregnancies, the upper reproductive tract, including the placenta, is devoid of a resident microbiome.

In contrast to term pregnancies, women experiencing PPROM had high prevalence of *L. iners* and high-diversity microbiota enriched with *G. vaginalis*. However, the species were rarely observed in matched upper reproductive tract samples, indicating that certain vaginal taxa may not have the ability to colonise the reproductive tract beyond the cervix. These results are consistent with recent findings demonstrating that the capacity of different vaginal bacteria to adhere and attach to key glycan structures involved in colonisation events are pH-mediated[41]. We also observed high heterogeneity in the compositional structure of different sample sites in those women experiencing PPROM who have vaginal microbiota dominated by *L. iners* and *G. vaginalis*. In these women, upper reproductive tract samples were commonly colonised by pathogenic bacteria including *S. agalactiae*, *S. anginosus*, *S. sanguinegens* and *Escherichia-Shigella*. It is possible that vaginal *L. iners* and *G. vaginalis* act as ecological drivers of microbial invasion into the FM by producing cytolysin, a pore-forming toxin that disrupts the integrity of host cell membranes[42].

Detection of bacterial sequences in placental samples was largely limited to pathogenic species previously associated with adverse reproductive tract health including *S. agalactiae*, *S. anginosus*, *S. sanguinegens* and *Escherichia-Shigella*[26,34,43–46]. These were detected as the dominant taxa in the FM and/or placental samples of 5/15 (33%) patients. Sequence reads mapping to pathogenic bacteria in the upper reproductive tract were also observed in women carrying an *L. crispatus*-dominated vaginal microbiota at the time of delivery. While a *L. crispatus*-dominated vaginal microbiome composition is associated with reduced risk of PPROM, our results caution against over-interpreting cross-sectional vaginal microbiota data in clinical contexts. For example, in this study we demonstrated that a specific strain of *S. anginosus* (mapped to SGB8028 in StrainPhlAn), which was first detected in an *L. crispatus* dominated vaginal microbiome sample 4 weeks prior to membrane rupture, became the dominant taxa in the FM at the time of delivery. In this patient, the vaginal microbiota at delivery was dominated by *L. crispatus*. These results are indicative of an ascending vaginal infection leading to membrane rupture, a mechanistically plausible notion considering *S. anginosus* produces streptolysin-S-like peptides encoded by the *sag* gene cluster, which act as broad-range beta-hemolysins, leading to epithelial cell lysis[44]. The

fact that we were able to detect potential pathogenic strains in the FM that were not detected early on in pregnancy maybe due to lack of sufficient number of vaginal samples to detect vaginal seeding that may have happened at an alternative time during pregnancy or may have occurred even earlier at the time of conception. This indicates that these type of interrelationships between the vaginal bacteria and upper reproductive track sites clearly needs more investigation.

We have recently shown that preterm birth associated with a high-diversity, pathogen-enriched vaginal microbiome is characterised by excessive local innate immune response involving activation of the complement cascade[19,47]. Consistent with cases of PTB, in PPROM, women with pathogen colonisation of the FM were found to have significantly reduced levels of C1q and MBL. Since C1q recognises and binds directly to the surface of pathogens or indirectly to the antigen-antibody complexes attached to pathogens and MBL binds to mannose and other carbohydrate moieties on the surface of pathogens[48], the reduced levels of C1q and MBL we observed here are consistent with consumption associated with activation of the classical and lectin complement pathways, respectively[49–52]. Reflective of complement pathway activation, we found that PPROM and pathogen detection was associated with increased levels of the potent complement anaphylatoxin fragment, C5a, which is generated through activation of the terminal complement cascade downstream of C3 cleavage. C5a has been shown to upregulate the expression of multiple inflammatory mediators[53–55], including the release of pro-inflammatory cytokines[56,57]. Production of C5a has also been reported to upregulate pro-labour mediators in human placental and FM explants[58] and in animal models, macrophage-release MMP-9 leading to collagen digestion and cervical ripening resulting in preterm birth[59]. Interestingly, we also observed a low C3:C5a ratio, indicative of complement pathway activation[60], at the rupture site of a patient with *L. crispatus* dominance. However, the adjacent mid-site FM sample was found to be dominated by *S. anginosus* (> 70% relative abundance), likely accounting for this observation.

