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The reproductive tract microbiota and miscarriage

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Dedicated to my love and the little blessings we share

Declaration of Originality

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This thesis presents original work of the titled author. That which is not is referenced accordingly.

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Thesis Abstract

Early miscarriage (pregnancy loss before 12 weeks) occurs in 20 % of pregnancies of which half are due to aneuploidy. However, the mechanisms that drive euploid miscarriage are poorly understood and, despite its prevalence, there are no interventions that prevent sporadic miscarriage. Emerging evidence supports the role of the vaginal microbiota in adverse pregnancy outcome. Our group has previously shown that miscarriage is associated with vaginal dysbiosis but the cytogenetic status of those miscarriages was unknown. The existence and origin of an early pregnancy placental niche and the interaction between the rectal and vaginal microbiota in early pregnancy loss has not been addressed.

Bacterial 16S rRNA gene based metataxonomic analysis was used to interrogate the vaginal microbiota in women who miscarried compared to term healthy pregnancies. The same approach was used to compare the trophoblast and rectal microbiota of miscarriage patients with termination of pregnancy patient controls. I have shown that, compared to aneuploid miscarriage, euploid miscarriage is associated with a significantly higher prevalence of *Lactobacillus* spp. deplete vaginal microbial communities. In women with *Lactobacillus* spp. deplete vaginal microbial communities, euploid miscarriage associates with higher concentrations of pro-inflammatory cytokines (measured using the Human Magnetic Luminex Screening 8-plex Assay) when compared to viable term pregnancy. *Prevotella bivia* and *Streptococcus* were identified as particularly common in euploid miscarriage and as drivers of proinflammatory cytokines. This shows that it is a combination of an adverse vagina microbiota and the maternal local immune response to it that predicts miscarriage. I have also demonstrated that there is a bacterial signal in trophoblast above the background contamination control, but it was difficult to account for cross contamination via the vaginal niche due to sample collection methods. In those that had an adverse vaginal microbiota significant rectal dysbiosis was seen in euploid compared to aneuploid miscarriage. Furthermore, a healthy gut microbiome appears to dampen the cervicovaginal immune response to *Lactobacillus* spp. deplete vaginal microbiota.

Overall the findings presented in this thesis support the hypothesis that the vaginal microbiota plays an important aetiological role in euploid miscarriage and that the rectal or gut microbiota may modulate the inflammatory reaction to vaginal dysbiosis. These findings suggest that modulation of the vaginal and or rectal/gut microbiota, using prebiotics or live biotherapeutics may reduce the risk of miscarriage.

CHAPTER 1: Introduction

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1 Introduction

1.1 Miscarriage

1.1.1 Definition

The term miscarriage refers to the loss of an intrauterine pregnancy before 24 completed weeks gestation. The term clinical miscarriage refers to cases that are confirmed by ultrasound or histology [1, 2]. The definition of recurrent pregnancy loss has recently been updated from the European Society of Human Reproduction and Embryology (ESHRE) guideline group and is characterised by the loss of two or more pregnancies [3]. Previously early miscarriage was defined as pregnancy loss before 12 weeks and late miscarriage losses between 12-24weeks. However, a recent consensus statement from the ESHRE early pregnancy specialist interest group claimed that this outdated distinction is not based on ultrasound findings or knowledge of the biological process. This group proposed that by the end of the ninth gestational week organogenesis is complete and this marks the end of the embryonic period. Therefore the term early miscarriage should be reserved for those miscarriages less than 10 weeks gestation and fetal miscarriage refers to those $\geq$10 weeks with a fetus $\geq$33mm on ultrasound scan [2].

1.1.2 Incidence

Spontaneous miscarriage is recognised as the commonest early pregnancy complication affecting 15-20% of all pregnancies recorded by hospital databases. The actual figure is likely to be as high as 30% given the number of unreported cases in the community [4]. Second trimester miscarriage is much less common with studies reporting incidences that vary between 1-4% and studies investigating recurrent pregnancy loss (RPL) identify an incidence of 1-2% [1, 3].

1.1.3 Diagnosing Miscarriage

The criteria for miscarriage diagnosis have been robustly interrogated in recent years. A prospective multi-centre observational study published in 2011 investigated the limitations of cut off values for mean gestational sac diameter (≥16mm) and crown rump length (≥5mm) and found unacceptably high false positive rates for miscarriage at 4.4% and 8.3% respectively [5]. Further work exploring gestational sac size and embryonic growth did not provide additional safe criteria for diagnosing miscarriage [6]. The data from these studies prompted national guidance to change and currently the Royal College of Obstetricians and Gynaecologists (RCOG) and the National Institute for Health and Care Excellence (NICE) have adopted the following criteria to define miscarriage [7]:

- Empty gestational sac of mean gestational sac diameter ≥25mm
  Or
- Embryo with a crown-rump length ≥7mm with no heartbeat

1.1.4 Aetiology of miscarriage

1.1.4.1 Cytogenetic Abnormalities

The most common cause of pregnancy loss in the first trimester is sporadic chromosomal error, which represents approximately 50% of pregnancies from which trophoblast has been cultured and karyotyped. However, chromosomal error contributes to less than 4% of losses between 12-22weeks [8, 9]. Recent work has focused on comparing molecular approaches to more traditional techniques that require chorionic villi to be separated from maternal tissue and grown in culture. Some of these studies found a higher detection rate with molecular techniques increasing the contribution of chromosomal anomalies in first trimester loss to nearly 70% [10]. In a study using chromosome microarray analysis 1861 samples of fetal origin cytogenetic abnormality was detected in 59.4% (aneuploidy in 85.4%, triploidy in 10.3%, structural anomalies or tetraploidy in 4.2%) [11]. Nonetheless each new technique has its own set of benefits and limitations and no specific approach is considered superior (Table 1.1) [12]. Despite the influence of chromosomal error in first trimester loss there is evolving evidence that most human embryos are mosaic as cleavage stage embryos but can endure aneuploidy via mitotic non-disjunction. A high frequency of chromosome instability was seen when 23 good quality embryos from women <35 years old without preimplantation genetic aneuploidy

screening indications were investigated. Only 9% were normal diploid in all blastomeres [13]. These studies are the best available representation of normal human embryogenesis [14]. There is a limited understanding as to when mosaicism becomes clinically relevant although it is likely dependent on when such aberrations are generated during human development [15]. A study that transferred mosaic embryos, confirmed by array comparative genomic hybridization, in 18 women who had no euploid embryos available for transfer, resulted in 6 healthy euploid babies [16]. Therefore, transient aneuploidy does not mean that a poor outcome is destined for all these embryos. Such embryos may drive adaptation, evade cellular stress and self-correct via apoptosis [17].

Table 1.1 Comparison of traditional and molecular cytogenetic techniques.

	Traditional	Molecular			
	Cytogenetics	**SNP Microarray**	**a-CGH**	**QF-PCR**	**MLPA**
Quick Turnover Time	No (4 Weeks)	Yes	Yes	Yes	Yes
Identifies Maternal Contamination	No	Yes	No	No	No
Fresh Tissue Only	Yes	No	No	Yes	Yes
Ability To Detect:					
Microdeletions/Duplication <5mb	No	Yes	Yes	Yes	Yes
Balanced Translocations	Yes	No	No	No	No
Triploidy	Yes	Yes	No	Yes	No
Tetraploidy	Yes	No	No	No	No
Mosaicism	Yes	Yes	No	Yes	No
Parental Origin Of Aneuploidy	No	Yes	No	Yes	No

Mb: million base pairs, aCGH: array comparative genomic hybridization; QF-PCR: quantitative fluorescent polymerase chain reaction; SNP: single nucleotide polymorphism array; MLPA: multiplex ligation probe amplification. *Source: Adapted from McQueen et al, Hardy et al, Wapner et al and Diego-Alvaraz et al. [9, 12, 18, 19]*

1.1.4.2 Infection

Current evidence suggests infection is implicated in 15% of early miscarriages and 66% of late miscarriages (12-24 weeks) [20]. It is not clear which mechanisms are activated to induce miscarriage, but these may involve disrupting the complex immunological state at the maternal-fetal interface that is required for a successful pregnancy. Screening patients for bacterial vaginosis (BV), which is microbiologically characterised by a loss of commensal vaginal *Lactobacillus* species and overgrowth of strict anaerobes, found no difference in the rate of conception between women with BV and normal flora. However, there was an

increased risk of miscarriage in the first trimester, independent of other known risk factors [21]. A recent meta-analysis also demonstrated an increased relative risk (1.68, 95% CI 1.24-2.27) of first trimester loss in patients diagnosed with BV compared to those with a normal vaginal microbiota. However, there was no significant impact on live birth rate or clinical pregnancy rate [22]. Furthermore, the impact of BV on first trimester miscarriage has not been reproduced outside of assisted conception units. Other studies have identified an association with second trimester miscarriage or preterm birth [23]. These studies also use classical microbiological methods rather than molecular based methods such as bacterial DNA sequence analysis. The role of infection in second trimester loss is considered even more significant. One study identified histological chorioamnionitis in the placenta in 77.2% of cases compared to no chorioamnionitis in the control group [24]. Furthermore, brucellosis, *Chlamydia trachomatis* and syphilis have all been implicated in miscarriage. Giakoumelou and colleagues explored the mechanism driving miscarriage in patients infected with *Chlamydia trachomatis* and reported that the expression of chemokines crucial to trophoblast invasion was reduced in infected decidualized cells. The impaired endometrial stromal cell decidualization contributed to miscarriage in these patients [25]. There is limited evidence identifying an association between viral infections such as herpes simplex 1, 2, human papillomavirus and parvovirus B19 and miscarriage. However, evidence does suggest that miscarriage is associated with human immunodeficiency virus (HIV) and this association persists after adjustment for confounding variables [20].

1.1.4.3 Uterine anomalies

Congenital uterine anomalies result from incomplete fusion of the Mullerian Ducts in embryological development. Meta-analyses have shown that arcuate uteri are associated with increased rates of second trimester miscarriage (RR 2.39; 95%CI, 1.33-4.27, *P*= 0.003). Septate and subseptate uteri are associated with reduced clinical pregnancy rates and increased rates of first trimester miscarriage. However, one of the main limitations with pooled data from these studies is the heterogeneity with regards to the classification systems used [26]. In terms of surgical intervention there are no randomized control trials in this field to support intervention [27]. A recent retrospective multicentre cohort study analysed cases where hysteroscopic metroplasty was used in patients with a dysmorphic uterus and unexplained infertility or RPL. The study found a potential increase in live birth rate in those who had a metroplasty (47.4% conceived spontaneously of whom 32.4% had failed one or more attempt of assisted conception). However, given the methodological flaws in this study

design there is still inconclusive evidence as to whether surgical intervention will benefit these patients [28].

1.1.4.5 Factors associated with recurrent miscarriage

Recurrent pregnancy loss is defined as two or more pregnancy losses [29]. To date, research interest has largely been focused on a spectrum of subclinical disorders that might underpin this condition. Studies have explored thrombophilia, anatomical, endocrine and immunological factors and the potential role that they have in increasing the risk of RPL. However, these studies are often underpowered and fail to fulfil Hill's criteria for causation (a set of well recognised criteria that need to be met to establish a causal link) [30]. The strongest body of evidence lies with antiphospholipid syndrome (APS). Metanalyses have demonstrated that lupus anticoagulant was associated with RPL (OR 7.79, 95% CI 2.30-26.45). The combination of all anticardiolipin antibody titres showed association with fetal loss <13 weeks (OR 3.56, 95% CI 1.48-8.59) and fetal loss <24 weeks (OR 3.57, 95% CI2.26-5.65) [31]. Furthermore, a metanalysis exploring the use of heparin in APS with RPL demonstrated a significant benefit with a combination of heparin and aspirin [32]. Hence in the recent ESHRE guidance the only strong recommendation for treatment is low dose aspirin prior to conception and prophylactic heparin once a positive pregnancy test is confirmed in patients with APS and three or more pregnancy losses [3].

Systematic reviews have shown that patients with subclinical hypothyroidism or thyroid antibodies (specifically thyroid peroxidase) have an increased risk of RPL [33]. However, a randomised control trial in 952 women evaluating the use of 50 µg per day of levothyroxine in euthyroid women with thyroid antibodies showed no improvement in the live birth rate [34]. Nonetheless, this study did not explore titrating the dose of levothyroxine based on thyroid stimulating hormone. The current ESHRE guidelines recommend investigating RPL patients for thyroid abnormalities and only treating if hypothyroidism is detected [3]. There is limited evidence on the value of immunological screening in RPL patients and no therapeutic interventions that should be offered [3]. It remains unclear if peripheral biomarkers reflect the maternal-fetal interface. In particular a meta-analysis examining uterine natural killer cells in the context of RPL showed no significant difference in the uterine NK levels in women with RPL compared to controls [35]. RPL couples do have an increased likelihood of parental karyotypic disorders such as balanced translocations that can manifest in an unbalanced fetal chromosomal abnormality and result in miscarriage. Nonetheless, studies have shown the

chance of having a healthy pregnancy in carrier couples with RPL is the same as RPL non-carrier couples [36]. Furthermore, with each additional miscarriage the frequency of euploid loss increases and the chance of successful pregnancy decreases. However, there is still a high cumulative live birth rate in the affected group regardless of medical intervention [37]. Although there remains a lack of understanding as to the mechanisms that underpin RPL, increasing loss of euploid embryos indicates that the uterus may have a stronger influence on the underlying pathology.

1.1.4.4 Endometrium as a biosensor

As discussed above, many pre-implantation embryos are mosaic or aneuploid and self-correction via apoptosis may occur to ensure survival and drive adaption. However, given the diversity of embryos other mechanisms must lie within the endometrium to sense and select out sub-optimal embryos to ensure maternal investment is reserved for successful pregnancies [17].

In vitro studies have shown that arrested embryos do not initiate the same inflammatory response required for implantation as the control or developing embryos. This work demonstrates how the endometrium is a selective biosensor of embryo quality [38]. Figure 1.1 describes several quality control checkpoints proposed by Jan Brosens' group that need to be met in the first 12 weeks to reduce the likelihood of failure thereafter. The inability of the endometrium to positively select good quality embryos or negatively select out poor quality embryos will lead to clinical miscarriage. Endometrial mesenchymal stem cells (eMSCs) are immune-privileged, highly regenerative and able to modulate the inflammatory response and promote wound healing [39]. The main feature of the human menstrual cycle is the cycling endometrium and the ability to undergo decidualisation in every ovulatory cycle regardless of embryo cues. The process of decidualisation encourages stromal cells to differentiate into decidual cells that are resistant to stress and create a robust interface in pregnancy [17]. The regular cycling nature of the endometrium demonstrates the need for endometrial regeneration via eMSCs. Recent studies have shown that RPL is associated with uterine stem cell deficiency and pro-senescent cycles in the peri-implantation endometrium. However, the reason behind why this deficiency occurs has yet to be explored [40, 41]. Furthermore, a recent randomized double-blind placebo-controlled study described the use of sitagliptin, a dipeptidyl-peptidase IV (DPP4) inhibitor to increase the endometrial plasticity during the implantation window in patients with RPL [42]. DPP4 inhibits stromal cell derived factor 1-

alpha (SDF-1) which is a potent chemotactic factor crucial in homing the bone marrow derived cells into the endometrium. The study found that inhibition of DPP4 by sitagliptin leads to enhanced eMSC in midluteal endometrium and reduced decidual senescence. However, the study was limited in that the measure of eMSC was carried out indirectly using colony-forming unit assays and it is unclear whether the endometrial response was transient or persisted in subsequent cycles. Also the study was not powered to assess the effect of preconception sitagliptin on live birth rate in RPL patients [42].

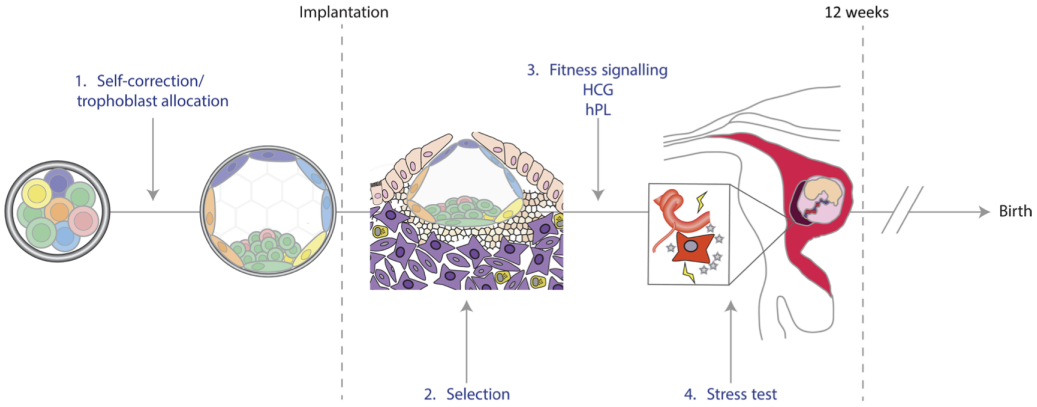

Figure 1.1 First trimester checkpoints controlling embryo selection, implantation and placentation.

