Review

New techniques to characterise the vaginal microbiome in pregnancy

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Abstract: Understanding of the vaginal microbiome in health and disease is essential to screen, detect and manage complications in pregnancy. One of the major complications of pregnancy is preterm birth, which is the leading world-wide cause of death and disability in children under five years of age. The aetiology of preterm birth is multifactorial, but a causal link has been established with infection. Despite the importance of understanding the vaginal microbiome in pregnancy in order to evaluate strategies to prevent and manage PTB, currently used culture based techniques provide limited information as not all pathogens are able to be cultured.

The implementation of culture-independent high-throughput techniques and bioinformatics tools are advancing our understanding of the vaginal microbiome. New methods employing 16S rRNA and metagenomics analyses make possible a more comprehensive description of the bacteria of the human microbiome. Several studies on the vaginal microbiota of pregnant women have identified a large number of taxa. Studies also suggest reduced diversity of the microbiota in pregnancy compared to non-pregnant women, with a relative enrichment of the overall abundance of *Lactobacillus* species, and significant differences in the diversity of *Lactobacillus* spp. A number of advantages and disadvantages of these techniques are discussed briefly.

The potential clinical importance of the new techniques is illustrated through recent reports where traditional culture-based techniques failed to identify pathogens in high risk complicated

pregnancies whose presence subsequently was established using culture-independent, high-throughput analyses.

Keywords: Preterm birth; genital infections; vaginal microbiota; high-sequencing throughput; metagenomics; *Lactobacillus*

1. Introduction

Understanding the vaginal microbiome in health and disease is essential to screen, detect and manage complications in pregnancy. A major complication of pregnancy is preterm birth (PTB), which is the leading world-wide cause of death and disability in children under five years of age [1–4]. Whilst the aetiology of PTB is multifactorial, a causal link has been established with infection. The rates of neonatal infectious diseases in mothers with chronic chorioamnionitis who deliver at term is 20%, and in mothers who deliver prematurely is 50% [5]. Despite the importance of understanding the vaginal microbiome in pregnancy in order to evaluate strategies to prevent and manage PTB, currently used culture-based techniques provide clinicians with limited information of bacterial communities present in the vagina as not all bacteria are able to be cultured.

Pathogens may gain access to the amniotic cavity and fetus by ascending migration of vaginal microflora, haematogenous dissemination through the placenta, retrograde seeding from the peritoneal cavity through the Fallopian tubes, or iatrogenic introduction at the time of invasive procedures [6,7]. Evidence obtained from studies culturing bacteria support the view that the most common pathway of microbial invasion of the intra-amniotic cavity is the ascending route [8]. Meta-analyses of antibiotic administration to women with bacterial vaginosis showed a significant reduction in the incidence of preterm deliveries and low weight babies associated with treatment [9]. A positive association between periodontal disease and uterine infection during pregnancy remains controversial [10], but a number of oral bacterial species have been identified in the intra-amniotic space suggesting haematogenous spread [11]. Thus, it is reasonable to hypothesise that preventing ascending genital tract infections and the initiation of inflammatory cascades in the uterus will reduce PTB, neonatal fever and other morbidities. Consequently, identification of the bacterial communities present in the vagina during pregnancy will help to achieve a comprehensive picture of its microbiota that can be exploited to promote health and prevent/combat disease.

The aim of the study is to provide a description of the vaginal microbiome in health and disease that has been achieved by specific application of new analytical and bioinformatics tools employed to investigate generally the human microbiome.

2. Methods

Searches of publications in PubMed performed with the key terms 'vagina' and 'microbiome' yielded 396 references. Adding the key words 'sequencing' or '16S rRNA' or 'metagenomics' produced 74, 68 and 19 references, respectively. If instead, the term 'pregnancy' was added to the

original search 'vagina and microbiome' the query returned 94 references, and further adding the term '16S rRNA' reduced this number to 16. An independent search with the key words 'microbiome' and 'new generation sequencing' returned 111 articles. A check of the articles retrieved indicated extensive redundancies that allowed purging duplications from the list. All the articles in the streamlined reference list underwent a preliminary analysis to identify studies that had primary data on the vaginal microbiome obtained by employing non-cultivation, high-throughput sequencing methods. The selected articles and references therein were chosen for this review.