In summary, our data provides evidence that the vagina is a likely source of pathogenic bacteria detected upper reproductive tract around the time of delivery in PPROM. While pathogenic bacteria can be frequently detected in the fetal membranes in PPROM, our data does not provide support for upper reproductive tract colonisation in healthy term pregnancy. Moreover, our results indicate that microbial driven PPROM involves activation of the complement cascade, particularly the classical pathway, leading to downstream production of pro-inflammatory cytokines, chemokines and MMP-9 resulting in the degradation of FM integrity. Although limited by a small sample size and relatively few longitudinal vaginal samples collected prior to the rupture event, our study provides new insight into the mechanism by which the reproductive tract microbiome contributes to the aetiology of PPROM and preterm birth. While high-diversity, suboptimal vaginal microbiota are a well-recognised risk-factor for PPROM, our findings demonstrate that pathogen colonisation of the fetal membranes can occur in the presence of a *Lactobacillus*-dominated vaginal microbiome, which suggests pathogen driven PPROM is likely to be more common than previously acknowledged. Future application of vaginal microbiome-based diagnostic approaches for patient stratification and therapeutic (e.g. antibiotics) interventions in PPROM

should be utilised cautiously and may benefit from temporal assessment of microbiota-host interactions throughout pregnancy.

Method Details

Ethics Statement

The study was approved by the NHS National Research Ethics Service (NRES) Committees London–Stanmore of the National Health Service (REC 14/LO/0328; and R-REC 3679). All participants provided written informed consent prior to sampling and experiments were performed in accordance with the approved institutional guidelines.

Study population and sample collection

A total of 39 women with and without PTB risk factors were recruited into this prospective cohort study from Imperial College Healthcare NHS Trust Hospitals (Queen Charlotte's and Chelsea and St Mary's Hospitals), London, UK. The inclusion criteria were pregnant women who experienced PPROM or had term pregnancies without labour but required caesarean-section (C-section) at time of delivery. Women were excluded if they were under 18 years of age or had vaginal bleeding within 72 hours of sampling, sexual intercourse, HIV or Hepatitis C-positive status.

Of the thirty-nine women included in the study, 15 experienced PPROM and 24 had an uncomplicated pregnancy and delivered at term (Fig. 1A). We collected matched sets of vaginal swabs, FM from rupture, mid, distal sites and placental tissue (Supplementary Fig. 2). The PPROM cohort were drawn from women recruited from the preterm birth surveillance clinics, these are women with risk factors for PTB including previous PTB, previous spontaneous miscarriage, previous rescue/ultrasound indicated cervical cerclage or previous LLETZ procedure. For a subset of these women, vaginal swabs taken during pregnancy prior to PPROM were available. The term control group were delivered by CS because of previous CS, previous traumatic vaginal delivery, or fetal macrosomia. Both cohorts were recruited with comparable maternal age, ethnicity, and BMI. Detailed maternal clinical and birth outcome metadata were collected for all participants.

This study consisted of two cohorts of patients who either had PPROM (n = 15) or C-section term deliveries with intact membranes (n = 24) (Fig. 1). PPROM was defined according to the guidelines of participating hospitals as pooling of amniotic fluid on speculum examination following rupture of FM occurring without labour and more than 24 hours prior to preterm (< 37 weeks gestation) delivery. The recruitment period for patients with PPROM was between October 2013 to June 2015 and for patients with term C-section deliveries between October 2020 to April 2021.