Embryo self-correction to ensure a good quality embryo advances (1), the decidualizing endometrium acts as a biosensor for the embryo (2), the embryo needs to ensure it produces fitness signals prior to placental hormone production at 10 weeks (3) and finally the 'stress test' demonstrated by the onset of placental perfusion and the significant maternal cardiovascular investment that ensues (4).
Source: Lucas et al Reproduction [17].

1.1.4.6 First trimester bleeding and the role of progesterone

The peak incidence of bleeding in the first trimester occurs between 6-7 weeks gestation. This time point correlates with the shift in progesterone production from the corpus luteum to the placenta. If the placenta fails to adequately produce progesterone at this stage bleeding can ensue [43]. Therefore, it has been proposed that prevention of this transient fall in progesterone may minimise the risk of miscarriage in patients with early pregnancy bleeding. A multicentre double-blind placebo controlled trial was conducted in patients with early pregnancy bleeding [44]. A total of 4153 women received either 400mg of progesterone as a vaginal suppository twice daily or a placebo. There was no significant difference between the live birth rates in either group. However, there was a subgroup of patients with early pregnancy bleeding and any number of previous miscarriages that showed a significant benefit in the live birth rate with the use of progesterone. Therefore, there was a proportionate benefit in women with increasing number of previous miscarriages. [44].

1.1.4.7 Risk Factors

A large scale, prospective register-based study from Norway between 2009-2013 examined risk of miscarriage in 421,201 pregnancies[45]. The study found that the risk of miscarriage was lowest in women aged 25-29 (10%) and increased after the age of 30 reaching 53% in women aged over 45. This study also demonstrated an age adjusted odds ratio that increased the risk of miscarriage with every previous miscarriage; after one, OR = 1.54, after two, 2.21 and after three, 3.97. Certain lifestyle factors have also been linked to risk of first trimester miscarriage. Studies have shown an increased risk of miscarriage with alcohol in a dose dependent manner (adjusted hazard ratio 1.66 and 2.82 with 2-3.5 drinks per week and >4 drinks per week respectively) [36]. A meta-analysis of prospective studies exploring the association between caffeine intake and pregnancy loss have shown high caffeine intake during pregnancy is associated with pregnancy loss. However, such studies are often affected by publication bias and residual confounding due to incomplete adjustment for smoking and pregnancy symptoms [46]. The relationship between smoking and poor obstetric outcomes has been well established. The association between smoking and miscarriage was detailed in a meta-analysis that reported that active smoking is associated with an increase in miscarriage (relative risk 1.23, 95% confidence interval 1.16-1.3) [47]. A meta-analysis investigating the association between obesity and miscarriage demonstrated that women with a body mass index $\geq$ 25 kg/m^2 have a significantly higher odds of miscarriage regardless of the method of conception (OR 1.67, 95% confidence interval 1.25-2.25) [48].

1.1.5 The Psychological Burden of miscarriage

There are profound psychological sequelae associated with early pregnancy loss (EPL). A recent review found evidence of significant depression and anxiety in the first month following EPL as well as evidence of post-traumatic stress symptoms [49]. A prospective multi-centre cohort study investigated the levels of posttraumatic stress, depression and anxiety in women at 1, 3 and 9 months after EPL [50]. Post-traumatic stress symptoms were seen in 29% of women 1 month after EPL and 18% after 9 months. Moderate/severe depression was reported in 11% of women after 1 month and 6% after 9 months. The high levels of post-traumatic stress, anxiety and depression after EPL decline over time but still remain significant even after 9 months. This illustrates the profound psychological morbidity associated with EPL and the impact this has on every aspect of life. An earlier prospective longitudinal study investigated the psychological conditions of patients four weeks and four months post diagnosis of miscarriage. The study identified patients reported less self-blame in the group that confirmed chromosomal abnormality as the underlying cause of the miscarriage. This sheds light on how understanding the underlying mechanisms are not only important for future intervention but also to help a woman come to terms with the loss she has suffered [51].

1.2 The Human Microbiome

The term ‘microbiome’ refers to the collection of all microorganisms (bacteria, archaea, lower and higher eukaryotes and viruses) as well as their genes and local environmental conditions within a specific niche. The term ‘microbiota’ describes the microbial communities living in a microhabitat (e.g. a body site) [52]. It is understood that microbial cells colonizing the human body out number host cells at a ratio of about 3:1 [53]. However, these microbes have coevolved with the host to form an important symbiotic relationship that has a crucial role in human health and disease [53, 54].

1.2.1 Approaches for characterisation of the microbiome

The evolution of microbiome research has rapidly developed over the last couple of decades as limitations of culture-based techniques have been superseded by advanced culture-independent approaches. Culture, used since the late 19th century, is labour intensive and provides only a limited view of the diversity of bacteria in a particular body site. Even though more sophisticated culture-based approaches permits isolation and growth of fastidious organisms, the growth of some depends on the metabolic activity of others. Therefore, until recently, the true compositional complexity of microbial communities was severely underestimated by culture.

Culture independent techniques often involve sequencing of bacterial DNA (Figure 1.2). These high throughput techniques characterise the microbiota by mapping DNA sequences against libraries and permit estimation of relative proportions of microorganisms in a sample. Shotgun metagenomic sequencing involves sequencing whole community DNA (bacterial, viral, fungal and host). Advantages of this technique include the ability to explore the genetic diversity and putative function of the microbiota and is not limited to bacteria. However, a significant proportion of the DNA sequenced is of host origin, which consumes sequencing power and requires bioinformatic techniques to separate bacterial from human sequence data. Therefore, a more widely used technique is metataxonomics or amplicon sequencing, which focuses on sequencing and amplifying specific regions of DNA such as regions of the bacterial 16S ribosomal RNA genes (16S rRNA). This allows efficient phylogenetic identification but is limited to the analysis of bacteria alone and depending on the quality of sequence data obtained, can be limited in taxonomic resolution [54, 55].

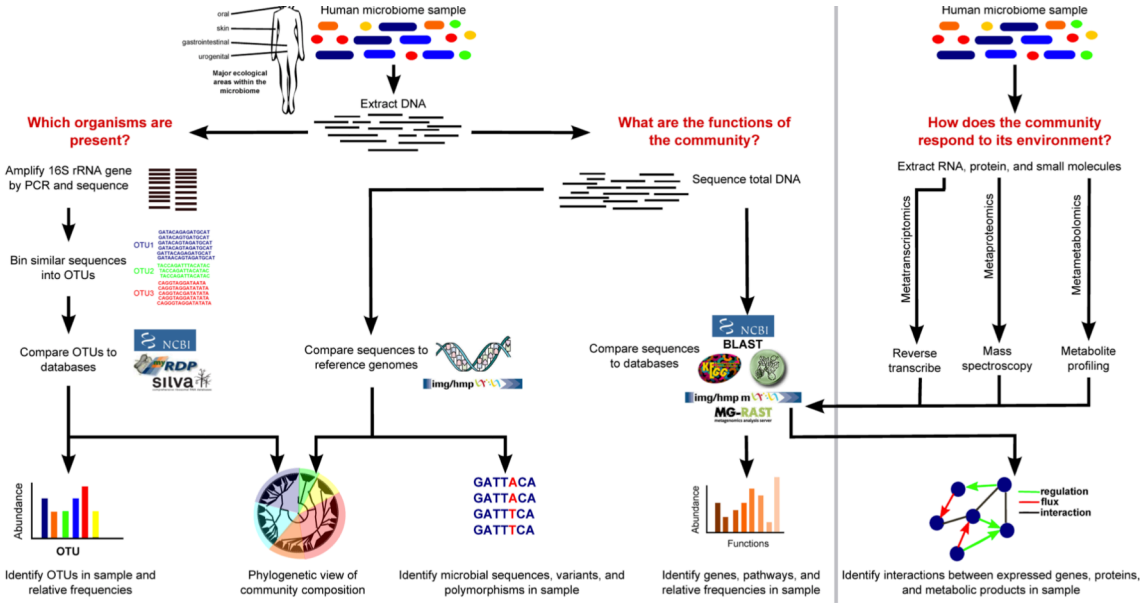

Figure 1.2 Approaches for profiling the human microbiome.

This figure demonstrates the current techniques used to profile the microbiome. One aspect is to identify which organisms are present in a given sample by amplifying sequences of the 16S rRNA gene. The function of the community is determined by sequencing total metagenomic DNA which is then compared to function orientated databases. Emerging methods such as metatranscriptomics and metaproteomics can integrate gene expression within the microbial communities. Abbreviations: OTU; operational taxonomic unit. This characterises bacteria based on sequence similarity depending on a particular threshold (e.g. >95%).
Source Morgan et al [56]

The ribosome is a ribonucleoprotein complex consisting of proteins and RNA which allows proteins to be synthesized. In a bacterial cell the 70S ribosomes are dispersed throughout the cytoplasm and consist of a large subunit (50S) and small subunit (30S). The small subunit (30S) is composed of 16S rRNA and 20 proteins. The 16S rRNA is encoded by a gene of 1500 nucleotides and nine hypervariable regions (Figure 1.3). One of the main functions of 16S rRNA is the initiation and extension of protein synthesis. It is present in all bacterial species in a variety of copy numbers. Its conservation between bacterial species allows amplification but the divergence in the variable regions produce a phylogenetic signal that allows 16S rRNA gene DNA sequence data to identify bacteria potentially to species level [55, 57].

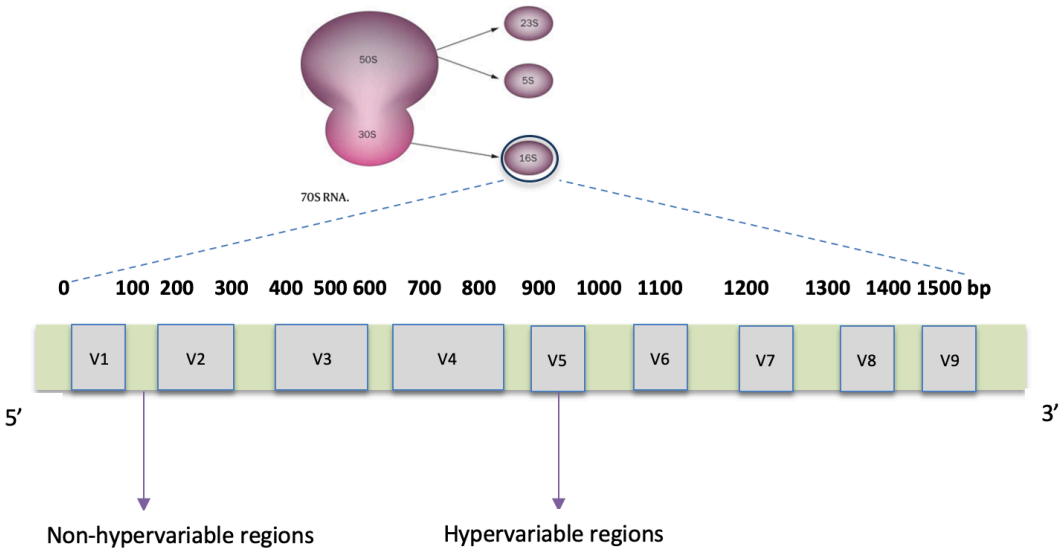

Figure 1.3 The 16SrRNA gene demonstrating the conserved regions and hypervariable regions.

Source: adapted from Renvoisé et al [57] and Fraher et al [55]

1.2.2 Bacterial Taxonomy

Taxonomy is the science of classifying and organizing living organisms in a process that catalogues biodiversity. There are three separate subdisciplines of taxonomy; classification; which groups organisms into taxa based on similarities, nomenclature; the assignment of names to taxonomic groups and identification; which determines which group new isolates belong to. In the past there has been limited agreement on the criteria required to distinguish between different bacterial species. However, the currently accepted approach is to classify genetically related phylogenetic groups in a hierarchical way [58, 59] (Figure 1.4).

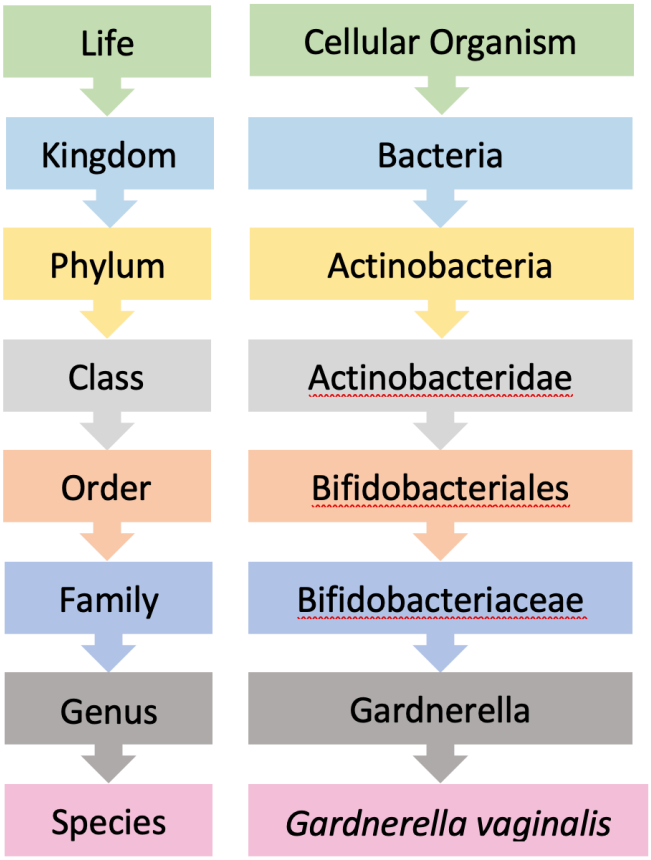

Figure 1.4 Example of bacterial taxonomy of Gardnerella vaginalis

1.3 The Human Microbiome Project

The National Institutes of Health Human Microbiome Project (HMP) was launched in 2007 and was one of the first initiatives to focus on characterising microbial communities from different body sites including the oral, nasal, vaginal, gut and skin of healthy adults. A key finding of this work was that identifying the microbial communities alone was not sufficient to explore the host phenotype. It was crucial to integrate molecular functional components. This gave rise to the second phase of the project which explored the host-microbiome interplay through investigating longitudinal samples, metabolomic profiles and host cytokine profiles [60, 61]. The second phase of the project focused on preterm birth, inflammatory bowel disease and pre-diabetes.

1.4 The vaginal microbiota

It has been long established that the vagina is colonized by microbiota throughout a women's lifespan. Gustav Doderlein first described Lactobacilli in 1892 and subsequently, these species were shown to be keystone members of vaginal microbial communities [62]. There is a large body of evidence that Lactobacillus colonisation of the vagina promotes homeostasis and has a substantial impact on reproductive health [63-65]. They are thought to be able to do this by utilizing glycogen within the vagina to produce lactic acid, which creates an acidic pH and deters the growth of many other bacteria. The production of lactic acid also contributes to the upregulation of autophagy which clears intracellular pathogens from vaginal epithelial cells [66]. Lactobacilli also inhibit pathogenic bacteria from binding to vaginal epithelial cells and produce bacteriocins to eliminate other bacteria and strengthen their dominance [67, 68].

Unlike other body sites, vaginal microbial communities can be readily classified into groups based upon relative abundance profiles. One of the first studies to explore this was by Ravel and colleagues who characterised the vaginal microbiota composition of 396 asymptomatic reproductively aged women. At species level the vaginal microbiota there were able to divide samples into 5 major groups termed community state types (CSTs) [69] :

CST I – *Lactobacillus crispatus dominated (L. crispatus)*
CST II – *Lactobacillus gasseri dominated (L. gasseri)*
CST III – *Lactobacillus iners dominated* (*L. iners)*
CST IV – *'high diversity' (Lactobacillus depleted, high diversity with enrichment of* anaerobic *organisms)*
CST V – *Lactobacillus jensenii dominated (L. jensenii)*

These groups likely play distinct functional roles in the cervicovaginal niche. *Lactobacillus crispatus, gasseri* and *jensenii* all produce both L and D isomers of lactic acid whereas *Lactobacillus* iners has a smaller genetic code and cannot produce D-lactic acid. The D isomer is crucial as it downregulates matrix metalloproteinase -8 (MMP-8) production which inhibits entry of bacteria to the upper genital tract [66]. Studies exploring the vaginal secretions in women with a vaginal microbiota dominant in *L. crispatus* demonstrate increased autophagy and lower cellular stress compared to women dominated by *L. iners* [70]. The restricted genome size of *L.iners* also makes it more permissive to co-colonisation with other bacterial

species which can provide it with cofactors, substrates and energy sources that it is unable to produce itself. Accordingly, *L.iners* colonisation of the vagina can precede a compositional shift towards increased microbial diversity of the niche [71].

The CST IV (high diversity) group is characterised by a low abundance of *Lactobacillus* spp. and an over representation of anaerobic bacteria such as *Atopbium, Prevotella, Sneathia, Gardnerella* and *Mobiliuncus* [72]. A number of these taxa have been associated with BV which is a polymicrobial disorder that is associated with preterm birth [73], higher risk of acquiring sexually transmitted infections [74], and late miscarriage [75]. Nonetheless there is limited understanding to the pathogenesis of BV and the varied clinical picture and reproductive outcomes suggest there is still a great deal to be uncovered [76-78].