The inclusion criteria were studies in English published in the last 15 years that identified specific taxa in the vaginal microbiome of non-pregnant women or pregnant women, employed cultivation-independent high-throughput sequencing methods, and demonstrated the power of the new techniques to contribute to the characterisation of this microbiota. Also included were two older papers that were seminal for the development of new bioinformatics tools.

3.   The Vaginal Microbiome

A growing understanding of the central role played by microbes in human health and disease as well as advances in techniques to identify microorganisms and bioinformatics tools to analyse very large data sets have provided the foundation to characterise and investigate the microbial communities that inhabit the human body: the human microbiome [12]. From a microbiological perspective the vagina is a complex and dynamic habitat that has a significant impact on the health of the woman. The changes in the structure of this ecosystem are influenced by many factors including age, menarche, time of the menstrual cycle, sexual activity, pregnancy, infections, and various habits and practices [13–17]. The composition of the vaginal microbiota has been investigated for over a hundred years, and up to 15 years ago, most conclusions about the vaginal microflora of post-pubertal women were based on methods that used cultivation of microbial populations [18], and more recently, on culture-independent targeted polymerase chain reaction (PCR) methods [19,20]. These approaches yield biased and incomplete assessments of the structure of microbial communities, because many members of these communities are not culturable *in vitro,* and a diverse array of bacteria other than those identified by targeted PCR may be present, and thus remain undetected. For instance, in current clinical practice microbiological analyses of the female genital microbiota focus on a number of species from about 25 genera including *Atopobium, Chlamydia, Clostridium, Escherichia, Gardnerella, Mycoplasma, Neisseria, Prevotella, Staphylococcus, Streptococcus, Ureaplasma.* In more complicated cases searches are conducted for extra genera such as *Dialister, Enterococcus, Fusobacterium, Haemophilus, Leptotrichia, Megasphaera, Mobiluncus, Peptostreptococcus,* and *Veillonella.*

Cultivation-independent broad-range PCR analyses of 16S rRNA gene sequences from microbial communities suggest only a small percentage of bacteria in nature have been identified, even in well-studied environments. Studies of the vaginal microflora employing these methods have revealed a richer microbiota with a much large number of taxa than those identified employing culturing methods [21–23] In particular, the identity and diversity of the vaginal bacterial populations during pregnancy remain largely unknown for various racial backgrounds, health status and lifestyle. Also, the complex interactions of the various members of the vaginal microflora are not

sufficiently understood to enable this knowledge to be clinically exploited to combat disease. This study offers a brief discussion of techniques and tools employed to elucidate the vaginal microbiome, and provides an overview of current knowledge of the vaginal microbiome in late pregnancy. Case examples highlight potential clinical applications of culture independent techniques.

4. Techniques and Computational Tools to Analyse the Human Microbiome

The advent of high-throughput sequencing (HTS) had a significant impact on disease diagnosis, particularly of human genetic diseases and cancers [24–27], and to a lesser extent on microbial-related illnesses. The effectiveness of HTS techniques has been demonstrated by identifying aetiological agents in samples where traditional bacterial culture techniques failed and in cases where multiple bacterial agents were involved. Key limitations to a wider use of the HTS techniques are the lack of ability of diagnostic centres to perform fast sample analyses, and the capacity to analyse the large datasets generated by such methods. Nonetheless, the potential of HTS is evidenced by the application of these techniques and subsequent statistical analyses of the data to identify bacterial species that may be involved in preterm birth. Such work may help refine more targeted clinical screening approaches. Two important methods for microbial identification and characterisation that use HTS are sequencing of 16S rRNA and metagenomics.