Cervico-vaginal fluid samples were collected from the posterior fornix under direct visualisation using a BBL CultureSwab MaxV Liquid Amies swab (Becton, Dickinson and Company, Oxford, UK) at the time of

presentation with ruptured membranes before erythromycin treatment or prior to C-section term delivery. Longitudinal vaginal samples were also collected during pregnancy, before membranes rupture, from a subset of women who subsequently experienced PPROM (Supplementary Fig. 2). The vaginal swabs were placed immediately on ice before being snap frozen and stored at -80°C within 5 min of collection. For 4 patients with PPROM, additional swabs were collected longitudinally at different timepoints during pregnancy, prior to rupture of membranes (Supplementary Fig. 2).

FM samples were collected following C-section delivery from rupture site (MR, typically an area overlying the cervix), distal site (MD, proximate to the placenta) and mid site (MM, between rupture and distal sites). Placental samples were collected from the intervillous space (maternal-fetal interface). The villous tissue was obtained from an area that had no visible damage, hematomas, or infarctions and after trimming to remove adhering decidua from the basal plate. Tissue samples were obtained within an average of 3 hours of delivery, rinsed in chilled sterile phosphate-buffered saline to remove maternal blood and snaped frozen on dry ice then stored at -80°C. Negative controls were included for each lot of laboratory reagents.

DNA extraction and sequencing of bacterial 16S rRNA genes

Extraction of DNA from the vaginal BBL™ CultureSwab™ was performed as previously described[61]. For tissue samples, approximately 0.25g of tissue was cut from the stored tissue, minced with single-use sterile forceps and scalpel. The entire experimental procedure was carried out in a Class 2 biological safety cabinet to reduce the risk of environmental contamination of the samples. Prior to DNA extraction, 400ul of sterile, filtered, UV treated PBS was added to the minced tissue and vortexed for 30 seconds followed by lysis in a Tissue Lyser LT for 2 min at 50 Hz. The extraction was then performed as previously described[62]. Sequencing was performed on an Illumina MiSeq platform (Illumina Inc.) at Research and Testing Laboratory, (RTL Genomics) in Lubbock, Texas, USA. The hypervariable regions V1-V2 of 16S rRNA were amplified using mixed universal primers. The forward primer set (28f-/YM) consisted of 28F-Borrellia GAGTTTGATCCTGGCTTAG; 28F- Chlorlex GAATTTGATCTTGGTTCAG; 28F-Bifido GGGTTCGATTCTGGCTCAG; 28F- YM GAGTTTGATCNTGGCTCAG mixed to a 4:1:1:1 ratio. This mixed formulation has been shown to maintain the rRNA gene ratio of Lactobacillus spp. to Gardnerella[63]. The reverse primer consisted of 388 R GCTGCCTCCCGTAGGAGT.

Shotgun sequencing

Whole-genome shotgun (WGS) sequencing was performed by CosmosID Inc., (Rockville, MD, USA) in a subset of the PPROM patients (n = 4) to generate higher resolution strain-level data. DNA samples were quantified using Qubit 4 fluorometer and Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Wilmington, DE, USA). As previously described[65], DNA libraries were prepared using the Illumina Nextera

XT DNA Library Preparation Kit and Illumina Nextera Index Kit[66] (San Diego, CA, USA) with total DNA input of 1ng. Genomic DNA was fragmented using a proportional amount of Illumina Nextera XT fragmentation enzyme. Combinatory dual indexes were added to each sample followed by 12 cycles of PCR to construct libraries. DNA libraries were purified using AMpure magnetic Beads (Beckman Coulter) and eluted in QIAGEN EB buffer. DNA libraries were quantified using Qubit 4 fluorometer and Qubit™ dsDNA HS Assay Kit. Libraries were then sequenced on an Illumina HiSeq X platform 2x150 bp end reads.

Quantitative real-time PCR (qPCR) of the 16S rRNA gene

The 16S rRNA gene copy number was quantified for all samples to determine the bacterial load across the RT at the various anatomical sites; the vagina, FM (at rupture, mid and distal site) and the placenta from women who experienced PPROM and women delivered at term. Real-time qPCR was performed using the BactQuant assay as previously described[67]. DNA from tissue samples was used neat but a tenfold dilution was used for vaginal samples.