1.4.1 Vaginal microbiota and reproductive health outcomes

Whilst the association between the vaginal microbiota and preterm birth has been widely studied there is paucity of data exploring the early pregnancy vaginal microbiota and adverse early pregnancy events. Our group recently conducted a nested case control study of 161 pregnancies and concluded that reduced abundance in *Lactobacillus* spp. and increased alpha diversity is associated with first trimester miscarriage, (P=0.005 and 0.003 respectively). One of the limitations of this study was the inability to define which miscarriages were euploid or aneuploid. This would have provided insight into which groups were most vulnerable to a suboptimal microbial composition [79]. Work has also focused on how the vaginal microbiota influences infertility patients. In a prospective study of 130 infertility patients undergoing in-vitro fertilization (IVF), the clinical pregnancy rate was significantly lower (P=0.004) in women with an abnormal microbiota (high concentrations of *Gardnerella vaginalis* and/or *Atopobium vaginae*) [80]. Other studies have also shown that a vaginal microbiota dominant in *Lactobacillus* spp. in infertility patients is associated with a positive pregnancy outcome. However, many of these studies have small samples sizes and a wide range of heterogeneity in the patient populations [81, 82]. Subsequent work exploring the bacterial communities in paired endometrial fluid and vaginal samples in infertile patients undergoing IVF demonstrated those with a non-*Lactobacillus* spp. dominant microbiota had decreased implantation, decreased ongoing pregnancy and lower live birth rates (P= 0.02, 0.02, 0.002 respectively) [83]. While additional studies have tended to corroborate these findings, most fail to address the cross contamination effect during sample collection of the low biomass in the endometrium passing through the high biomass vaginal microbiota [84].

A strong body of evidence exists with regards to the risk of preterm birth and vaginal microbial composition. Our group characterised the vaginal microbial communities longitudinally from 6-weeks gestation in women high and low risk for preterm birth. Those patients with a microbial composition dominated by non-*Lactobacillus* spp. were associated with preterm pre-labour rupture of membranes (PPROM) at all gestational age time points [85]. This work highlighted the early pregnancy microbial composition can influence outcomes that occur at a later timepoint. Women who deliver at term are more likely to have a vaginal microbiota dominant in *L. crispatus* and patients who deliver preterm consistently show increased richness and diversity within the vaginal communities [86-89]. A prospective study examining the vaginal microbiota from patients with a dilated cervix pre and post rescue cerclage, identified reduced *Lactobacillus* spp. abundance in patients with premature cervical dilation. Furthermore, this study also demonstrated that *Gardnerella vaginalis* was associated with unsuccessful rescue cerclage (*P*=0.017) [90]. Subsequent work that looked at specific taxa associated with preterm birth demonstrated a higher prevalence of Mollicutes in preterm birth compared to those who delivered at term [64]. A recent study analysing the vaginal samples of 45 spontaneous preterm birth and 90 pregnancies that delivered at term also confirmed women who delivered at term had a predominance in *L. crispatus* (P=0.014). This study also found specific taxa were more abundant in preterm birth, including, BV associated bacterium 1 (BVAB-1), *Prevotella* and *Sneathia amnii.* Furthermore, detailed analysis was performed using metagenomic and metatranscriptomics to interrogate the genes encoding for bacterial secretion systems, which are key in pathogenicity. There were more transcriptionally abundant genes that encoded secreted proteins in the preterm birth cohort that were linked to the taxa identified by metataxonomics [91].

1.4.2 Vaginal microbiota and pregnancy

The composition of the vaginal microbiota in pregnancy displays decreased diversity, stability and increased levels of *Lactobacillus* spp.. One of the first longitudinal studies that characterised the vaginal microbiota using sequencing techniques in pregnant and non-pregnant women found that high diversity communities were rarely seen in pregnant women who delivered at term [72]. Even though bacterial communities in pregnancy did appear to shift between CSTs dominant in *Lactobacillus* spp. they rarely transitioned to CST IV. It is proposed that *Lactobacillus* spp. stability in pregnancy is an evolutionary adaptation to enhance reproductive fitness and protect against ascending infection [72]. The stability may

be driven by high oestrogen levels which encourage glycogen deposition in the vaginal epithelium and support the colonization of *Lactobacillus* spp. [92] . Work has shown that the post-partum state is characterised by a dramatic shift to less *Lactobacillus* spp. dominant communities with increased alpha diversity [93]

1.4.3 Factors that influence the vaginal microbiota

1.4.3.1 Ethnicity

One of the first studies to explore the vaginal microbiota in asymptomatic reproductively aged women highlighted that ethnicity significantly contributed to differences in vaginal microbial composition. Vaginal bacterial communities dominated by *Lactobacillus* spp. were found in 80.2% and 89.7% of Asian and White women respectively, whereas these communities only dominated 59.6% and 61.9% of Hispanic and Black women respectively. Furthermore, there was an overrepresentation of the high diversity group (CST IV) in the Black and Hispanic group compared to Asian and White women [69]. Subsequent work has corroborated these findings. A Dutch study of 610 patients demonstrated that a vaginal microbiota dominated by non-*Lactobacillus* spp. was most common in women of sub-Saharan African descent, independent of modifiable behaviours such as sexual risk behaviours, vaginal cleansing practices and hormonal contraceptive use [94].

A recent collaborative study from the HMP focused on analysing the vaginal microbiota in a cohort of 300 pregnant women and 300 non-pregnant women of African, Hispanic and European ancestry. This study showed a shift towards a *Lactobacillus* spp. dominant profile in pregnancy at the expense of anaerobic communities. These changes, which were confirmed by metagenomic and metatranscriptomic data, occurred in early pregnancy, and were most significant in women of African and Hispanic ancestry (P=0.01). No significant effect was seen on the prevalence of BVAB-1 (often associated with dysbiosis) in pregnancy. BVAB-1 is often seen in women of African ancestry and therefore resisting transition to *Lactobacillus* spp. dominance may explain the increased risk of adverse pregnancy outcomes in this ethnic group. Interestingly no significant differences were seen in the shift of vaginal communities of pregnant and non-pregnant women of European ancestry. These results are unsurprising given that non-pregnant European women commonly already have a *Lactobacillus* spp. dominance and that hormonal changes in pregnancy contribute towards vaginal communities enriched in *Lactobacillus* spp. However, this study did not take socioeconomic status and age into account, both can adversely impact the vaginal microbiota [95]. Nonetheless, in previous

studies the association between race and diversity in the vaginal microbiota was present irrespective of socioeconomic status [96].

1.4.3.2 Hygiene practices

Hygiene practices have a considerable influence on the vaginal microbiota. A study reporting the temporal dynamics of the vaginal bacterial communities in 32 reproductively aged women over 16 weeks showed fluctuation in composition affected by time of menstrual cycle and sexual activity [97]. The practice of vaginal douching is also thought to contribute to the disruption of the vaginal communities. An *in-vitro* study investigating the effects of specific vaginal douching products demonstrated the ability of all products to limit the growth of Lactobacilli strains [98]. A small study investigating the influence of tampons on the vaginal microbiota showed no significant impact. However, this study was small involving only seven women without a true control group and was funded by a tampon manufacturer [99].

1.4.3.3 Endogenous and exogenous female hormones

The fluctuation of hormonal levels at different reproductive stages of a woman's life can alter the vaginal microbial composition. The production of glycogen in the vaginal epithelium is under the control of oestrogen and supports the colonization of *Lactobacillus* spp. It is the presence of α-amylase in the lower genital tract that metabolises glycogen into smaller breakdown products that allows the growth of *Lactobacillus* spp. [100, 101]. A recent metanalysis explored the specific structure of the vaginal microbial communities at different gynaecological stages and correlated this with female sex hormones. They found the predominance of *Lactobacillus* spp. changes according to different physiological states. High bacterial diversity is seen at early stages of puberty, but this decreases as gonadal hormone levels rise. The rising hormonal levels in pregnancy, especially in the second and third trimester also correlates with a predominance of *Lactobacillus* spp. [102]. The stability of the vaginal microbial composition fluctuates during the menstrual cycle and this is also thought to be due to the changing hormonal levels in addition to bleeding which can lead to a change in vaginal pH and alter substrate availability in the niche [97]. The reduced oestrogen levels in post-menopausal women that cause vulvovaginal atrophy have also shown to increase levels of bacterial diversity [103]. Studies exploring the vaginal microbial composition in the hypo-oestrogenic postpartum period demonstrated dramatic changes and a shift to less *Lactobacillus* spp. dominance [93]. The use of hormonal contraceptives also influences the vaginal microbial composition. A randomised trial identified the protective role of combined

oral contraceptives in reducing bacterial diversity and inflammatory taxa [104]. Other studies have also shown the role of combined oral contraceptives in decreasing BV association bacterial taxa. However, this is not the case for progesterone only contraceptives or non-hormonal contraceptives [105].

1.4.3.4 Antibiotics

Given the large body of evidence that shows changes in the vaginal microbial composition can influence reproductive outcomes, it is crucial to explore the role of therapeutics that can alter the composition. Most studies to date have examined the role of antibiotics in pregnant patients with BV in relation to the risk of preterm birth, and the evidence is conflicting. The use of antibiotics is thought to prevent colonization of pathogenic bacteria and combat ascending vaginal infection. The largest randomized control trial, which screened 84,530 women in early pregnancy and randomised 3105 women with BV to an oral clindamycin treatment arm and placebo arm, found no risk reduction for late miscarriage (16-22 weeks) or spontaneous very preterm birth (22-32weeks) [106]. A subsequent metanalysis confirmed these findings [107] although it highlighted heterogeneity of the studies included, with different patient cohorts and antibiotic regimes being compared.

Certain antibiotics themselves appear to be harmful in early pregnancy and can increase the risk of spontaneous miscarriage. A systematic review found macrolides, quinolones and tetracyclines all increased the risk of miscarriage and should be given with caution [108]. Therefore, the use of antibiotics in early pregnancy needs to have an appropriate risk benefit analysis and should only be given when there is clear evidence of an infectious process. Antimicrobial resistance genes are present in the vaginal microbiome of patients with BV symptoms and therefore this could influence the use of antibiotics in this field [109]. The formation of biofilms are now implicated in BV and the inability of antimicrobials to penetrate this matrix is also likely to result in treatment failure and resistance [110]. The impact of antibiotics on the vaginal microbial composition and subsequent reproductive outcome is unclear. In patients with PPROM, prophylactic antibiotics is often given to combat ascending vaginal infection. However, studies have shown that *Lactobacillus* spp. depletion is present prior to PPROM and is linked to increased early onset neonatal sepsis. The use of certain antibiotics can exacerbate this dysbiosis especially in those with a favourable *Lactobacillus* spp. dominant vaginal microbiota. It is essential we have well designed trials in the future that use the appropriate antibiotics on the correct group of individuals and probiotics where

appropriate to eradicate pathogenic or BV associated bacteria and ultimately improve patient outcome [111, 112].

1.4.3.5 Probiotics and live biotherapeutics

There is a growing interest in modulation of the vaginal microbiota using probiotics or live biotherapeutic products. A systematic review of oral probiotic use in pregnant women at low risk for preterm birth did not find a reduction in the incidence of preterm birth (<37 weeks) [113]. Recent studies have shown that oral probiotics administered in early pregnancy do not modify the vaginal microbiota [114, 115]. Subsequently a systematic review evaluated the use of vaginal probiotics in BV and vulvovaginal candidiasis. The use of vaginal probiotics was promising in BV cure and prevention, but of the 13 studies included five had medium and eight had high overall risk of bias. There was also minimal detection of probiotic strains after the dosing period, implying a lack of colonization. There is a considerable heterogeneity in these trials in terms of probiotic strain, length of use and duration between last probiotic insertion and vaginal sample collection [116]. There is now a drive to conduct rigorous trials especially in pregnancy, to address these issues and improve patient outcome. A recent randomized double blind placebo controlled trial in 228 women found that vaginally administered L. *crispatus* prevented BV recurrence after metronidazole treatment [117]. This work encourages future trials to focus on vaginal administration of *L. crispatus* in pregnancy to alter the vaginal composition and improve pregnancy outcome.

1.5 Gut microbiota

The gut microbiota differs significantly from the vaginal microbiota as it constitutes a more diverse and rich bacterial community. Generally, gut diversity is been associated with a healthy host; whereas a loss of diversity has been often linked to a variety of disease states including inflammatory bowel disease and obesity [63].

Firmicutes, Bacteroidetes, Actinobacteria and *Proteobacteria* are the most abundant phyla in the human gastro-intestinal tract. Many species have been identified as having a crucial role in modulating and influencing the host immune response [112]. Disruption of the commensal gut microbiota can trigger inflammation and alter health outcomes. A central component of the immune system that limits intestinal inflammation is colonic regulatory T cells (Tregs). Certain bacteria such as *Bacteroides fragilis* and Clostridial species induce Treg responses. Humans also rely on bacteria in the gut to breakdown undigestible dietary components.

Bacterial fermentation products such as short chain fatty acids have also been reported to modulate cytokine production and T-reg expression [118].

1.5.1 Gut microbiota in pregnancy

In pregnancy the bacterial load in the gut has been reported to increase throughout the gestational course [119]. The largest study to characterise the changes in gut microbiota composition across each trimester was conducted in 91 women. This study found the gut microbiota dramatically changes from the first trimester to the third trimester with increased diversity between women and increasing *Proteobacteria* and *Actinobacteria*. The stool samples of third trimester women also had increased inflammatory profiles. Furthermore, when the third trimester microbiome was transferred into germ free mice a mild inflammatory response was induced. Therefore, the gut microbiota may change over the course of pregnancy to allow for immunomodulation, successful pregnancy and timely parturition [120]. Since the gut microbiota influences the immune response in pregnancy, a premature alteration can instigate adverse pregnancy outcome. There is also a risk of microbial seeding between the gut and vagina given the anatomical proximity. A study which examined day 4 postpartum faecal samples from 10 preterm birth women and 102 term women found a reduction in OTUs from *Bifidobacterium* and *Streptococcus* [121]. Another smaller study comparing the gut microbiota of term pregnancy and preterm birth found lower levels of *Clostridium* spp. and *Bacteroides* spp. in the preterm group. *Bifidobacterium* is known to have anti-inflammatory properties invitro and *Clostridia* spp. induces T-reg cell activation. Therefore, altered gut composition could trigger inflammation that induces preterm birth and other poor reproductive outcomes. However, there is still a great deal of work that needs to be carried out in this field to generate conclusive evidence [112].

1.5.2 Factors that influence gut microbiota

There are many factors that influence the gut microbiota but understanding their underlying roles is difficult given the impact of confounders. A study that analysed the faecal microbial community of adult female monozygotic and dizygotic twin pairs (154 individuals) found a shared gut microbiota among family members but difference in the specific bacterial lineages [122]. Diet, has a major role in shaping gut microbiota composition. For example, *Prevotella* is enriched in those with high fibre diets in particular countries, whereas individuals from the

US have more *Bacteroides* which correlates with a diet rich in animal protein and saturated fats [123]. Studies have also shown the adult gut microbiota is not resilient to repeated antibiotic administration. Interestingly antibiotics in early life may influence the gut microbiota and the development of obesity. Lifestyle traits such as sleep deprivation can elevate the Firmicutes to Bacteroidetes ratio which may also contribute to poor health outcomes [54].

1.6 Placental microbiota

A large amount of research has been conducted to establish if there is an underlying placental microbiome that contributes to adverse pregnancy outcome. The belief that reproduction occurs in a sterile environment was supported by studies that used traditional culture-based techniques that failed to detect bacteria in the placentas of healthy pregnancies [124]. However, several studies using DNA sequencing techniques have since been employed, which report detection of a microbial signature. Aagaard et al published the first such study that collected placental samples from 320 subjects and reported a unique non-pathogenic placental microbiota niche [125]. Subsequent studies also reported a specific placental microbiota that differed between term and preterm deliveries [126-128]. While there are some reports that these organisms contribute to metabolic function, their low biomass in the placenta questions the physiological significance. Some microbes that have been reported to exist in the placenta are most commonly isolated from soda lakes and marine environments [112]. Therefore reagent and laboratory contamination has been suggested as a potential source of microbial signatures putatively attributed to the placental microbiome [129]. Recent studies have attempted to address this issue by extensively matching the cases being investigated with detailed laboratory controls. These studies could not identify a microbiota within the placenta that was distinguishable from background technical controls [130, 131]. A recent study by Goffau et al found no evidence of bacterial signals from placental samples of pregnancies complicated by preterm birth, pre-eclampsia and small for gestational age infants (n=318) or uncomplicated pregnancies (n=219). This study found the main source of bacterial DNA was from the laboratory reagents. However, *Streptococcus agalactiae* (Group B Streptococcus) was identified in the placenta of 5% of women prior to the onset of labour, although there was no association with complicated pregnancies. The detection of this organism was present using 16S rRNA amplification and verified by metagenomics and quantitative PCR. Therefore, while the study concluded a resident placental microbiome does

not exist, bacterial placental infection can still be present although it is unlikely to contribute to the majority of complicated pregnancies [132]. Studies that have examined pathogens such as Salmonella in human placental villous explants from different gestational ages demonstrated that the bacterial burden was highest in the first trimester explants. Therefore, the first trimester may be a more vulnerable time and placental infection needs to be carefully considered in relation to poor outcomes [133].