4.1. Sampling technique

The choice of sampling site of the vagina with swabs should be considered, since there has been controversy about the microbial diversity in different regions of the vagina. To investigate the vaginal microbiota, a study of pregnant healthy Chinese women collected three repeated swabs at the cervix, posterior fornix and vaginal canal and different gestational ages. For each individual woman there was high vaginal microbiome homogeneity across the three sampling sites. The results revealed different beta diversity (differences between women) at various gestational ages [28]. In contrast, a study that included women of several race/ethnic backgrounds with pregnancies both healthy term and preterm birth found that sampling site was an important variable [29].

4.2. Genomics employing the 16S rRNA gene

The 16S rRNA gene is a universal component of the DNA transcriptional machinery of bacteria and archaea. This gene has both conserved and hypervariable regions; the former makes universal amplification possible, and sequencing the latter allows discrimination between different microorganisms. These characteristics make the 16S rRNA gene well suited as the basis to identify, classify and quantitate microorganisms in complex biological mixture in samples containing up to thousands of different species [30].

From the DNA extracted from samples, specific fragments of the 16S rRNA gene are amplified employing the polymerase chain reaction (PCR) technique in a series of cycles. The amplified gene segments are then sequenced using HTS developed to sequence in parallel large numbers of individual DNA fragments.

Detecting 16S rRNA sequences of bacteria directly from samples as a phylogenetic marker has served to discover their presence in environments where they were previously unknown, to identify new taxa, and to establish phylogenetic relationships between them. In recent years, the use of cultivation-independent methods based on broad-range PCR analyses of 16S rRNA sequences have increased the understanding of the composition of vaginal bacterial communities.

The application of 16S rRNA analysis to samples that contain tens or hundreds of bacterial communities allows deep views into the diversity of these populations. Nonetheless, the method has limitations. There are three important sources of error. These are: (a) bias towards some species owing to unequal amplification of different species' 16S rRNA genes; (b) uncertainty in choosing the hypervariable region that will provide the maximum discriminating power for a given sample, since no single region is able to distinguish between all bacteria; and (c) complications of 16S rRNA-based analyses by artifacts such as chimeric sequences caused by PCR amplification and sequencing errors [31,32].

For example, the potential bias introduced by sample processing, sequencing and taxonomic classification in 16S rRNA studies was investigated employing samples of a 80 bacterial mock communities comprised of prescribed proportions of cells from seven vaginally-relevant bacterial strains, and two additional sets of 80 mock communities by mixing prescribed quantities of DNA and PCR products. Different DNA extraction kits can produce dramatically different results and the effects of DNA extraction and PCR amplification for the protocols employed were much larger than those owing to sequencing and classification. The work concluded that due attention should be given to sample processing notwithstanding advances in sequencing technology [33].

Another recent study found that the 8F-534R primer pair assigned more sequences to *Lactobacillus* spp. (65.5% vs. 25.4%) and less sequences to *Sarcina* spp. (9.6% vs. 22.1%) compared to the 968F-1401R primer pair [34]. Other bacterial taxa with inconsistent results across the 8F-534R and 968F-1401R primer pairs include *Bacillus* spp., *Fusobacterium* spp., *Lactococcus* spp., *Streptococcus* spp., *Clostridium* spp., *Gemella* spp., *Lachnospira* spp., *Leuconostoc* spp., *Microbacterium* spp., and *Weissella* spp. [34]. In an attempt to correct these types of errors, Klindworth and colleagues conducted a comprehensive analysis of overall coverage and phylum spectrum of 175 primers and 512 primer pairs with respect to the SILVA 16S/18S rRNA non-redundant reference dataset [35]. In addition to providing a guideline for primer selection based on application, the authors put forward a selection of primer pairs that are considered optimal for the amplification of bacterial and archaeal rRNA genes at different sites [35].

4.3. Metagenomics of whole genomes

Metagenomics studies of microorganisms refer to non-culture based approaches for collectively studying sets of genes or genomes from mixed populations of microbes. These studies are grouped according to different screening methods: (a) shotgun analysis using mass genome sequencing; (b) genomic activity-driven studies designed to search for specific microbial functions; (c) genomic sequence studies using phylogenetic or functional gene expression analysis; and (d) next generation sequencing technologies for determining whole gene content in environmental samples [36].