Luminex immunoassays

A total of 0.25g of FM and PL tissue samples were thawed and minced on ice then re-suspended in 400 µL of phosphate-buffered saline solution containing protease inhibitor (5 µL/ml; Sigma-Aldrich) before being homogenised on ice and centrifuged at 10.000 rpm at 4°C for 10 min to remove cellular debris. Customized Luminex® multiplex assays (R&D Systems/Bio-Techne) were used together with a Bio-Plex 200 system (Bio-Rad Laboratories Ltd) to quantify levels of interleukin (IL)-1β (IL-1β), IL-6, IL-10, granulocyte colony stimulating factor (G-CSF), tumour necrosis factor-α (TNF- α), C-X-C Motif Chemokine Ligand 10 (CXCL10) and matrix metalloproteinases 9 (MMP9) with no dilution required. IL-8 was measured on a single plex Human Premixed Analyte Kit (R&D Systems/Bio-Techne), following a 100-fold dilution using Calibrator Diluent RD6-52. The Human Complement Magnetic (Milliplex® Merck/Millipore) Bead panel 1 was used for measurement of C2, C4b, C5, C5a and mannose binding lectin (MBL), while panel 2 was used for measurement of C1q, C4, C3 and C3b/iC3b. Complement C3b/iC3b, C4b, C4 & C2 required a 100-fold dilution with Universal Assay Buffer included in the assay kit (Supplementary Table 1). All samples were analysed on 96-well plates in duplicates. The coefficient of variation was calculated across all plates for standards and a common pooled sample analysed on all plates as a calibrator to assess for plate-to-plate variation. Results were expressed in picograms per ml of supernatant volume for cytokines, MMP9, C4 and C5a and nanograms per ml of supernatant volume for all other analytes. A relatively very small proportion of concentrations that are not extrapolated (mostly in placental samples), were substituted with either the lowest or highest value calculated for our data set from the standard curve. This was applied to 32/507 (6.3%) of all the cytokine assays and 3/384 (0.8%) of all the complement assays.

Bioinformatics and statistical analyses

The sequencing data were processed using the QIIME2 amplicon pipeline (*v*2024.10.1)[68], after removal of primer and adapter sequences with *cutadapt* (*v4.9*)[69]. Amplicon sequence variants (ASVs) were identified with dada2[64] and annotated using a bespoke naïve Bayes classifier trained[70] on the whole SILVA SSU Ref NR99 database (release 138.2)[71]. Bioinformatics and statistical analyses were performed in R (v4.0.0). Low abundance taxa accounting for less than 0.1% within-sample relative abundance and present in less than 1% of samples were filtered using phyloseq (v1.32.0)[72] and genefilter (v1.70.0) packages. Negative controls accounting for environmental, reagent and kit contamination were used to identify potential contaminants using the Decontam R package (v1.9.0) with a prevalence threshold of 0.5[73]. Conversion of raw counts to relative abundance was performed using the funrar (v1.4.1) package.

Shotgun metagenomic reads were pre-processed and quality controlled using the pipeline available at https://github.com/SegataLab/preprocessing. Briefly, reads were quality controlled and those of low quality (average quality score <Q20), fragmented (< 75 bp) and with more than two ambiguous nucleotides were removed with Trim Galore (v.0.6.6). Contaminant and host DNA was identified with Bowtie2 (v.2.3.4.3)[74] using the parameter '-sensitive-local,' allowing confident removal of the phiX 174 Illumina spike-in and human reads (hg19 human genome release). Remaining high-quality reads were sorted and split to create forward, reverse and unpaired reads output files for each metagenome.

Taxonomic composition was determined with MetaPhlAn version 4.1.0 and Jun23 database using parameter stat_q 0.1 to increase the sensitivity to low abundant species. Phylogenetic tree of Streptococcus anginosus strains was constructed with StrainPhlAn 4.1.0 using an expanded set of 401 species-specific marker gene[75] and parameter min_base_coverage 2 to reduce the noise of sequencing errors and insure confidence base calls.