Even in studies where distinct bacteria have been detected by DNA sequencing, it is unclear if these are viable organisms or dead material. The placenta has a role in removing offending organisms and there is a risk that such sensitive techniques are amplifying these cleared microbes. Other arguments have also been made against a placental microbiota due to anatomical, physiological and immunological barriers that exist at the maternal fetal interface that prevent microbial invasion. If a unique microbiome existed in the placenta an immunologically naïve fetus could be overwhelmed [124]. Therefore, there may be bacteria present at a low level in the placenta but given the function of the placenta, bacterial colonization and development of a placental niche is unlikely [134]. Figure 1.5 summarises the potential routes of contamination and the many difficulties encountered in this field of research.

In the case of spontaneous preterm birth associated with chorioamnionitis, certain organisms such *Mycoplasma* spp. and *Ureaplasma* spp. have been located in the placenta [135, 136]. Therefore, while the evidence to support a functionally relevant placental microbiota is weak, placental colonization may occur in pathogenic situations as a result of ascending vaginal infection or haematogenous spread. To date, most studies have focused on term and preterm placentas collected vaginally or by caesarean section. There is paucity of data exploring the early pregnancy microbiome in trophoblast samples and how that relates to reproductive outcome. One recent study has evaluated the role of *Mollicutes* in miscarriage by evaluating endocervical swabs and placental tissue of miscarriage patients (n=89) and controls (n=20) using quantitative PCR. *Mollicutes* in the placenta increased the chance of miscarriage by sevenfold and there was also a significant increase in microbial load of *M. hominis, U. urealyticum* and *U. parvum* in miscarriage patients compared to the control group. It has been proposed that ascending microbes invaded the placental tissue and trigger the adverse event but given the fact that these microbes can be ready detected in cervical swabs there remains

a possibility that their detection in placenta is a result of contamination at sample collection [137].

If specific bacteria can colonize the placenta it is important to understand what potential effect this may have on decidualized endometrial cells. A recent study investigating this *in vitro* found that *L. crispatus* was significantly more successful at attaching to the host cells compared to other Lactobacillus strains [138]. Importantly, this study also showed that the interaction between *Lactobacillus* and placental cells did not cause inflammation or host cell death. These initial findings are promising if live biotherapeutics suggested for use in pregnancy are able to translocate and colonize the placental membranes. It is important that future research elucidates the interplay between different strains of bacteria and the placenta [138]. Table 1.2 summarises the current evidence regarding the placental microbiome in pregnancy at any gestation.

Table 1.2 Current evidence to date of the human placental microbiota at any gestation

Author/year	Sample	Sample size	Mode of delivery	Techniques	Contamination Control	Findings
Aagaard et al/2014 [125]	Villous tree	Term (N=231) Preterm (N=89)	Term Caesarean (N=53) Term Vaginal (N=178) Preterm Caesarean (N=20) Preterm Vaginal (N=69)	16S rRNA gene sequencing V1-3 Metagenomics (subset N=48)	1 blank extraction kit/11 placental samples (no bands routinely amplified). Reagents sequenced when non-human sources were identified but details not provided.	There is a unique low abundance placental microbiome. Observed similarities in non-pregnant oral and placental microbiomes. The placental microbiome differs between preterm and term women and in those with and without antenatal infection.
Doyle et al/2014 [127]	Placental membranes (chorion and amnion)	Spontaneous preterm birth (N=14) Term (N=10)	Preterm Vaginal (N=14) Term Caesarean (N=4) Term Vaginal (N=6)	16S rRNA gene sequencing V1-2 and V5-7	No	Bacterial DNA present in preterm and term placental membranes irrespective of mode of delivery. A consistently identifiable bacterial species in preterm labour.
Antony et al/2015 [128]	1x1x1cm cuboidal section excised from different areas of placenta	Term (N=175) Preterm (N=62)	Caesarean (N=54) Vaginal (N=183)	16S rRNA gene sequencing V1-3	No	Excess gestational weight gain associated with altered placental microbiome and metabolic profile in preterm birth patients.
Zheng et al/2015 [139]	Placenta 4x 1cm^3 cuboidal sections (decidua and fetal chorion discarded)	Low birth weight <3kg (N=12) Normal birth weight ≥3kg - <4kg,(N=12)	Vaginal (N=24) Caesarean (N=0)	16S rRNA gene sequencing V3-4	No	There is a placental microbiome. The placentas of low birthweight neonates had lower bacterial richness and evenness compared to normal birthweight neonates.
Bassols et al/2016 [140]	Villous tree	Gestational Diabetes (N=11) Without Gestational Diabetes (N=11)	Vaginal (N=22) Caesarean (N=0)	16S rRNA gene sequencing V3-4	No	A distinct microbiota profile is present in the placental samples of patients with gestational diabetes.

Author/year	Sample	Sample size	Mode of delivery	Techniques	Contamination Control	Findings
Collado et la/2016 [126]	Placenta Amniotic Fluid Colostrum Meconium	Infant mother pairs (N=15)	Term Caesarean (N=15)	16S rRNA gene sequencing V1-3 Anaerobic culture of placenta and amniotic fluid samples	No	Placenta and amniotic fluid harbour unique microbial communities. Meconium shares features with the microbiota in placenta, amniotic fluid and colostrum. Fetal intestinal colonization could be initiated in-utero. *Staphylococcus* and *Propionibacterium* were cultured from placenta.
Lauder et al/2016 [130]	Placenta (basal plate biopsy and fetal side biopsy)	Term (N=6)	Caesarean (N=1) Vaginal delivery (N=5)	16S rRNA sequencing V1-2 qPCR	Laboratory air swabs (N=11) Sterile swabs (N=8) Blank extraction kits (N=8)	Microbial signatures in placental tissue could not be distinguished from technical controls.
Prince et al/2016 [136]	Swabs from chorion or villous membrane adjacent to fetal side.	Term (N=27) Preterm (N=44) Term Chorioamnionitis (N=12) Preterm Chorioamnionitis Mild (N=11) Severe (N=20)	Term Caesarean (N=7) Term Vaginal (N=20) Preterm Caesarean (N=7) Preterm vaginal (N=37)	Metagenomics Culture for *Ureaplasma* or *Mycoplasma spp.*	No Only yields with high reads were included in analysis without concern for contamination	Spontaneous preterm birth patients have a placental microbiota that differed by severity of chorioamnionitis.
Doyle et al/2017 [141]	Amnion and Chorion	1097 subjects *Rural Malawi setting	Unreported vaginal, caesarean, preterm and chorioamnionitis cases	16S rRNA gene sequencing V5-7 qPCR	Reagents from blank extraction kit sequenced for every 10 extractions. Only placental samples that were positive for bacterial DNA (defined as 40 CFU/µl) were sequenced. Sample processing delays increased the chance of positive qPCR	A distinct placental microbiome exists. 68.1% of amnion-chorion and 46.8% placental samples had positive qPCR. A varied placental microbial structure is associated with severe chorioamnionitis. The source of bacteria in the placenta overlapped with the vagina and not the oral cavity.
Gomez-Arango et al/2017 [142]	Placental biopsy from fetal side. Matched oral and faecal samples	37 patients Overweight (N=13) Obese (N=24)	Term Caesarean (N=17) Term Vaginal (N=20)	16S rRNA gene sequencing V6-8	Reagent, DNA extraction and PCR control pooled and sequenced for each kit type. Any OTUs detected were removed from analysis.	A placental microbiome was identified irrespective of mode of delivery. Placental microbial communities shared a higher similarity to oral microbiome than gut but this declined with each taxonomic level.

Author/year	Sample	Sample size	Mode of delivery	Techniques	Contamination Control	Findings
Parnell et al/2017 [143]	Placenta : Basal plate Villous tree Fetal membrane	57 Term Women	Term Caesarean (N=34) Term Vaginal (N=23)	16S rRNA gene sequencing V1-9 (V7/8 did not amplify. V1,5,9 amplified less than half and V2 showed environmental contaminants) qPCR conducted on V4 region	Water Control N=5 and Regent test blanks N=8 Negative controls occasionally had 34 copies/ µl. Only positive qPCR in placental samples were included (if >34 copies/µl)	Tissue specific profiles identified in placental microbiome. Variation is seen in the placental microbiota between amnion-chorion and basal plate.
Leon et al/2018 [135]	Placental Villous tree	256 patients Term (N=165) Preterm (N=91)	Caesarean Term (N=81) Vaginal Term (N=84) Caesarean Preterm (N=55) Vaginal Preterm (N=36)	16S rRNA gene sequencing V5-7	Negative extractions and PCR blanks were examined. Samples ≥500reads were analysed (N=19)	Low level relatively diverse placental microbial signature is present in normal and complicated pregnancies. There was overlap between technical controls and placental samples. A unique preterm placenta did not exist but *Ureaplasma* and *Mycoplasma* enriched the spontaneous preterm birth cohort.
Zheng et al/2017 [144]	Placenta 4x 1cm^3 cuboidal sections (decidua and fetal chorion discarded)	Term without macrosomia (N=10) Macrosomia Birth weight>4kg (N=10)	Caesarean (N=20)	16S rRNA gene sequencing V3-4	No	Distinct placental microbiota profile in fetal macrosomia
Seferovic et al/2019 [145]	Placental villous tree	53 patients Term (N=26) Preterm (N=26) 1 Positive control with histological chorioamnionitis	Term Caesarean (N=22) Term Vaginal (N=4) Preterm Caesarean (N=8) Preterm Vaginal (N=18)	In Situ Hybridization against conserved region of 16 S ribosome. 16S rRNA sequencing V4 Metagenomics	Environmental swab cultures (inside and outside placental containers). Kit-negative extractions N=6	Very low biomass bacteria were observed by histological and 16S rRNA gene sequencing distinct from environmental controls. Unclear if commensal microbial abundance varies in preterm and term pregnancies. Viability of organisms unknown

Author/year	Sample	Sample size	Mode of delivery	Techniques	Contamination Control	Findings
De Goffau et al/2019 [132]	Placental terminal villi	537 women Adverse pregnancy outcome (N=318) Controls (N=219)	Caesarean SGA (N=20) PET (N=20) Control (N=40) Vaginal and Caesarean SGA (N=100) PET (N=100) Control (N=198) Preterm (N=100)	16S rRNA V1-2 Metagenomics qPCR for *Streptococcus agalactiae*	Positive control using *S. bongori* to compare 16S rRNA with metagenomics For each DNA isolation kit extraction blanks were carried out.	No evidence to support a placental microbiome. No relationship between placental infection and SGA, PET or preterm birth. The major source of bacterial DNA was contamination from laboratory reagents. The only organism consistently present in the placenta of 5% of women prior to labour (detected by three methods) was *Streptococcus agalactiae*.
Theis et al/2019 [131]	Amnion-chorion plate Villous tree	Healthy Term women (N=29)	Term Caesarean (N=29)	16S rRNA V4 qPCR Metagenomic surveys Bacterial culture	DNA extraction kits (N=6) Laboratory environmental controls (N=16) Operating rooms (N=21)	No consistent evidence the placenta harbours a unique microbiota. 28/29 placental samples did not yield bacterial cultivars. 18 prominent OTUs accounted for 90% of placental tissue and 86.4% of background technical controls. There were no consistent differences in the composition of placental samples and technical controls.
Gschwind et al/2020 [146]	Chorionic villi Umbilical cords Fetal membranes	Healthy Term pregnancy (N=38)	Caesarean (N=29) Vaginal (N=9)	16S rRNA V8-9 qPCR V4 Bacterial culture Metagenomics	16 Extraction blanks (N=16) Reagent extraction kit controls (N=3) Culture media and incubation condition controls (N=38)	Placenta does not harbour specific consistent functional microbiota. No significant viable bacteria or bacterial DNA in the in utero samples collected from caesarean section.

Author/year	Sample	Sample size	Mode of delivery	Techniques	Contamination Control	Findings
Sterpu et al/2020 [134]	Placenta (maternal, middle and fetal side). Saliva Vaginal Rectal Amniotic fluid Vernix	76 Term pregnancies	Term Caesarean (N=50) Term Vaginal (N=26)	Metagenomics qPCR 16S rRNA V6-8 Bacterial culture	PCR reagents DNA extraction controls	16S rRNA gene sequencing and qPCR found bacterial signals that were not distinguishable from background controls. No meaningful comparisons could be made to oral, faecal or vaginal samples. Very few genera detected by16S rRNA sequencing could be confirmed by culture.
Olomu et al/2020 [147]	Parenchymal placental tissue Vaginal Rectal Maternal blood Cord blood	Term patients (N=47) GDM (N=16) Obese (N=16) Normal weight (N=15)	Term Caesarean (N=47)	16S rRNA V3-4 qPCR	Multiple negative or blank controls. Sterile swabs exposed to operating rooms or air in sampling room. Reagent, Kit and sequencing reaction controls.	No distinct microbiome existed in placental samples that differed from blank controls. An additional source of cross contamination was identified from high biomass samples being analysed adjacent to low biomass samples.
Oliveira et al/2020 [137]	Endocervical swabs Placental tissue	Miscarriage patients (N=89) Control with no history of miscarriage (N=20)	Miscarriage patients undergoing curettage 8-20 weeks gestation (N=89) Term pregnancies vaginal deliveries (N=20)	qPCR to detect *M. genitalium, M. hominis, U. parvum, U. urealyticum* and *N. gonorrhoeae*	No	Women with *Mollicutes* detected in placenta had a seven-fold higher chance of miscarriage. A positive association between *U. parvum* in placental tissue and miscarriage.

Abbreviations:
OTU: Operational Taxonomic Unit
CFU: Colony Forming Unit
qPCR: Quantitative Polymerase Chain Reaction
SGA: Small for Gestational Age
PET: Pre-eclampsia
GDM: Gestational Diabetes Mellitis

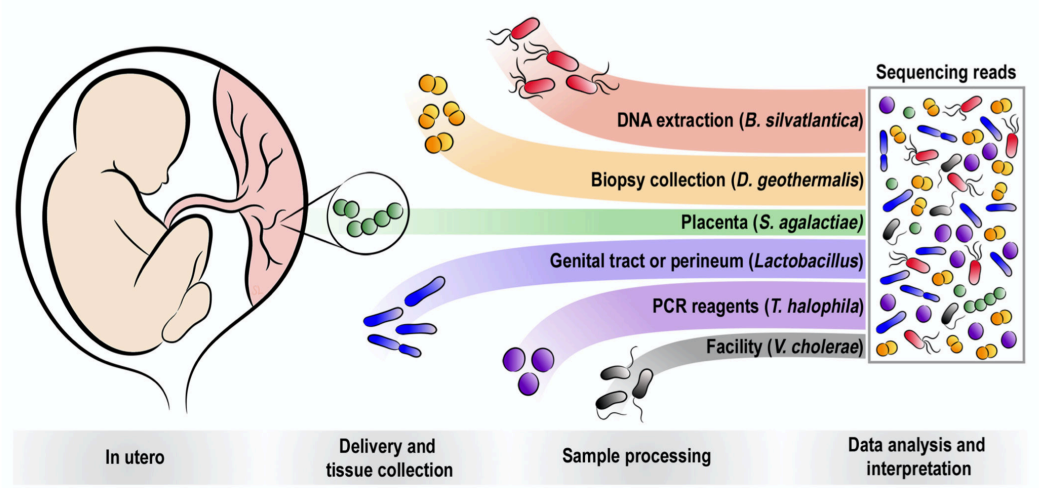

Figure 1.5 Potential sources of bacterial signals and contamination seen in placental samples

The source of bacteria in the placenta could be present in utero, but the delivery and method of collection and sample processing could also contaminate the placenta and alter the bacterial signals. *Source: Goffau et al [132]*

1.7 Adaptive and Innate Immunity in Pregnancy

The human immune system consists of innate and adaptive components that have a coordinated approach to defend the body from pathogens. The adaptive immune system responds to antigens by a specific antibody or T cell response that has the capacity for memory in future encounters. Figure 1.6 details the different cytokines produced by each differentiated T cell. The innate immune system is highly conserved and provides rapid response with macrophages, granulocytes and pathogen recognition receptors (Toll-like receptors and acute phase response proteins) [148]. The concept that the pregnancy represents a form of host graft model in which there needs to be an immunosuppressive state to prevent rejection of the semi-allogenic fetus is now considered to be an outdated concept. A successful pregnancy requires a dynamic and responsive immune system that can recognize danger signals and promote repair if required [149]. Therefore, the immunological milieu of pregnancy is unique and active. The different immunological stages of pregnancy evolve over the course of each trimester. Initially a pro-inflammatory environment supports implantation and placentation, then an anti-inflammatory shift supports fetal growth and finally a proinflammatory event promotes the onset of labour [150]. These three immunological stages are explained in further detail below.

The human blastocyst consists of the inner cell mass which gives rise to the embryo and the external layer (trophectoderm) which forms the placenta. Successful implantation occurs

when the trophoblast breaches the decidual lining and invades a receptive maternal decidua. The invasive trophectoderm restructures the decidua and mimics the process of tissue injury and repair. Inflammation at the implantation site is characterised by upregulation of Interleukin-6 (IL6), IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor (TNF) which are derived from endometrial stromal cells and infiltrating immune cells [150]. After implantation there is rapid fetal growth and development, and the predominant state is a Th2 type anti-inflammatory environment. It is thought that ongoing pro-inflammatory signals in response to infection could increase risk of miscarriage or preterm birth [151].