To conduct these analyses, DNA or RNA isolated from a sample is randomly sheared, the

fragments are clonally amplified employing PCR, and then sequenced using one of the various HTS developed to sequence in parallel large numbers of individual DNA or RNA fragments. The sequence data are then processed for assembly using one of the two strategies, either reference-based assembly (co-assembly) or de novo assembly. The information on DNA sequences is sorted into taxonomic groups that may represent individual or closely related genomes. Generally, metagenomic sequences are annotated in two steps: (a) feature prediction is performed by identifying characteristics of interest within genes; and (b) functional annotation is performed by assigning putative gene functions and taxonomic neighbours.

4.4. Some computational tools to analyse large sequencing data sets

To analyse large raw reads data sets generated by HTS of universal genes, several computational tools have been developed that can be employed as barcodes to classify microbes (e.g. 16S rRNA gene and *hsp60*). Two of the most commonly used tools to classify reads into operational taxonomic units (OTU) are MOTHUR [37] and Quantitative Insights Into Microbial Ecology (QIIME) [38] MOTHUR integrates and streamlines a number of algorithms employed for microbial classification (e.g. NAST, PyroNoise, Classifier and UChime) into an open-source stand-alone program, while QIIME acts as an interface that connects a number of programs used for microbial classification (e.g. pynast and uclust). More recently, the algorithm UPARSE [39] was developed to improve the accuracy of OTU clustering; both MOTHUR and QIIME are able to run UPARSE to classify OTU. Furthermore, the software package microbial Profiling Using Metagenomic Assembly (mPUMA) [40] utilises *de novo* assembly of OTU to enable the analysis of microbial communities.

A new method called STIRRUPS employs the USEARCH algorithm with a curated reference that can be used for rapid species-level classification of 16S rRNA partial sequences. It was developed to construct a vaginal 16S rRNA sequences reference database for bacterial taxa likely to be associated with vaginal health. The method and database provide accurate species-level classifications of metagenomics 16S rRNA sequence reads that will be useful for analysis and comparison of microbiome profiles from vaginal samples [41].

Other tools have been designed as pipelines for more complex data sets arising from whole genome sequencing approaches (metagenome analysis) such as MetaGenome Rapid Annotation using Subsystem Technology (MG-RAST) [42], QIIME [38], Metagenomics Platform for Sequence Analysis and Management System (MetaSAMS) [40], and EBI Metagenomics [44].

The statistical analyses of sequence data sets requires both simple and multivariate statistical techniques including Principal Component Analysis (PCA), non-metric Multi-dimensional Scaling (MDS) and Permutational Multivariate Analysis of Variance (PERMANOVA) [44,46]. Principal Component Analysis can determine if a sample clusters with or away from others, and identify what microbial taxa contribute to differences in microbial composition. Multi-dimensional Scaling is an alternative ordination method to PCA. The relative abundance of bacterial taxa can be compared with PERMANOVA using a Bray-Curtis similarity measure to construct distance matrices. This procedure is a multivariate analogue of ANOVA except that pairwise distances/similarities between sampling units (in this case using the Bray-Curtis similarity coefficient) are used to calculate multivariate averages (centroids) and test statistics (pseudo-F). Probabilities are obtained by

comparing the pseudo-F value to a distribution of test statistics generated by random permutations of the data.

5.   The Vaginal Microbiome of Pregnant Women

5.1. *Comparison of the vaginal microbiome of healthy non-pregnant and pregnant women*

Fewer bacterial species inhabit the vagina in comparison with the gastrointestinal tract, although DNA sequences from more than 80 bacterial genera corresponding to more than 950 taxa have been identified [23]. Many vaginal bacterial taxa are yet to be characterised [41].

Bacteria of the genus *Lactobacillus* are the most abundant colonisers of the vagina of healthy women. A culture-independent, universal PCR amplification of the 16S rRNA gene investigation of the microbiome of 396 reproductive-age asymptomatic women found their vaginal bacterial communities clustered into five vaginal groups (VG). Four of these groups were dominated by *Lactobacillus* spp.: *L. crispatus, L. gasseri, L. iners,* or *L. jensenii,* albeit they co-inhabited with other bacterial taxa [21]. The fifth group was characterised by a greater abundance of other bacteria.