Data visualisation including bubble plots, stacked bar charts, lollipop plots and dot plots were performed using the ggplot2 package (v3.5.1). The top 20 taxa shown in stacked bar charts and bubble plots was based on the mean relative abundance for each species. Cytokines and complements were compared according to pregnancy outcome and according to bacterial composition using Mann-Whitney Test. Fisher's Exact Test was used to determine statistical significance for categorical variables. For all tests, the level of statistical significance was taken as a p-value ≤ 0.05. β- diversity was calculated using the Bray-Curtis index and compared using PERMANOVA of MicrobiomeAnalyst 2.0[76].

Declarations

Acknowledgements

We acknowledge and thank the participants involved in this study. Thanks to Imperial Women's Health Research Centre staff for their assistance in coordinating participant recruitment and sample collection. We also thank the Parents as Partners public involvement group for their contributions to the study

design. We gratefully acknowledge the financial support of the Daphne Jackson Trust Fellowship, co-sponsored by the Medical Research Council, the Genesis Research Trust and the Department of Metabolism, Digestion and Reproduction at Imperial college London. This research was funded by March of Dimes Prematurity Research Centre at Imperial College London. Infrastructure support was also provided by the Tommy's National Preterm Birth Centre, The Parasol Foundation Centre for Women's Health and Cancer Research, and the NIHR Imperial Biomedical Research Centre.

Author contributions

DAM, PRB, RGB, TOB, and LS conceived the study and designed the experimental approach. Clinical sample collection and metadata management were undertaken by RGB, TOB and MA. Experimental work was performed by TOB, EH and YSL. Data processing, analysis and interpretation were carried out by TOB, DAM, SN, GDSC, MP, NG and PRB. TOB, SN, and DAM generated all figures and tables. The initial manuscript was drafted by TOB and DAM. All authors reviewed the manuscript and approved the final version.

Declaration of interests

PRB and DAM hold a patent to the use of *Lactobacillus crispatus* in pregnant women (International Application No. PCT/GB2022/050453; International Publication No. WO 2022/175680). DAM is a Scientific Advisory Board member of Calla Lily Clinical Care (UK) and Freya Biosciences APS (Denmark).

Data availability

Raw data files for the sequencing data used in this study are available through the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena) in ENA project; (PRJEB124241).

Code availability

Analysis code and processed datasets are available in the GitHub repository; https://github.com/sherrianne/Beleil-et-al_VAP.