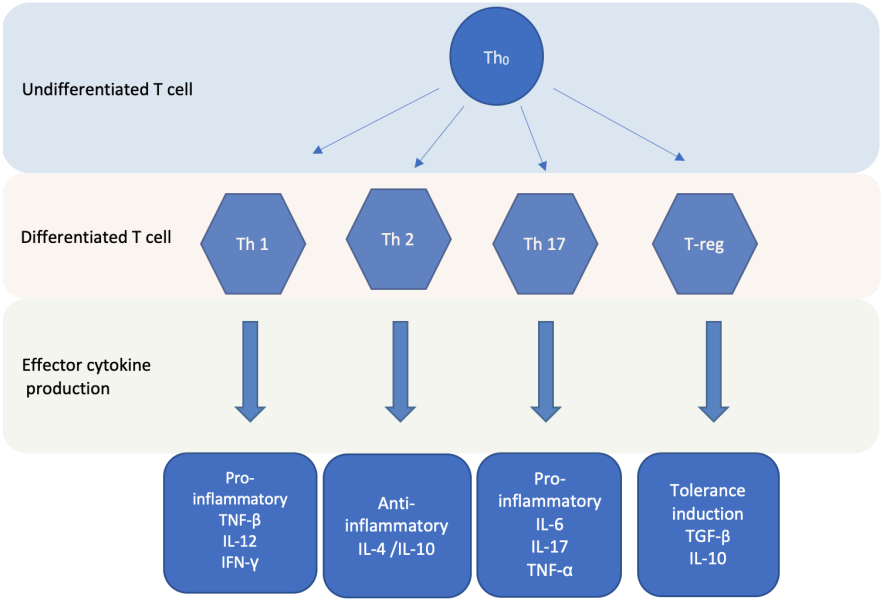

Figure 1.6 depicts differentiated T cells and the effector cytokine production of each.

Source adapted from Omenetti et al [152] and Al-Nasiry et al [153]

The anti-inflammatory environment is mediated by several immune cell types including decidual NK cells and T-reg cells. Decidual NK cells play an important role in tissue renewal and recent studies have shown they are involved in clearing senescent cells which stimulate tissue breakdown[41]. A failure to clear these senescent cells results in pro-senescent cycles that certain studies have linked to recurrent pregnancy loss. A switch to a pro-inflammatory environment occurs once the nuclear factor-κB (NF-κB) signalling pathway is initiated prior to the onset of labour and delivery. The influx of immune cells further simulates inflammation and a production of pro-labour pathways leading to the onset of uterine contractions.

Therefore, the process of pregnancy has many unique immunological profiles and the inability to maintain the correct balance at each stage could alter the outcome [150].

1.8 The microbiota and immune regulation

Immune regulation in pregnancy is a complex network of different processes that involve epigenetic modifications, microRNAs, autophagy and histocompatibility genes. An evolving concept is that microbiota also contributes to the immunological milieu of the reproductive tract and if disrupted can alter reproductive outcomes.

Trophoblast cells have an active role in altering the immune response by attracting and educating immune cells and shaping their response to external stimuli. Cell populations that are promoted by trophoblasts such as NK cells and M2 macrophages support vascular and tissue remodelling whereas T-reg cells promote tolerance at the maternal fetal interface. Trophoblasts also sense and respond to pathogen associated molecular patterns (PAMPs) that are present in microorganisms [150]. Therefore pathogen invasion is sensed by the trophoblast cells and can disrupt tolerance at the maternal-fetal interface triggering pregnancy complications such as chorioamnionitis and preterm birth [154]. Bacteria may gain access to the maternal-fetal interface by ascending infection or haematogenous spread. The controversaries surrounding the presence of a resident normal microbiota in the uterus or placenta have been discussed above.

The role of the microbiota in the receptivity of the immune system and how it might alter pregnancy outcome is unclear. Studies have shown that when exposing both human and mice trophoblast cells to bacterial products an induction of IFN-β by the placenta modulates the maternal immune system and promotes tolerance [155]. The question remains whether certain types of bacterial taxa or an altered microbial composition can modify the tolerance at the maternal-fetal interface and provoke inflammatory complications. Other studies have considered the possibility of concurrent viral infections that sensitise the pregnant mother to bacterial products [151, 156].

1.9 Localised proinflammatory cytokine expression in miscarriage

Many studies have reported that cervicovaginal and decidual cytokines are dysregulated in miscarriage. However, the timing of sample collection and understanding if the miscarriage itself has induced an inflammatory response or if it is part of the pathophysiology that triggers the condition remains unclear. The study of cytokines is itself a complex area given the pleiotropy and redundancy of the network [157]. A Swedish study that performed a cervical biopsy prior to surgical management of miscarriage found an increased cervical tissue level of IL8 in both incomplete or missed miscarriage patients [158]. Further studies have also shown that higher levels of inflammatory cytokines are seen in decidual stromal cells from miscarriages than in normal pregnancy. Many postulate that excessive inflammation could initiate pregnancy loss via stromal cell apoptosis [159, 160]. Studies have also analysed the levels of IL-6 and IL-8 in early pregnancy cervical mucus samples prior to miscarriage in those with a history of recurrent pregnancy loss. Interestingly the cervical mucus showed significantly higher concentrations of pro-inflammatory cytokines in those that subsequently miscarried compared to those that had a live birth [161]. It remains unclear if altered inflammatory expression in pregnancy loss may reflect host response to microbiota. However, there is a correlation between vaginal communities deplete in *Lactobacillus* spp. and local pro-inflammatory cytokines within the cervicovaginal fluid (CVF). This indicates that at least in the vaginal nice, microbiota can disrupt local inflammatory mediators and trigger adverse outcome [162-164].

Table 1.3 The role of each analyte chosen in early pregnancy and relevant biological function

Cytokine	Role in early pregnancy	Levels in miscarriage	Function
IL-6	Present in human endometrium and early pregnancy decidua. Helps to influence trophoblast invasion[157].	Increased levels at maternal- fetal interface (second trimester miscarriage)[157].	Pleiotropic mediator in acute phase response to injury and infection[165].
IL-8	Stimulate trophoblast invasion in vitro models[157].	Uterine-cervical samples significantly increased in first trimester miscarriage compared to controls[157].	Pro-inflammatory multifunctional chemokine [157].
TNF-α	Studies have shown it inhibits trophoblast invasion[157].	Decidual stromal cell secreted high levels of TNF-α T in spontaneous miscarriage [157].	Pro-inflammatory[157].
IL-1β	IL-1β regulates human cytotrophoblast at the maternal-fetal interface. It has a key role in trophoblast invasion and tissue repair[166].	Higher IL-1β seen in placental tissue of recurrent miscarriages compared to controls [167].	Th-1 Proinflammatory cytokine[168].
IFN-γ	Regulates the expression of intercellular adhesion molecule-1 in the epithelium and endothelium. Involved in implantation process [169].	Higher levels in the blood and endometrial tissue of recurrent miscarriage patients[169].	Th-1 Proinflammatory cytokine[169].
IL-18	Expressed by human chorion and decidua and present at the maternal fetal interface. Shown to influence implantation process [170].	Increased levels in early pregnancy led to miscarriage[171].	Induces pro-inflammatory cytokines [172] But is unique in inducing Th-1 or 2 differentiation depending on the immunological milieu[170].
IL-10	Produced by villous cytotrophoblasts. Levels are increased in early pregnancy [173].	Decreased decidual levels in the first trimester of missed miscarriages compared to elective termination of pregnancy [174].	Anti-inflammatory cytokine[173].
IL-4	Produced by the placenta and amnion. Inhibits Th1 response and cytokine production. Significantly elevated by progesterone [168].	Decreased production in recurrent miscarriage compared to normal pregnancy[175].	Th2 anti-inflammatory cytokine[168].
IL-2	Upregulated in pregnancy and promotes T cell proliferation. Promotes angiogenesis. Regulates the dynamic immune system[176].	Increased expression in decidual and villous samples of spontaneous miscarriage cases [177].	Complex signalling depending on receptor activation. [178].

1.10 Project aims and hypotheses

Emerging evidence implicates the reproductive tract microbiota as a key modulator of local inflammatory and immune pathways. However, there is limited understanding of the association between first trimester miscarriage and the reproductive tract microbiota. Furthermore, studies to date have not characterised the underlying phenotype of the miscarriage or explored the relationship between the vaginal microbiota in miscarriage and local inflammatory mediators. While aneuploid miscarriages are thought to be lost due to reasons intrinsic to the pregnancy, external factors are more likely to play a role in euploid miscarriage. The work in this thesis aims to address this by investigating the vaginal microbial composition and local immune responses in chromosomally normal and abnormal miscarriages. The discovery of a link between suboptimal vaginal microbial composition and local inflammatory responses in chromosomally normal miscarriage would provide a new avenue for therapeutic intervention designed to modulate microbiota composition and reduce the risk.

Hypotheses

1. A suboptimal vaginal microbial composition alongside localised pro-inflammatory cytokine expression is associated with chromosomally normal miscarriage.
2. There is a unique early pregnancy placental niche compared to laboratory contamination controls.
3. There is an altered gastrointestinal tract microbiota in euploid miscarriage compared to aneuploid miscarriage.

Aims

1. To characterise the vaginal microbial composition and local immune response in chromosomally normal and abnormal miscarriages and in healthy controls.
2. To determine if the early pregnancy placenta harbours a microbiota signature distinct from background and contamination controls.
3. To characterise the gastrointestinal tract microbial composition in chromosomally normal and abnormal miscarriages and correlate this with paired vaginal samples.

CHAPTER 2: Materials and Methods

2 Materials and Methods

2.1 Study design

2.1.1 Recruitment

The work described in this thesis was a prospective observational study in which patients were recruited from two hospital sites across Imperial College Healthcare NHS Trust: St Mary's Hospital (SMH) and Queen Charlotte's and Chelsea Hospital (QCCH) from March 2014-Februrary 2019.

Participants were recruited into the following groups:

1. Women attending an ultrasound scan at the Early Pregnancy Assessment Unit at QCCH in the first trimester of pregnancy less than 14 weeks gestation, with singleton intrauterine pregnancies
2. Patients at the point of diagnosis of missed miscarriage, or with retained products conception requiring surgical, medical or conservative management, at the Early Pregnancy Assessment Unit at QCCH.
3. Women undergoing surgical termination of pregnancy under clause C of the abortion act were recruited from day theatres at SMH.

2.1.2 Diagnostic Criteria

The first trimester was defined as <14 weeks' gestation by last menstrual period (LMP) or, where LMP was not known, ultrasound scan dating based on crown-rump length measurements (CRL). An intrauterine pregnancy was defined based on an ultrasound scan showing an intrauterine gestation sac with or without a visible embryo and heartbeat. Missed miscarriage was confirmed when an empty gestation sac was present with a mean sac diameter of 25mm or more, if an embryo with CRL measurement of 7mm or more was identified without an embryonic heartbeat or if the embryonic heartbeat was absent irrespective of the size of the CRL, where one had previously been observed [5, 179]. A diagnosis of incomplete miscarriage was made when a transvaginal ultrasound demonstrated irregular heterogeneous tissue in the endometrial cavity in keeping with retained products of conception after a previous ultrasound scan had shown an intrauterine pregnancy [1].

A detailed questionnaire including demographic information, past medical, gynaecological and obstetric history was completed. Validated symptom scores were used to assess vaginal bleeding based upon a pictorial blood assessment chart score at the time of sampling [180].

2.2 Eligibility criteria

2.2.1 Inclusion criteria

- Patient undergoing a termination of pregnancy ≤20 weeks gestation
- Patient at the point of miscarriage diagnosis ≤20weeks gestation (with part or all the pregnancy in situ)

2.2.2 Exclusion criteria

- Younger than 18 years presenting with miscarriage or termination of pregnancy.
- A termination of pregnancy or a miscarriage greater than 20weeks.
- Women receiving antibiotic treatment within the preceding 2 weeks.
- Women who had sexual intercourse within the last 72 hours.
- Women who in the opinion of the researcher by virtue of language or learning impairment would be unable to give fully informed consent to the study.

2.3 Ethical Approval

This study was favourably reviewed by National Research Ethics Service (NRES) Committee Health and Care Research Wales Castlebridge Committee (reference number 16/WA/0357). Healthy pregnancies recruited in group 1 were part of the Early Pregnancy Outcome Study (Reference 14/LO/0199). All patients were given patient information leaflets (Appendix 1) and provided informed and written consent (Appendix 2).

2.4 Sample Collection

Informed consent was taken. Sample collection took place at one time point in each group. Figure 2.1 highlights the recruitment groups, research time point and data collection. Negative control swabs were also collected by exposing swabs to clinic and laboratory environments prior to freeze storage. The degree of vaginal bleeding was assessed at the time of surgical evacuation. Most women with miscarriage were recruited if they planned surgical

management of their miscarriage. Table 2.1 demonstrates the sample collection techniques and the immediate sample processing that occurred.

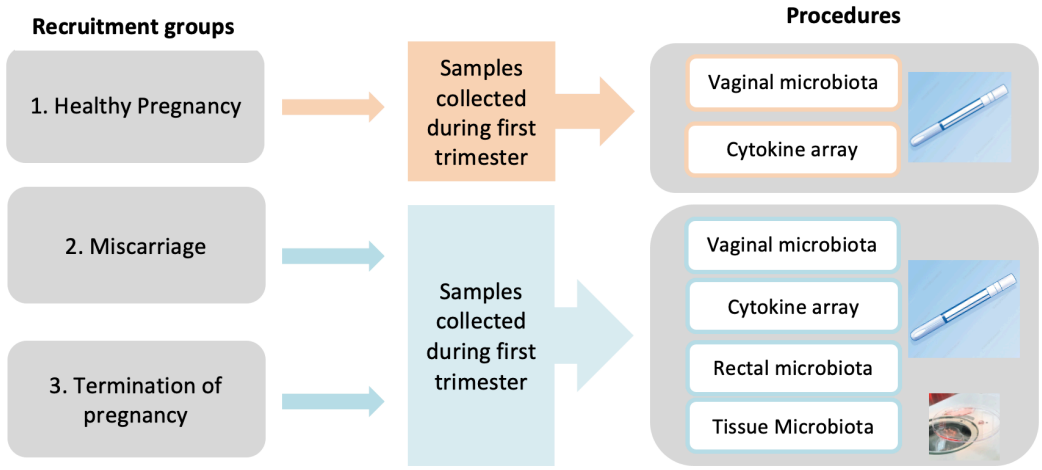

Figure 2.1 Diagram of study structure detailing study groups, sample time point and procedures conducted.

Table 2.1 Sample collection techniques and immediate processing

Sample Collection			
Purpose	**Sample type**	**Sample Site**	**Processing**
Vaginal microbiome	BBL™ CultureSwab™ containing liquid Amies (Becton Dickinson, Oxford, UK)	Posterior vaginal fornix	Swab inserted into posterior fornix for 30 s and directly inserted into transport medium. Swab stored on ice and transferred to -80°C, within 30min of collection. All swabs were weighed before and after collection to determine the wet weight.
Cytokine analysis	A Transwab® MW170 with rayon bud type (Medical Wire & Equipment, Corsham, UK)	Posterior vaginal fornix	Swab inserted into the posterior fornix for 30 seconds and placed directly into empty amber eppendorf (VWR) and stored on ice and transferred to -80°C within 30 minutes of sampling.
Rectal microbiota studies	BBL™ CultureSwab™ containing liquid Amies (Becton Dickinson, Oxford, UK)	1.5-2cm beyond the anal sphincter	A swab was carefully inserted in the rectum and then gently rotated to touch anal crypts and directly inserted into transport medium. This was collected while the patient was under general anaesthetic. The swab was stored on ice and transferred to -80°C, within 30minutes of collection
Placental microbiome studies	Trophoblast Tissue	Direct extraction of tissue using polyp forceps or taken from suction pot.	Wearing sterile gloves and working in a sterile field the tissue is washed with normal saline and placed into three separate amber microtubes and stored at -80°C, within 1 hour of collection.
Cytogenetics	Miscarriage Trophoblast Tissue	Suction collection pot.	If clinically indicated the samples were sent to Kennedy Galton genetics lab for QF-PCR. Otherwise samples were stored at -80°C and processed using Bacs on Beads at a later date.
Urine Metabolome	Urine	Mid-Stream urine	A mid-stream urine sample was collected by the patient and placed on ice. 1000µl was separately aliquoted into 4 amber microtubes and stored at -80°C, within 1 hour of collection.
Plasma Cytokines	Blood	Venous sample	10 ml collected into lithium heparin tubes (green top for plasma) and yellow top for serum via a vacutainer. Tubes were inverted three times and placed on ice. Samples were centrifuged and aliquoted into three microtubes and stored at -80°C, within 1 hour of collection.