In healthy pregnancy, there is a decrease in the diversity of bacterial taxa in the vagina [47,48] and in the dominance of some VG.
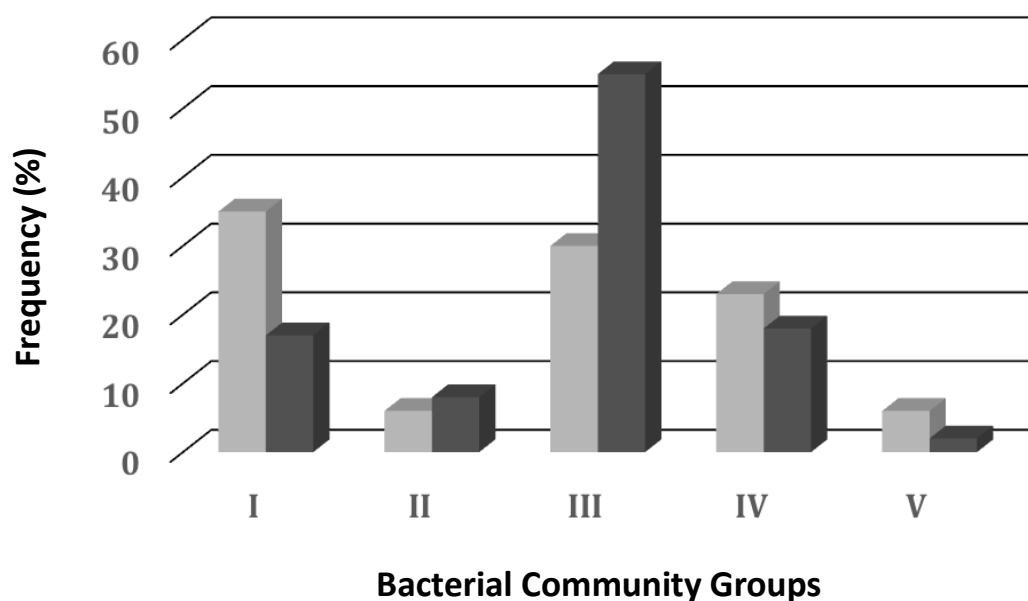
Figure 1. Frequencies of bacterial communities in non-pregnant women (light grey) and pregnant women (dark grey). The groups are dominated by *L. crispatus* (I), *L. gasseri* (II), *L. iners* (III), other bacteria (IV), and *L. jensenni* (V).

In a study of 24 pregnant women with uncomplicated pregnancy at term compared to a cohort of non-pregnant subject, differences were observed. There was a reduction in diversity, and an absence of specific taxa, as well as a relative enrichment of *Lactobacillus* species including *L.*

crispatus, L. iners, L. jensenii and *L. johnsonii.* [11]. The dominant orders during pregnancy were Lactobacillales, Clostridiales, Bacteroidales and Actinomycetales. Differences in the microbiome composition between pregnant and non-pregnant women were also observed in a retrospective case-control longitudinal study of 32 non-pregnant and 22 pregnant women. *Lactobacillus* spp. were the predominant members of the microbial community in normal pregnancies [49]. Figure 1 summarises the frequency of the five VG from three studies comprising 589 healthy non-pregnant women [21,22,44] compared to frequencies from 251 healthy pregnant women [29,49–51]. The frequencies in the groups dominated by *L. crispatus* and *L. iners* are different.

Current data suggest normal pregnancy induces changes in vaginal bacterial populations to a microbiome of low diversity. *Lactobacillus* species strongly dominate the vaginal environment during pregnancy, but changes also occur also in other colonising taxa.

5.2. *The microbiome of pregnant women with vaginal infections*

Ascending vaginal infections in pregnancy may lead to chorioamnionitis, PTB and adverse pregnancy outcomes [52]. These infections are postulated to arise predominantly through ascending pathways from the vagina, through the cervix and across the placental barrier. They contribute to 25% of cases of PTB [53].