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Figures

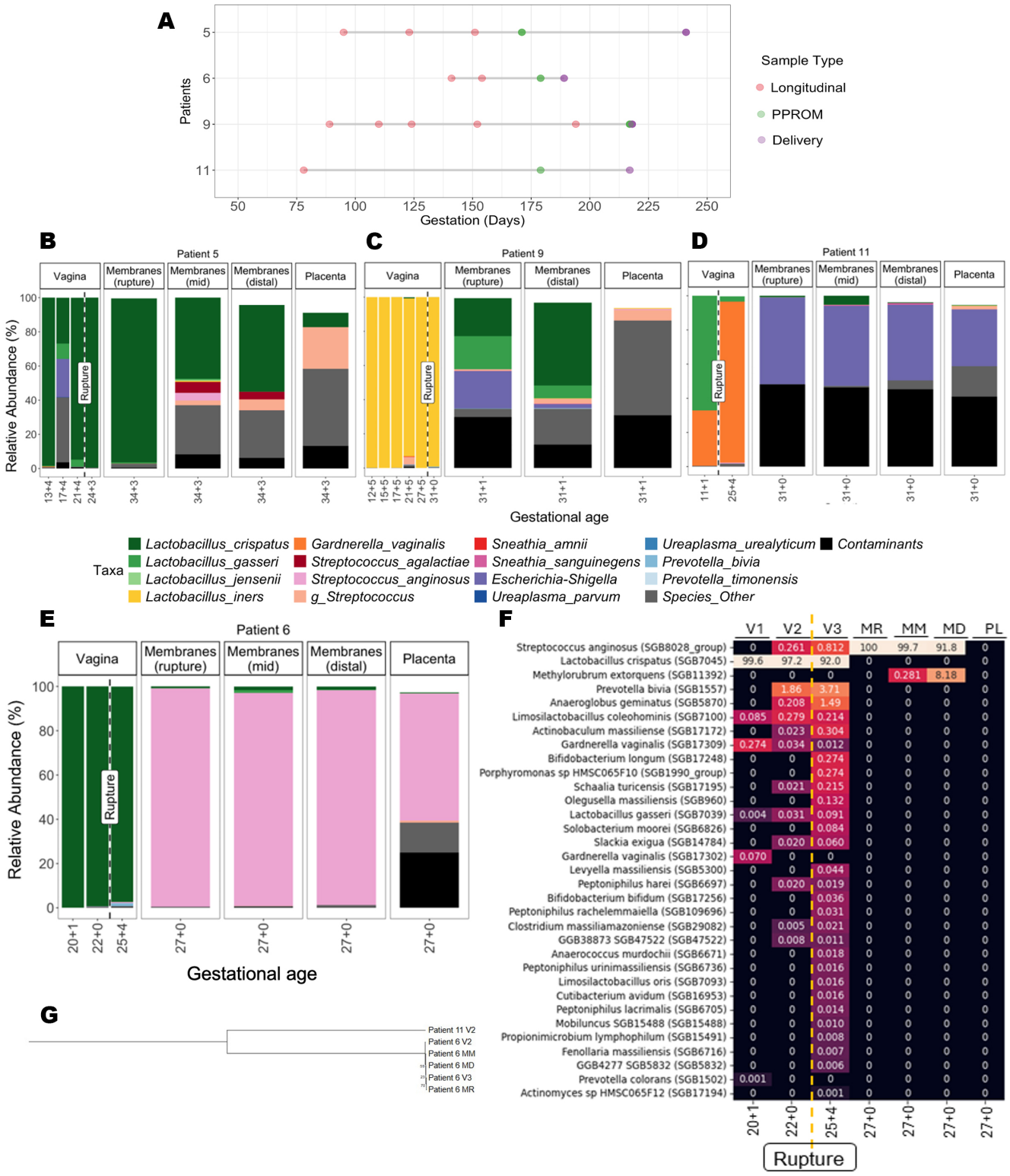


Figure 6

Longitudinal profiling of vaginal and upper RT bacterial composition. (A) Dumbbell plot showing gestational age for longitudinal vaginal samples collected during pregnancy, PPROM, and delivery for a subset of PPROM patients (n=4). (B) Metataxonomic profiling of longitudinal vaginal samples collected during pregnancy at 3 time points and at presentation after PPROM, as well as fetal membranes and placental samples collected at delivery from patient 5. (C) Metataxonomic profiling of longitudinal

vaginal samples collected during pregnancy at 5 time points and at presentation after PPROM, as well as fetal membranes and placental samples collected at delivery from patient 9. (D) Metataxonomic profiling of longitudinal vaginal samples collected early in pregnancy at 11+1 weeks gestation and at presentation after PPROM, as well as fetal membranes and placental samples collected at delivery from patient 11. (E) Metataxonomic profiling of longitudinal vaginal samples collected during pregnancy at 5 time points and at presentation after PPROM, as well as fetal membranes and placental samples collected at delivery from patient 6. (F) Shotgun metagenomics-based bacterial composition of patient 6 samples, from vagina pre-rupture (V1, V2), vagina at rupture (V3) and from fetal membranes sites (MR, MM, MD) and placenta (PL). (G) Strain-level phylogenetic tree analysis using StrainPhlAn indicating that the *S. anginosus* strain (SGB8028) detected in the vagina of patient 6 at time point V2 prior to membranes rupture, was the same as that detected in the vagina at presentation after rupture (V3) and in the fetal membranes samples collected following delivery. This strain differed from that detected in Patient 11.

Supplementary Files

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