2.5 Molecular Cytogenetics for miscarriage cohort

Patients who experienced three consecutive miscarriages qualified for NHS cytogenetic analysis. Chorionic villous material was collected at the time of surgical evacuation of the uterus and a fresh specimen was sent to the Kennedy Galton Laboratory where the tissue was analysed using quantitative fluorescent polymerase chain reaction (QF-PCR). In these cases, DNA was amplified using two multiplexes that include a total of 31 markers; assay 1 contains primers for chromosomes 13, 18, 21 and 22, and assay 2, primers for chromosomes 14, 15 and 16 and the X and Y chromosomes. Supplementary markers were used as required. PCR products were separated on an ABI 3100 capillary genetic analyser, and results were analysed using ABI Genotyper software [181]. In cases that did not qualify for NHS cytogenetics the samples were stored in amber microtubes at -80°C and processed later at The Doctors Lab (TDL) Genetics using KaryoLite BACs (bacterial artificial chromosomes) on Beads molecular karyotyping technology. The KaryoLite bacterial artificial chromosomes-on-Beads (KL-BoBs™) assay was performed using a prenatal chromosome aneuploidy and microdeletion detection test kit (Perkin Elmer, Waltham, MA, USA), according to the manufacturer's instructions. Briefly, genomic DNA from specimens as well as reference DNA were biotinylated and purified. The genomic DNA and BoBs™ was then subjected to single-cell hybridization overnight before washing and incubation with streptavidin-phycoerythrin, which was used as the reporter. Fluorescence of DNA bound to the microbeads was measured using a Luminex 200 (Austin, TX, USA) and BoBsoft™ analytical software (Perkin Elmer) was used for data analysis. A ratio of specimen fluorescence to reference fluorescence greater than 1.0 indicated the chromosome fragments were repeated and a ratio less than 1.0 indicated a deletion [182].

2.6 Vaginal, rectal and trophoblast microbiota: 16S ribosomal RNA gene sequencing

Vaginal, rectal and trophoblast tissue samples stored at -80°C were defrosted and the bacterial DNA isolated before the hypervariable V1-V2 regions of the 16S rRNA gene were amplified and sequenced as described below.

2.6.1 Materials

Table 2.1 Details of materials needed for different experimental techniques

Experimental process	Chemical/Kit/Buffer	Supplier
16s rRNA extraction	Liquid Amies swab	BBL™Culture Swab™, Becton, Dickenson and Company
	Sterile knife and Petri Dish	Corning Incoporated No 430167
	QIAmp DNA Mini Kit	Qiagen Catalogue No 51306
	Tissue Lyser LT	Qiagen
	Lysostaphin	Sigma L9043
	Lysozyme	Sigma L6876
	Mutanolysin	Sigma M9901/10KU
	TE50 (10mM Tris-HCl, 50mM EDTA at pH 7.4)	In House
	Ethanol 100%	VWR
	DNas/RNase free water	Sigma
	Filter sterilized PBS (phosphate-buffered saline)	In House
	0.1mm zircona/silica beads	BioSpec
PCR	NEB One Taq® DNA polymerase	BioLabs
	Deoxynucleotide mix (dNTP) 10mM	Sigma
	Agarose	Electrophoresis grade from Invitrogen
	5 x Buffer	BIO-37045
	6x Blue/Orange Loading Dye	Invitrogen
	Molecular weight marker	Bioline Hyperladder 100bp
	TBE buffer (Tris/Borate/EDTA)	In house
	16s rRNA forward & reverse primers	Invitrogen
Software	Excel	Microsoft
	PRISM	GraphPad
	Statistical Analysis of Metagenomic Profiles (STAMP)	Oximetrics
	ClustVis	GitHub
	Linear discriminative analysis (LDA) Effect Size (LEfSe)	Galaxy/Hutlab
	R	Qgraph package

Table 2.2 Components of enzyme cocktail mix for the PCR

Enzyme cocktail mix	Volume per sample
Phosphate-buffered saline (PBS)	170 µl
Lysozyme (prepared for concentration 10mg/ml in filter sterilized 10mM Tris.HCl pH8.0)	50 µl
Mutanolysin (prepared for concentration 25U/µl by dissolving 10,000 units in 400 µl sterile water)	6 µl
Lysostaphin (prepared from concentration 4000U/ml by dissolving 23915units in 20mM sodium acetate).	3 µl
TE 50 (10mM Tris-HCl, 50mM EDTA at pH 7.4)	41 µl
12% Triton	30 µl

	Forward	Reverse
Mixed Universal Primers	8F-YM GAGTTTGATYMTGGCTCAG, 28F-Borrellia GAGTTTGATCCTGGCTTAG, 28F-Chloroflex GAATTTGATCTTGGTTCAG and 28F-Bifdo GGGTTCGATTCTGGCTCAG at a ratio of 4:1:1:1	388R

2.6.2 Bacterial DNA extraction for vaginal and rectal swabs

Bacterial DNA was extracted from the BBL™ CultureSwab™ swabs using a QiAmp Mini DNA kit (Qiagen, Venlo, Netherlands). The methods used were based upon the Manual of Procedures for the Human Microbiome Project with minor modifications [183]. Briefly, the swabs were removed from the -80°C freezer and thawed slowly on ice. Approximately 500µl/swab was collected from the Amies transport solution into a 2ml centrifuge tube using a 5ml syringe. The samples were then centrifuged (8000rpm for 10mins) to form a pellet and the supernatant was removed and stored at -20°C for cytokine studies. The cell pellet was re-suspended in an enzyme cocktail containing: 50µl lysozyme(10mg/ml) (Sigma, Dorset, UK), 6µl mutanolysin (25,000 U/ml) (Sigma), 3µl lysostaphin (4,000U/ml in sodium acetate) (Sigma), 41µl TE (10mM Tris, 50mM EDTA at pH7.4), 170 µl phosphate buffer solution (PBS) and 30 µl 12% Triton. The samples were digested in a water bath at 37°C for 1 hour. Following this mechanical lysis was carried out by bead beating by adding 100mg bleached and rinsed 0.1mm zircona/silica beads to each sample and oscillating for 2 minutes at 25Hz in a Tissue Lyser LT (Qiagen). After brief centrifugation to bring down the beads, the supernatant lysate was transferred to fresh 2ml centrifuge tubes and 200µl supernatant aspirated and applied to the QIAMP DNA Mini Kit according to the manufacturers protocol. At the final step 100µl elution buffer (supplied with kit) was added to give 100µl template DNA. This was frozen at -20°C until

use in further experiments. In each batch of samples being extracted a negative control swab (waved in air) went through the entire protocol.

2.6.3 Bacterial DNA extraction of trophoblast tissue

Extraction of bacterial DNA from trophoblast tissue followed a similar protocol to the vaginal and rectal swabs with chemical and mechanical lysis. However, prior to chemical lysis tissue was defrosted on ice and dissected using a sterile knife and petri dish. Approximately 0.25g of tissue was then suspended in 500 µl PBS and vortexed for 30 seconds and placed in the Tissue Lyser at 50 Hz for one minute. The supernatant (250 µl) was extracted and stored at -80 °C. Extraction was performed as per described for vaginal and rectal swabs.

2.6.4 16S rRNA Polymerase Chain Reaction (PCR)

Following bacterial DNA extraction, presence of bacterial DNA was confirmed by Polymerase chain reaction (PCR) using universal primers targeting the 16S rRNA gene (Invitrogen, Carlsbad, CA, USA) as a quality control step. PCR master mix was made as per Table 2.4, containing 5µl template DNA, to give a total of 50µl per reaction. PCR was run on a BioRad Tetra 2 machine (BioRad) using the conditions detailed in Table 2.5. Gel electrophoresis was performed using 1-1.5% Agarose gels. These were prepared by microwaving agarose in 1xTBE buffer until dissolved before Sybr Safe gel stain was added (1µl per 100ml Agarose gel solution) and the gel was allowed to set. Nine microliters of Tritrach loading dye (Invitrogen) was added to each DNA extract and a molecular weight marker was included on each gel. A sample known to contain DNA was used as a positive control, PCR-grade sterile water and a blank swab extraction were used as negative controls. The gel was transilluminated and photographed under ultraviolet (UV) light (Figure 2.2).

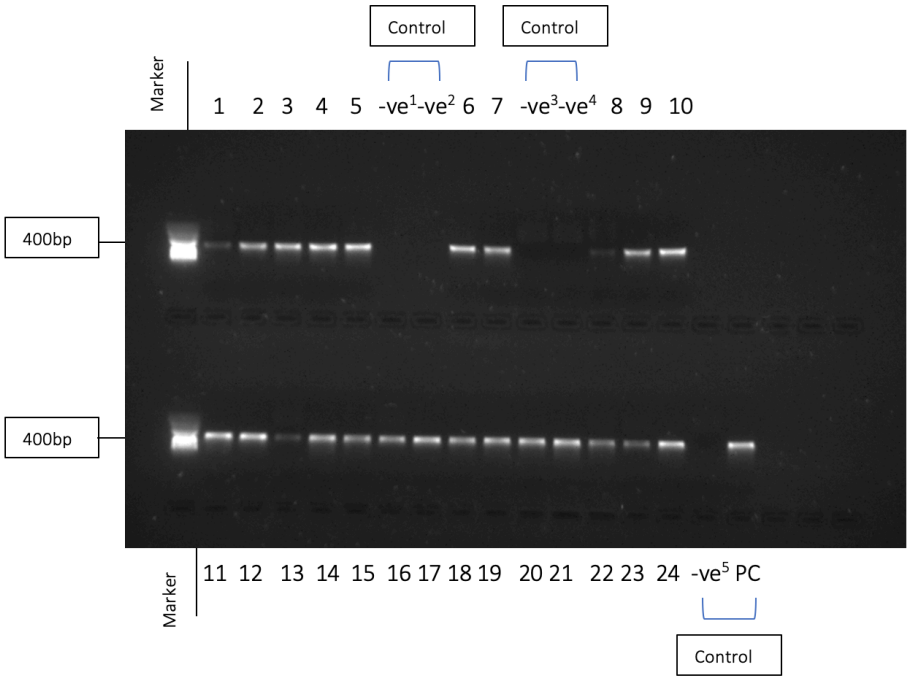

Figure 2.2 PCR Gel

PCR gel demonstrating molecular weight marker, extracted DNA rectal samples 1-24 and controls (PC=positive control, -ve^{1}=water, -ve$^{2\text{-}5}$ =blank swab). This process was performed for all samples prior to 16S rRNA gene sequencing to confirm the presence of extracted DNA.

Table 2.3 Bacterial PCR Mastermix (Total 50µl per reaction)

	Volume per reaction
5x Reaction Buffer	10µl
AmpliTaq Gold DNA Polymerase (ThermoFisher Scientific)	0.25µl
16s rRNA forward primer (Invitrogen) Sequence: 3' GCC TTG CCA GCC CGC TCA GTC AGA GTT TGA TCC TGG CTC AC	1µl
16s rRNA reverse primer (Invitrogen) Sequence: 5' GCC TCC CTC GCG CCA TCA GCA CTG CAT GCT GCC TCC CGT AGG AGT	1µl
Deoxynucleotide mix (dNTP) 10mM (ThermoFisher Scientific)	1µl
PCR-grade sterile water (Sigma)	31.75µl
Template DNA (from bacterial DNA extraction)	5µl

Table 2.4 16S rRNA PCR reaction conditions

Step 1. Incubate at 95°C for 5 minutes Step 2. Incubate at 95°C for 30 Seconds Step 3. Incubate at 55°C for 30 Seconds Step 4. Incubate at 72°C for 1 minute 30 Seconds Step 5. Cycle to step 2 for 30 more times Step 6. Incubate at 72°C for 7 minutes Step 7. Incubate at 4°C till removed

2.6.5 Sequencing of 16S rRNA gene amplicons

Microbial profiling using a MiSeq platform (Illumina, San Diego, CA, USA) was performed commercially by Research and Testing (Lubbock, TX, USA [93]. Twenty microliters of bacterial DNA per sample was separately aliquoted to send for sequencing using an Illumina MiSeq platform. Mixed universal primers 28F-YM GAGTTTGATYMTGGCTCAG, 28F-Borrellia GAGTTTGATCCTGGCTTAG, 28F-Chloroflex GAATTTGATCTTGGTTCAG and 28F-Bifdo GGGTTCGATTCTGGCTCAG at a ratio of 4:1:1:1 with 388R TGCTGCCTCCCGTAGGAGT reverse primers were used to amplify the V1-V2 region of 16S rRNA. The detailed steps are outlined in the following article [93].

2.6.6 Processing of sequence data

The data was processed and analysed using the MiSeq SOP Pipeline of the Mothur package [184]. Sequence alignment was performed using blastn (16SMicrobial.tar.gz) and classification used RDP (Ribosomal Database Project)[185]. To account for sequencing depth bias, data were resampled and normalized to the lowest read count, rarefaction curves were investigated after sub-sampling to confirm coverage was maintained at >96%. Singleton operational taxonomic units (OTUs) and OTUs<10 reads in any sample were collated and labelled as OTU_singletons and OTU_rare phylotypes respectively, to maintain normalisation and to minimise artefacts. The first batch of sequencing involved 112 vaginal samples, 110 rectal samples and 107 tissue samples. To test run-to-run sequencing reproducibility and ensure results were comparable a selection of samples were re-sequenced alongside additional miscarriage vaginal swabs in the second run.

2.6.7 Statistical analysis of microbiota sequencing data

Assignment of vaginal microbial profiles into composition groups analogous to community states types was performed using ClustVis, https://biit.cs.ut.ee/clustvis/ [186]. For this, Ward linkage hierarchical clustering analysis (HCA) by nearest neighbour linkage was performed using genera and species taxonomic data. This enabled genera sequence data to be classified into *Lactobacillus* spp. dominant and deplete (vaginal microbiome grouping 1). The *Lactobacillus* spp. deplete cluster was further divided into Gardnerella dominant and Non-Gardnerella (VMG sub-grouping 1) and these clusters could be further divided into Gardnerella dominant, mixed Lactobacillus/Gardnerella, Prevotella or Streptococcus dominated (VMG sub-grouping 2). Differences in prevalence of these microbial groups

between patient groups was examined using Fischer's exact test. Richness (species observed and Chao1 [187]) and diversity (Inverse Simpson) indices were calculated for each patient group and compared using a non-parametric Mann-Whitney U test using PRISM (Version 8, GraphPad, 1994-2020 GraphPad Software, LLC.).

Linear discriminant analysis (LDA) effect size (LEfSe) modelling was used to identify discriminating features from different taxonomic levels (phylum to species) based on different clinical outcomes [188]. This analysis was performed using taxonomic relative abundance, with per-sample normalization and default settings for alpha values (0.05) for the factorial Kruskal-Wallis test among classes and pairwise Wilcoxon test between subclasses. A logarithmic LDA score greater than 2 was used to determine discriminative features.

To infer the pattern of microbial co-occurrence relationships in the vaginal microbiome we used Bayesian Analysis of Compositional Covariance (BAnOCC) [189] to construct correlation matrices at the genus and species level. We limited the latter to the top 50 species with the greatest overall abundance across the entire cohort. The MCMCs were run for 10000 iterations and four chains (runs were checked for convergence) and subsequent analyses was restricted to edges with [r]>0.3. Co-occurrence networks were drawn using these correlation matrices in the Qgraph package in R[190].

The trophoblast microbial sequencing data underwent unsupervised multivariate analysis using principal components analysis (PCA) to provide an overview of the different body sites sampled using ClustVis, https://biit.cs.ut.ee/clustvis/ [186].

The rectal microbiota was analysed using Statistical Analysis of Metagenomic Profiles (STAMP) package (Version 8, OxMetrics Timerlake, London, UK [191]. Correlation between rectal and vaginal samples was carried our using a two group comparison and Welch's t-test. The significant rectal taxa associated with different pregnancy outcome groups were also calculated using corrected P values using the Welch's t-test. Richness (species observed and Chao1 [187]) and diversity (Inverse Simpson) indices were calculated for each patient group and compared using a non-parametric Mann-Whitney U test using PRISM (Version 8, GraphPad, 1994-2020 GraphPad Software, LLC.).

2.7 Cytokines in cervico-vaginal secretions

To assess the influence of the different vaginal microbial compositions and pregnancy outcomes on cervicovaginal inflammation, vaginal swabs were assessed for inflammatory cytokine concentrations.