New techniques have added to our understanding of these pathogenic pathways and their potential causative agents. The use of 16S rRNA gene sequence-based analyses have revealed the presence of anaerobic taxa not previously detected by culture. An increase in the abundance and diversity of some anaerobic taxa have been linked with vaginal infection [54]. The commonest taxa identified are from genera such as *Gardnerella, Megasphaera, Prevotella,* etc., as well as taxonomically "undetermined" taxa such as "bacterial vaginosis associated bacteria (BVAB)" [55].

Two studies [21,56] reported changes in the relative abundances of *L.iners,* the *Lactobacillus* found most commonly in healthy pregnancies, and in three anaerobic taxa associated with vaginal infections (Figure 2). In another study of 374 pregnant women, the presence of specific vaginal bacterial taxa was correlated against the risk of preterm birth. Culture-independent targeted PCR of the 16S rRNA gene of 12 bacterial taxa was carried out on fluid collected from the upper vagina. Among African-american and Hispanic women, even after controlling for selected maternal behavioural and biological characteristics, the bacterial community in the vagina in the second trimester of pregnancy was an independent correlation with adverse pregnancy outcome. *Mycoplasma* taxa were positively associated with PTB in both these groups of participants. However, the association was not observed in Caucasian participants. Surprisingly, a specific Group B *Streptococcus* taxon associated with bacterial vaginosis showed a negative association with PTB [20]. Another study of 88 women from five racial groups using universal 16S rRNA amplification found vaginal microbiome diversity in human pregnancy correlated with PTB. Race, ethnicity and sampling site were also important variables. The abundance of *Lactobacillus* spp. was higher among women at low risk of PTB relative to those at high risk, but there was no correlation between *Lactobacillus* abundance and PTB [29].
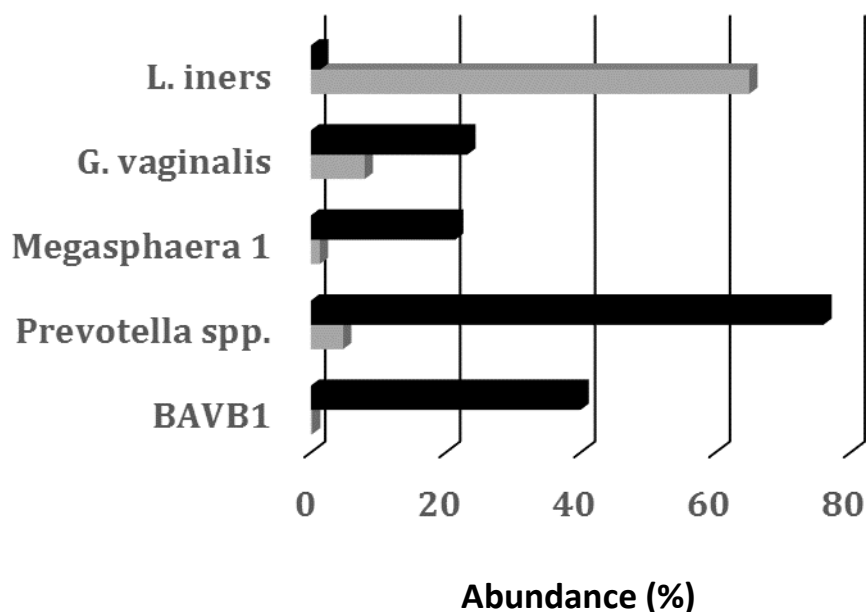
Figure 2. The relative abundances of *L. iners*, *Gardnerella vaginalis, Megasphaera 1, Prevotella* spp. and BVAB1 in healthy women (light grey) and in women with vaginal infection (dark grey) [21,56].

In a case-control study, pyrosequencing of 16S rRNA genes was used to investigate differences in the vaginal microbiome of women giving birth at term or preterm. It comprised 18 women with pregnancy complicated by spontaneous preterm labour and 72 controls with uncomplicated pregnancy. No differences were found in the relative abundance of microbial phylotypes, and there were no differences in the frequency of the vaginal community states between groups [49].