2.7.1 Magnetic Luminex Screen Assay for Cytokines

A Human Magnetic Luminex Screen Assay (R&D Systems-biotechne brand, MN, USA) was used to detect 9 pre-specified analytes in the cervicovaginal fluid. Analytes were selected according to their role in early pregnancy and adverse pregnancy outcome. A table justifying the choice of each analyte is provided in Table 1.3 . Multiplex bead array assays use a 96-well plate format to produce a quantitative measurement of multiple analytes. Luminex identifies the presence of a target antigen by binding to analyte specific antibodies that are pre-coated onto magnetic microparticles. It is advantageous to traditional assays such as ELISA (enzyme linked immunosorbent assays) as it requires smaller volumes, can test multiple analytes and detect target analytes that are present at lower concentrations [192]

2.7.3 Analyte extraction

2.7.3.1 Materials

Table 2.6 Materials used for cytokine analysis

Experimental process	Chemical/Kit/Buffer	Supplier
Luminex Assay	Human Pre-mixed Multi-Analyte kit. Components include: • Premixed cocktail of antibody-coated Magnetic beads • Premixed cocktail of biotinylated detection antibody • Standard Cocktail(s) • Bead Diluent • Biotin Antibody Diluent • Standard/Sample Diluent • Wash Buffer • Streptavidin-PE • One flat-bottom 96-well Microplate • Foil Plate Sealers (4) • Mixing Bottle • Certificate of Analysis	R&D Systems Inc, MN USA. Catalog No. LXSAHM-08
	Protease inhibitor	Sigma-Aldrich P8340
	Phosphate Buffered Solution	In house

2.7.3.2 Methods for cytokine analysis

Vaginal swabs were thawed slowly on ice and vortexed. For a subset of swabs (n=74) not stored in amies solution, a total of 500 µl of PBS supplemented with 2.5 µl protease inhibitor (PI) was added to the swab to provide a comparable dilution volume as those stored in Amies transport solution. A constant volume was used as the mean wet weight for all swabs were highly comparable (mean 0.07grams +/-0.02). The samples were then centrifuged (8000rpm for 10 minutes) and the supernatant removed and stored at -20 °C for cytokine studies. In cases where supernatant was not collected and only dry swabs were available, we suspended the swab in protease inhibitor (PI) and PBS (5µl PI/1 ml PBS) to the same volume as of those stored in Amies transport solution. We then inverted the swab and transferred to a separate microtube and centrifuged at 10,000rpm for 5minutes to release the biological material into

the microtube. We combined the supernatants and repeated the centrifugation step at 10,000rpm for a further 10minutes to ensure removal of all cellular debris. Supernatants were analysed by Human Magnetic Luminex Screening Assay (8-plex, R&D systems, Minneapolis, MN, USA) to measure the concentration of the following analytes; IL2, IL4, IL6, IL8, TNF-alpha, IFN-γ, IL-1beta, IL18 and IL10 using a Bioplex®200system (Biorad technologies). The cytokines were analysed as per manufacturer guidelines, standards were prepared by reconstituting each standard cocktail with the specific volume of calibrator diluent that was detailed in the certificate of analysis. The standards were serially diluted by reconstituting 100µl of each standard cocktail with Calibrator Diluent RD6-52 to create a standard curve. The 6 standards (50µl of each) were loaded in duplicate to a 96-well plate as well as two blanks (50µl of calibrator diluent) and 41 samples (50µl) in duplicate. A 1:1 dilution was used for IL2, IL4, IL6, TNF-alpha, IFN-γ, IL-1beta, IL18 and IL10 and a 1:10 dilution was used for IL-8 based on previous experience with the Luminex platform to ensure detection within the standard ranges [163]. The microparticle cocktail was diluted by reconstituting 5ml of Diluent RD2-1 with 500µl of the microparticle. This cocktail consisted of beads coated with the pre-specified analyte-specific capture antibodies. It was crucial to protect the microparticles from light at this handling step. A total of 50µl of this cocktail was added to each well which contained 50µl of the cell free supernatant. The plates were sealed with foil and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set to 800rpm. A magnetic plate holding device (Bio-Rad) was used and the 96 well plate was washed 3 times with 100µl wash buffer. The biotin antibody cocktail (50 µl) was then added to well and the plate was secured with a foil cover again and incubated for 1 hour at room temperature on the shaker set at 800rpm. Following this the plate was washed 3 times using 100µl wash buffer as previously described to eliminate unbound antibody. A total of 50µl Streptavidin-phycoerthyrin (-PE) was added to each well which allows the contents of each well to bind to the biotinylated antibody. The plate was then covered and incubated at room temperature for 30min at 800rpm. The plate was then washed three times as previously described. Finally 100µl of wash buffer was added to each well and placed on a shaker for 2minutes at 800rpm, this was not discarded to resuspend the microparticles for analysis. Plates were read on a Biorad Bioplex®200system which contained one microparticle specific laser to determine the biomarker being detected and another which identified the magnitude of the phycoerthyrin signal which corresponds to the biomarker bound. Sample concentrations below the limit of detection of the assay were assigned a value of 0.01. Analytes that were detected outside the standard range concentrations were calculated by extrapolation of the standard curve. It is important to

appreciate that the extrapolation could alter the validity of the results and future work should dilute samples to ensure the analyte concentrations are within the standard curve.

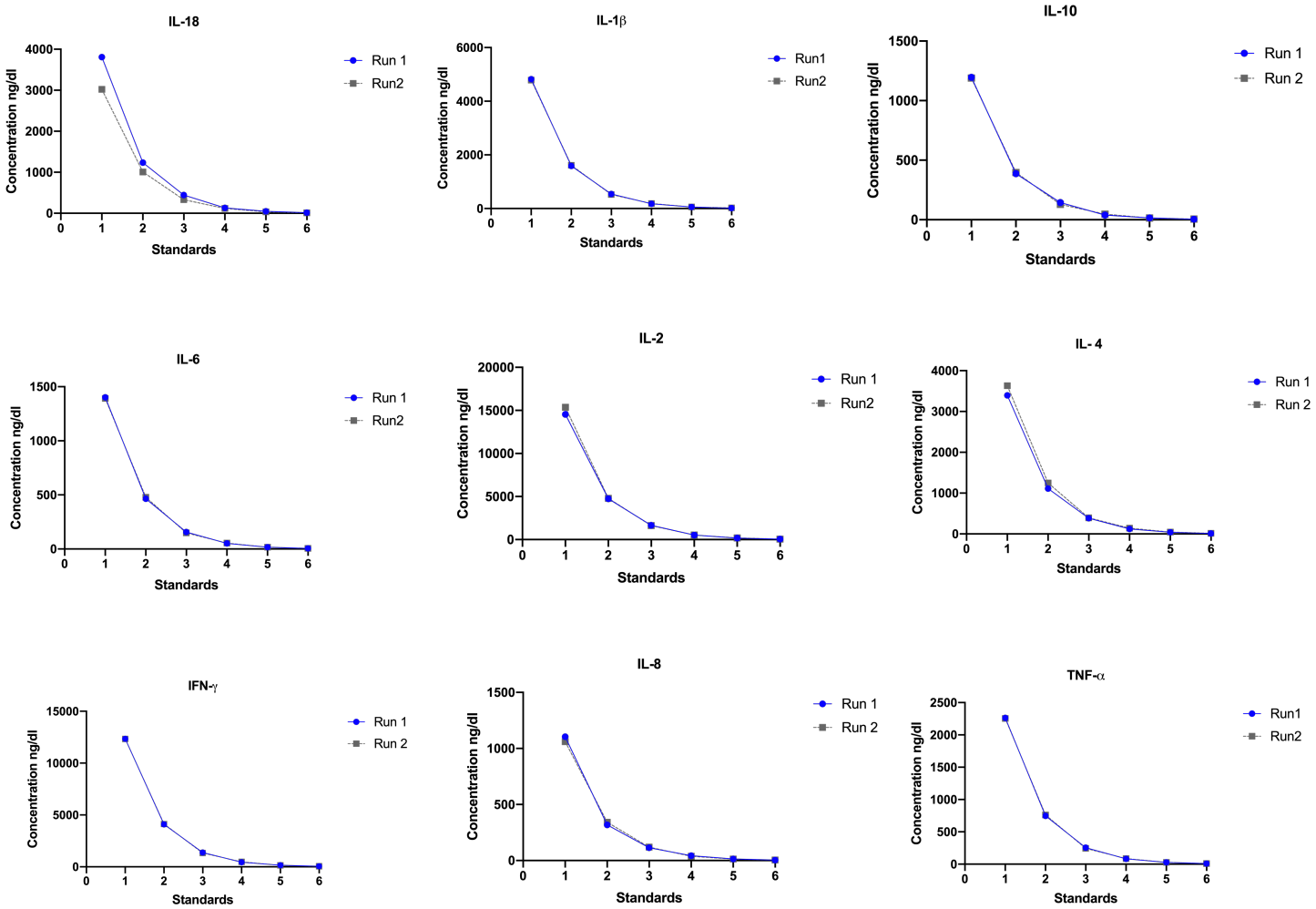

Figure 2.3 Comparison of standard curves from two different runs on different days using multiplex Luminex plates to show consistency.

2.7.3.3 Statistical analyses cytokine data

Statistical analysis was performed in GraphPad Prism v.8.4.3 (GraphPad Software Inc., California, USA). Data was represented as a clustered bar chart with mean +/- standard error of the mean for each cytokine by vaginal microbiome groupings. The percentage of activated cytokines was also compared by vaginal microbiome group. Activation was defined by

cytokines expressed in the upper quartile. The appropriate statistical test was carried out depending on normality and the number of groups analysed. P value <0.05 was considered significant.

2.8 Dipeptidyl peptidase-4 (DPP4)

DPP4 is a transmembrane glycoprotein that regulates the bioactivity in multiple peptides. In order to establish the role in miscarriage and the relationship with the vaginal microbial composition we measured the DPP4 activity in cervico-vaginal fluid samples from euploid miscarriages, aneuploid miscarriages and live viable term pregnancies.

2.8.1 Materials

Table 2.7 Materials for DPP4 assay kit

Experimental process	Chemical/Kit/Buffer	Supplier
DPP4 Activity Assay Kit	Components include: • DPP4 Assay Buffer (25mL) Catalog Number MAK088A • DPP4 Substrate, H-Gly-Pro-AMC (0.2mL) Catalog Number MAK088B • DPP4 Positive Control (20 µL) Catalog Number MAK088C • AMC Standard, 1 mM (0.1 mL) Catalog Number MAK088D • DPP4 Inhibitor, Sitagliptin (1 mL) Catalog Number MAK088E	Sigma-Aldrich (Catalog Number MAK088)
	96 well-flat bottom plate	
	Fluorescence multi-well plate reader	OPTIMA

2.8.2 DPP4 Methods

In this assay, DPP4 cleaves a non-fluorescent substrate, H-Gly-Pro-AMC, to release a fluorescent product. One unit of DPP4 is the amount of enzyme that will hydrolyse the DPP4 substrate to yield 1 mole of AMC/minute at 37°C. The supernatant from the cervicovaginal samples was defrosted and centrifuged at 13,000 rpm for 10minutes. The AMC standards

were prepared for fluorometric detection. Dilution of 10µL of the 1mM AMC standard solution with 990 µL of water was carried out to give the standard solution (10pmole/µL). We added 0,2,4,6,8 and 10 µL of this standard solution to a 96 well plate alongside 100 µL of DPP4 assay buffer. Following this we loaded the samples (50µL) to the remaining wells in duplicate and in addition 10 µL of DPP4 buffer to one sample well and 10µL of DPP4 inhibitor to the other. The plate was then covered and placed on a heat block for 37°C for 10minutes. We then made the master mix by mixing 38 µL of DPP4 buffer with 2 µL DPP4 substrate (40 µL is required for each sample and added to the 96 well plate but not in the standard curve wells). The plate was then placed on a horizontal shaker to ensure adequate mixing and further incubated at 37°C for 5minutes. Following this the initial fluorescence intensity $(FLU)_{initial}$, λ_{ex}=360/ λ_{em} =460nm was measured ($T_{initial}$) using the OPTIMA microplate reader. The plate was protected from light and measurements were taken every 5minutes until the value of the most active sample was greater than the value of the highest standard (100pmole/well). The final measurement (T_{final}) for calculating the enzyme activity was the penultimate reading before the most active sample exceeds the standard curve. The standard curve was plotted by subtracting the final measurement of the blank AMC standard from the standards and samples. The change in measurement for each sample was calculated from $T_{initial}$ to T_{final}. The change in fluorescence of each sample was compared to the standard curve to determine the amount of AMC released by the DPP4 assay between the initial and final timepoint which gives you B in the equation below. The DPP4 activity was calculated using the following equation:

$$\text{DPP4 Activity} = \frac{B \times \text{sample dilution factor}}{(\text{Reaction Time}) \times V}$$

B=Amount (pmole) of AMC released between $T_{initial}$ and T_{final}

Reaction time= $T_{final} - T_{initial}$ (minutes)

V=sample volume in mL added to each well

Sample dilution factor in this experiment is 1

DPP4 activity is reported as microunit/mL, where one unit of DPP4 is the amount of enzyme that will hydrolyse the DPP4 substrate to give 1 µmole of AMC per minute at 37°C.

CHAPTER 3. Euploid miscarriage is associated with *Lactobacillus* spp. depletion

Content from this chapter was published as:

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3 Euploid miscarriage is associated with *Lactobacillus* spp. depletion

3.1 Background

Early miscarriage (pregnancy loss before 12 weeks) occurs in one in five pregnancies of which 50% are thought to be due to chromosomal abnormalities [8]. Infection is implicated in 15% of early miscarriages and 66% of late miscarriages (12-24 weeks). However, the mechanisms driving miscarriage in these groups are poorly defined [20]. There are no interventions that prevent sporadic miscarriage and treatments such as progesterone supplements only modestly reduce the recurrence risk of miscarriage in subsequent pregnancies [44].

It is understood that pregnancy has a unique and dynamic immunological milieu that is required to support a healthy pregnancy [193]. Initially, a pro-inflammatory state is required for implantation which involves an influx of inflammatory mediators inducing tissue injury and repair [194]. Infection may disrupt the immunological synergy at implantation and trigger adverse outcome [195]. Emerging evidence implicates the reproductive tract microbiota as a key modulator of local inflammatory and immune pathways.

Healthy pregnancy is associated with increased *Lactobacillus* spp. dominance and stability [93, 196]. This is attributed to increasing oestrogen in pregnancy is thought to promote glycogen deposition in vaginal epithelia that is then converted into complex carbohydrates that can be preferentially utilized as carbon sources by *Lactobacillus* spp. [197]. Stable dominance of the vaginal niche by *Lactobacillus* spp. during pregnancy provides protection against pathogenic bacteria by producing lactic acid and antimicrobial compounds such as bacteriocins [66]. In contrast, depletion of vaginal *Lactobacillus* spp. has been linked to adverse pregnancy outcomes, including preterm birth and preterm prelabour rupture of fetal membranes (PPROM) [111, 198, 199].

As described in the introduction of this thesis, taxonomic profiles of vaginal microbial communities can be sorted into a discrete number of categories based on hierarchical clustering of the pairwise distances between samples. One of the first studies to apply this approach to vaginal microbial communities was by Ravel and colleagues who examined samples taken from 396 asymptomatic reproductively aged women. At species level,

hierarchical clustering analysis characterised the vaginal microbiota into five community state types (CSTs), four of which were characterised by high relative abundance of specific *Lactobacillus* species [69] : CST I – *Lactobacillus crispatus (L. crispatus)*, CST II – *Lactobacillus gasseri (L. gasseri)*, CST III – *Lactobacillus iners* (*L. iners)*, CST IV – *'high diversity'*, CST V – *Lactobacillus jensenii (L. jensenii).* The CST IV (high diversity) group was characterised by a low abundance of *Lactobacillus* spp. and an over representation of anaerobic bacteria such as *Atopobium, Prevotella, Sneathia, Gardnerella* and *Mobiluncus* species [72]. These taxa are associated with bacterial vaginosis (BV), a polymicrobial disorder that is associated with preterm birth [73], higher risk of acquiring sexually transmitted infections [74], and late miscarriage [75-78]. Recent work has identified the issue between study comparisons and how difficult it is to compare individual classification systems. Therefore to address these issues, the Ravel group [200] recently created the VALENCIA centroid-based tool (VAginaL community state typE Nearest Centroid clAssifier). This classifies samples based on their similarity to a set of racially, ethnically and geographically diverse reference data sets. This approach allows any individual microbiota community to be assigned to one of 13 community state types. There are six *Lactobacillus* spp. dominant community state types, I-A, I-B, II, II-A, II-B, and V which correspond to the original CST defined by Ravel et al [69], but with the designation expanded to allow for community states that contain a combination of organisms. The original *Lactobacillus* spp. depleted CST IV is expanded in VALENCIA into CST IV-A, IV-B and five CST IV-C (0-4), to account for the variety of different *Lactobacillus* spp. deplete bacteria communities. The main advantage of this classification system is that it can characterise the vaginal microbiome in a standardised way to allow comparison of different study datasets.

We previously mapped the longitudinal changes in vaginal microbiota during pregnancy, starting at 6 weeks of gestation, in women at high or low risk of preterm birth. Women with a vaginal microbial composition dominated by species other than *Lactobacillus* spp. at any point during gestation were at increased risk of PPROM [85], demonstrating that the early pregnancy vaginal composition influences adverse outcomes. We have recently shown that a suboptimal vaginal microbiota, deplete in *Lactobacillus* spp. is a risk factor for first trimester miscarriage [79]. However, this study did not distinguish between euploid and aneuploid pregnancy losses. While aneuploid pregnancies are lost mainly because of intrinsic developmental errors, euploid miscarriages may be caused by inflammatory signals triggered by an adverse vaginal microbial composition.

3.2 Aims

- To characterise the vaginal microbial composition in chromosomally normal and abnormal miscarriages and in healthy controls.
- To identify specific bacterial taxa related to different phenotypes of miscarriage.

3.3 Results

Two hundred women were recruited, of whom 119 eventually miscarried and 81 went on to a viable term pregnancy. Of the women who miscarried, 92 were recruited at first consultation, the time of miscarriage diagnosis. A live pregnancy was initially diagnosed in 108 cases of whom 27 subsequently went on to miscarry and 81 ultimately had a successful pregnancy and delivered at term. Molecular cytogenetic analysis was unavailable in 26 cases (in 24 cases because of inadequate numbers of villi and in two cases because of technical failure). Of the 81 cases, which ultimately had a viable pregnancy, 7 swabs were unavailable for microbiota analysis. The final study cohort consisted of two patient groups, one miscarriage group (n=93) and one term pregnancy group (n=74) (Figure 3.1).