The results to date suggest the vaginal microbiota in pregnancy is more complex in the presence of infection, and an increase in the abundance of anaerobic species is linked to adverse pregnancy outcomes. Larger studies involving women with geographic, racial and ethnic diversity are required to tease out key associations [47].

6.   Clinical Applications of New Technologies

Employing HTS of the 16S rRNA gene and statistical analyses on DNA extracted from vaginal swabs, bacterial taxa can be identified in the vagina of women with a complicated pregnancy. Recent cases report that taxa belonging to the genera *Acinetobacter, Bacteroides,* and *Hafnia,* and the species *Campylobacter curvus* and *Haemophilus parainfluenzae* were potentially involved in preterm, very preterm and extremely preterm births [57–59]; Table 1 summarises data from these reports. Of note is that none of the taxa identified by 16S rRNA techniques, nor any other pathogens, including Group B *Streptococcus*, were found employing standard hospital cultures of vaginal swabs.

These examples demonstrate how current culture-based methods of detection of bacterial infections do not reveal the entire microflora present in the female genital tract, even when they may dominate the microbiome in disease states diagnosed with histological clinical chorioamnionitis.

Cultivation-independent universal PCR analyses can detect potentially pathogenic species in cases when standard culture-based techniques are negative. The cases in Table 1 provide also new insights into pathogenic taxa in the vaginal microbiome of pregnant women, and demonstrate the need to review clinical practices employed to identify pathogens in maternal infections.

Table 1. Predominant bacterial communities in three premature births.

Mother (age in years, gravidity, parity)	Gestational Age (weeks)	Diagnosis	Hospital Microbiology	Taxa (sequence reads % supporting the presence of taxa)	Reference
29, G3, P2	26	PPROM, HCA	Negative	*Campylobacter curvus* (LVS: 1.4%; HVS 61.3%) *Haemophilus parainfluenzae* (LVS: 56.1%; HVS: 18.2%)	Mendz *et al.*, 2014
29, G1, P0	27	HCA	Negative	*Hafnia* spp. (50%) *Bacteroides* spp. (32%)	Kaakoush *et al.*, 2014
38, G2, P1	34	HCA, Vasculitis	Negative	*Acinetobacter* spp. (68.2%)	Quinlivan *et al.*, 2014

PPROM: pre-partum rupture of membranes; HCA: chorioamnionitis demonstrated by histopathology of the placenta; vasculitis of the umbilical cord. LVS: low vaginal swab; HVS: high vaginal swab.

7. Conclusion

The use of new technologies has advanced out understanding of the vaginal microbiome. Key findings are that: (1) species diversity is reduced during pregnancy; (2) patterns of vaginal populations are different in pregnant and non-pregnant women; (3) *Lactobacillus* spp. dominate the vaginal microbiome of healthy pregnant women, with varying relative abundances of different species, and with *L. iners* as the most frequent predominant species; and (4) accumulating evidence supports a role for alteration in the vaginal microbiome in PTB.

Sequence-based analyses of the 16S rRNA gene revealed also the presence of anaerobic species in the vagina not previously detected by culture [55], and allowed associations to be made between specific taxa and PTB.

Considering the limitations of studies to date to reveal all the microflora present in the genital tract of pregnant women, more work is required to understand what are the differences in the microbiome of women owing to race, age and lifestyle. Research employing non-culturing methods and state-of-the-art sequencing analyses will be needed to delineate the "entire picture" of the vaginal and uterine microbiomes and to determine their relationships.

A comprehensive view of the genital microflora will serve also to identify new bacterial taxa

involved in urogenital infections, and to elucidate whether colonisation of the uterus is primarily via ascending infection, and the role or other routes of access to the amniotic cavity.

Future efforts to reduce PTB depend upon a better knowledge of the taxa found in pregnant women in health and disease. This information will underpin the development of earlier and more specific methods to diagnose maternal genital infections, and to reduce mortality and morbidity in fetuses and neonates.

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Conflicts of interest

All authors declare no conflicts of interest in this study.

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