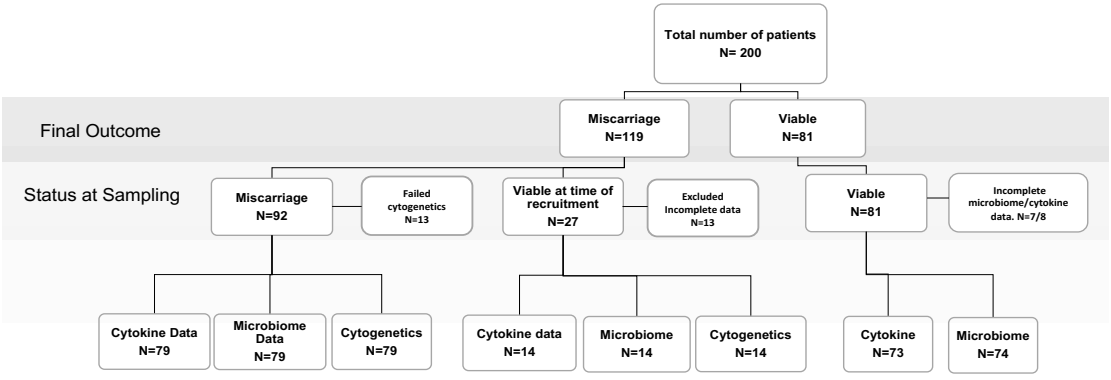

Figure 3.1 Flowchart detailing entire study cohort including status at sampling and experiments performed.

Detailed clinical and demographic characteristics were collected and compared for the three patient groups (Table 3.1). Maternal age was significantly higher in the aneuploid miscarriage group (*P*=0.0044, Kruskal-Wallis test). Women in the term pregnancy group were significantly less likely to have had one or more previous miscarriages (*P*=0.0084, Chi-squared test).

Table 3.1 Clinical and demographic characteristics of 167 patients included in study cohort

	Euploid miscarriage	Aneuploid miscarriage	Control (Term pregnancy)	p value
Number of women	54	39	74	
Maternal age, years	32	36	32	
Median (range)	(17-46)	(27-45)	(20-44)	†0.0044
BMI (kg/m^2)	25.1	24.4	24.5	
Median (range)	(17.7-35)	(18.7-39.4)	(18-38.4)	†0.205
Smokers (%)	7	2	3	
	(13)	(5.1)	(3.7)	††0.189
Ethnicity (%)				
White	30 (56)	24 (62)	54 (73)	
Asian	15 (28)	11 (28)	10 (13.5)	†††0.1271
Black	7 (13)	4 (10)	10 (13.5)	
Mixed	2 (3)	0 (0)	0 (0)	
Previous miscarriage (%)				
0	30(55.6)	22 (56.4)	31 (42)	
1	9(16.7)	6 (15.3)	29 (39)	††††0.0084
2	9 (16.7)	7 (18)	10 (14)	
≥3	6 (11)	4 (10.3)	4 (5)	
Gestational age group at sampling (%)				
5-8 weeks	14 (26)	14 (36)	7 (10)	
8-10 weeks	24 (44)	19 (49)	41(55)	
10-14 weeks	16 (30)	6 (15)	26 (35)	†††0.0083
Bleeding score				
0	28	24	65	
1	13	11	9	
2	8	2	0	†††0.0002
3	3	2	0	
4	2	0	0	
Status at sampling				
Missed	40	35		
Incomplete	4	0		†††0.1
Viable	10	4		

†Kruskal-Wallis Test, ††Fisher's exact (two-tailed), ††† Chi-squared. ††††Chi-squared combining euploid and aneuploid miscarriages and comparing with viable term pregnancies

Gestational age at sampling was categorized as 5-8, 8-10, or 10-14 weeks post last menstrual period (LMP). There were no significant differences in gestational age at the time of sampling between aneuploid and euploid miscarriage group, but the term pregnancy group were

sampled significantly later (*P*=0.0083, Chi-squared test). Bleeding scores were similar between euploid and aneuploid miscarriage groups and higher bleeding scores were more common in the miscarriage groups compared to the term pregnancy group (*P*=0.0002, Chi-squared test). There were no significant differences in BMI, smoking status or ethnicity between the groups.

3.3.1 Baseline vaginal microbiota composition

In total 4,523,582 sequence reads were obtained from 167 samples with an average of 27,087 reads per sample and a median read length of 370 bp after bar code removal. Following removal of singletons and rare operational taxonomic units (OTUs), a total of 128 taxa were identified in the vaginal microbiota of the study cohort. To avoid sequencing bias, OTUs were randomly sub-sampled to the lowest read count of 1402 which maintained a minimum coverage of 96% (range 96%-99.9%) for all samples. Further analysis was restricted to the top 50 taxa which accounted for 98% of the total sequence reads in the dataset.

3.3.2 Analysis of genus-level microbiota data

Ward hierarchical clustering of genus level relative abundance data for the whole cohort identified two major vaginal microbiome groups (VMG), which were characterised by *Lactobacillus* spp. dominated or *Lactobacillus* spp. depleted (Vaginal microbiome grouping 1; Figure 3.2). These were observed in 75% (125/167) and 25% (42/167) of samples respectively. The *Lactobacillus* spp. dominated group had a mean *Lactobacillus* content of 94.2%. The *Lactobacillus* spp. depleted group had a mean *Lactobacillus* content of 18.5%. The *Lactobacillus* spp. depleted cluster was further divided into Gardnerella dominated and Non-Gardnerella (Vaginal microbiome sub-grouping 1) and these clusters could be further divided into Gardnerella dominant, mixed Lactobacillus/Gardnerella, Prevotella or Streptococcus dominant (Vaginal microbiome sub-grouping 2).

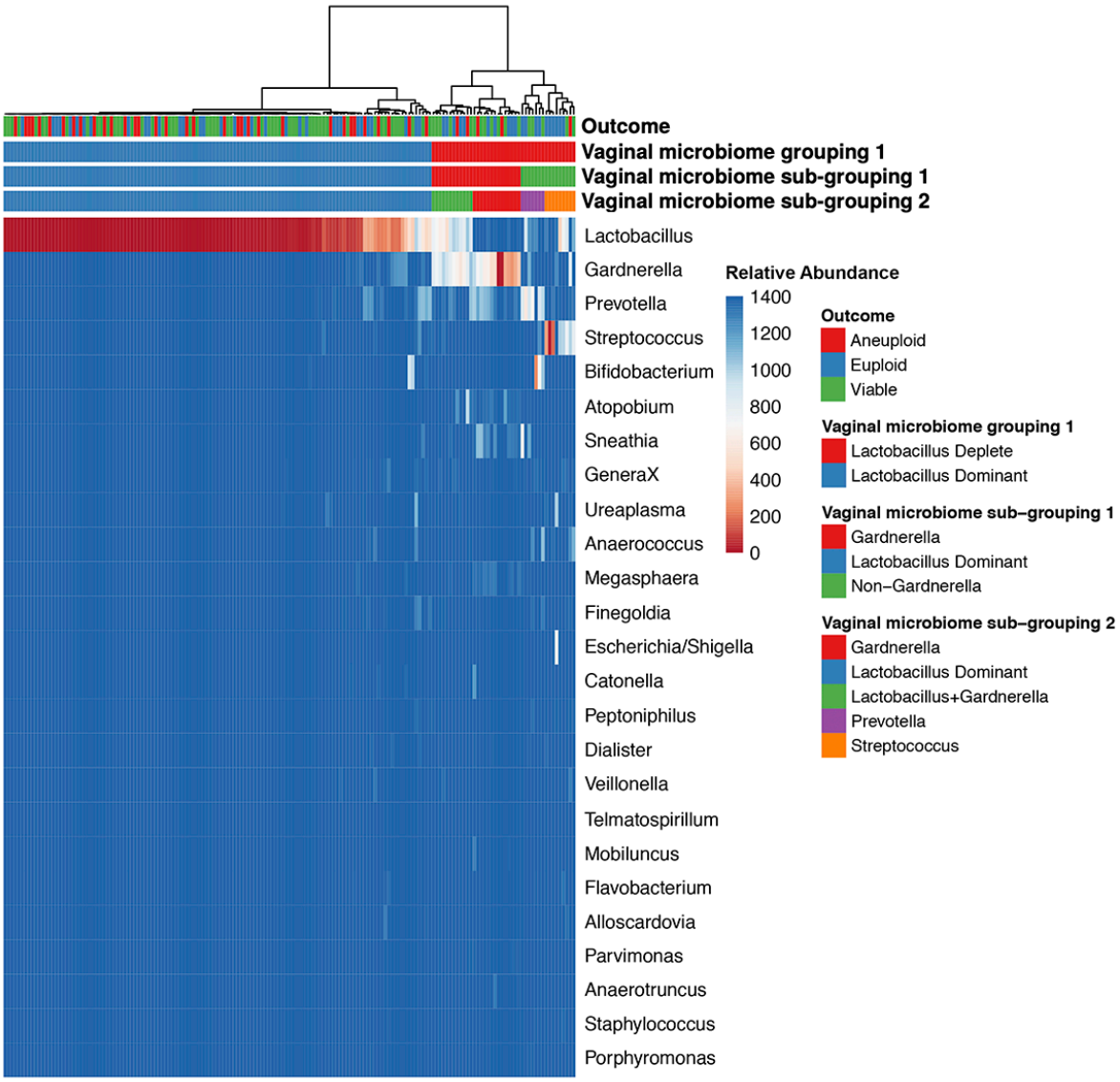

Figure 3.2 Heat map of relative abundance data for the top 50 most prevalent vaginal bacterial genera and relationship with different clinical outcomes.

This is a heatmap which uses Ward hierarchical clustering to construct a dendrogram and divide the cohort on the basis of different groups. Each column represents a different patient. The relative abundance key demonstrates the abundance of each genera for each patient. The red colour represents the highest relative abundance of that particular genera. The cohort consists of 167 individual samples and the dendrogram identified two major vaginal microbiome groups (VMG), characterised by *Lactobacillus* spp. dominance 75% (125/167) and *Lactobacillus* spp. depletion 25% (42/167) The *Lactobacillus* spp. deplete cluster was further divided into Gardnerella dominant and Non-Gardnerella (VMG sub-grouping 1) and these clusters could be further divided into Gardnerella dominant, mixed Lactobacillus/Gardnerella, Prevotella or Streptococcus dominated (VMG sub-grouping 2).

3.3.3 Analysis of bacterial species-level microbiota data

Similar analyses were performed at species level to identify the principal *Lactobacillus* spp. present in the *Lactobacillus* spp. dominant group of each individual patient. Ward hierarchical clustering separated patient samples into clusters that were dominated by either *Lactobacillus crispatus* (37%), *Lactobacillus iners* (19%), *Lactobacillus gasseri* (11%), *Lactobacillus jensenii* (10%), *Lactobacillus acidophilus* (2%), *Gardnerella vaginalis* (15%), and a highly diverse group (8%), Figure 3.3. This classification system did not identify a particular *Lactobacillus* spp. that was associated with subsequent miscarriage (chi-squared *P*=0.3, Table 3.2).

We also used the recently developed VALENCIA tool [200] to classify the vaginal microbiota and incorporated this into Figure 3.3. This tool indicated broad agreement between the two clustering approaches (84%, 140/167). In the remaining 27 samples where classification differed, low similarity scores indicated poor fitting to the CSTs pre-defined by the VALENCIA algorithm. For example, 7 were classified as *Gardnerella* dominant in our classification and *Lactobacillus* dominant by VALENCIA however when we examined composition of these samples in more detail, predominance of *Gardnerella* was confirmed. Therefore, the remaining analyses were therefore performed using our defined community clusters.

Table 3.2 Number of cases dominated by major *Lactobacillus* species or non-*Lactobacillus* species in each patient group.

	Euploid	**Aneuploid**	**Term**
L. crispatus	20	15	26
L. gasseri	3	5	10
L. iners	12	9	10
L. jensenii	2	4	10
G.vag/other	17	6	18
P value (Chi squared)	0.301817		

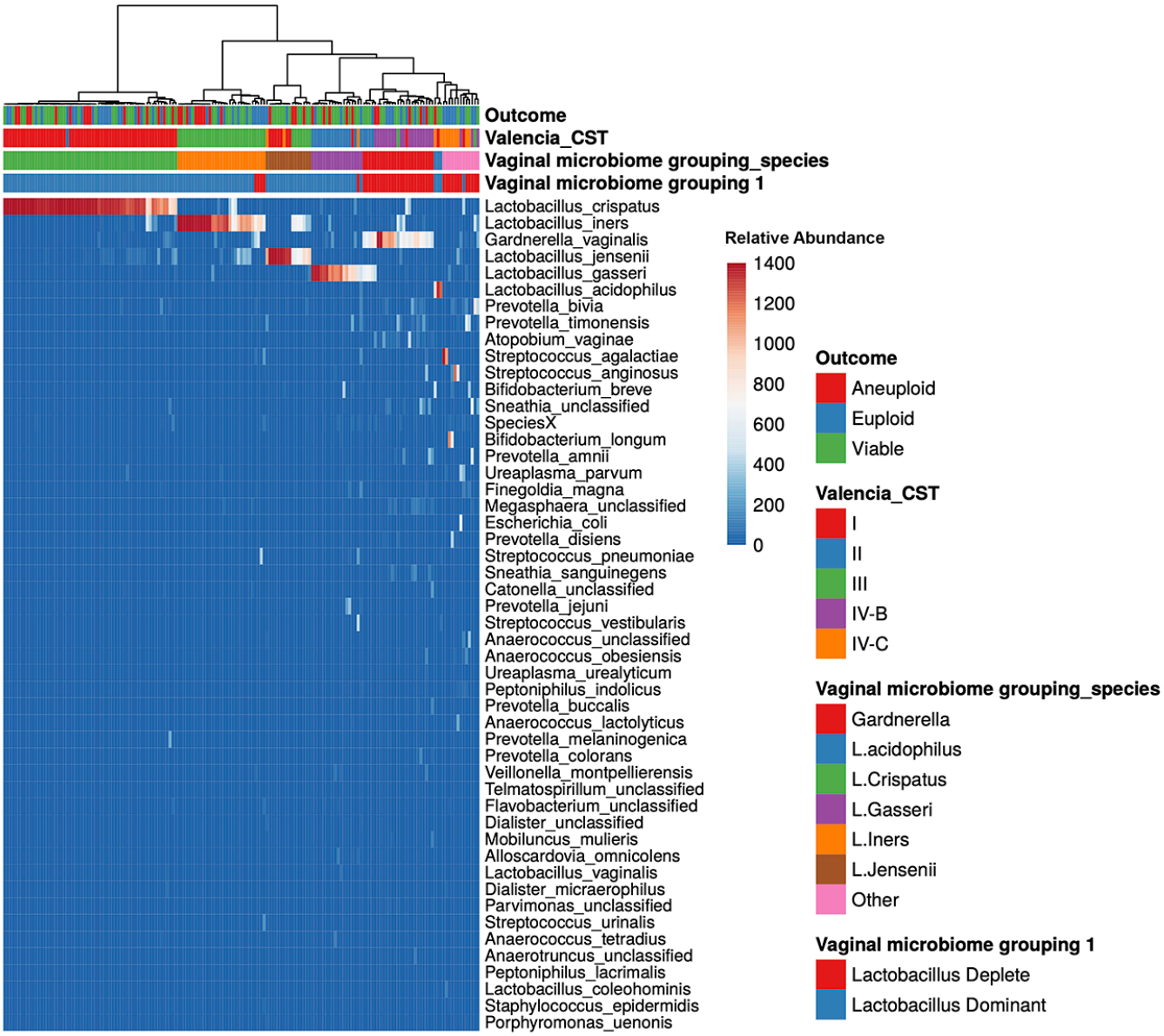

Figure 3.3 Heat map of relative abundance data for the top 50 most prevalent vaginal bacterial species and relationship with different clinical outcomes (n=167).

This heatmap uses Ward hierarchical clustering to separate patient samples into clusters that were *Lactobacillus crispatus* (37%), *Lactobacillus iners* (19%), *Lactobacillus gasseri* (11%), *Lactobacillus jensenii* (10%), *Lactobacillus acidophilus* (2%), *Gardnerella vaginalis* (15%), and a highly diverse group (8%).Each column represents a different patient and the groups are created based on the clustering of each species and the relative abundance level for each patient. The dark red represents the highest relative abundance and is shown in the key.

3.3.4 Vaginal microbial composition in relation to pregnancy outcome

Vaginal microbiota composition was compared between women who miscarried according to the pregnancy karyotype. Euploid miscarriage was associated with a significantly higher proportion of *Lactobacillus* spp. deplete VMG when compared to aneuploid miscarriage (*P*=0.01, two-tailed Fisher's exact test; Figure 3.4a). This difference remained significant when correcting for bleeding score, which was achieved by removing patients with a bleeding score >1 (*P*=0.02, two-tailed Fisher's exact test; Figure 3.4b). Euploid miscarriage was characterised by a significantly higher proportion of non-Gardnerella *Lactobacillus* spp. depleted VMG (*P*=0.02, two-tailed Fisher's exact test, Figure 3.4c) which was enriched for *Streptococcus* spp. in 60% of cases and *Prevotella* spp. in 40% of cases (Figure 3.4d). Consistent with these findings both the species richness and alpha diversity were higher in the euploid compared to aneuploid miscarriage (Figure 3.5 a and b).

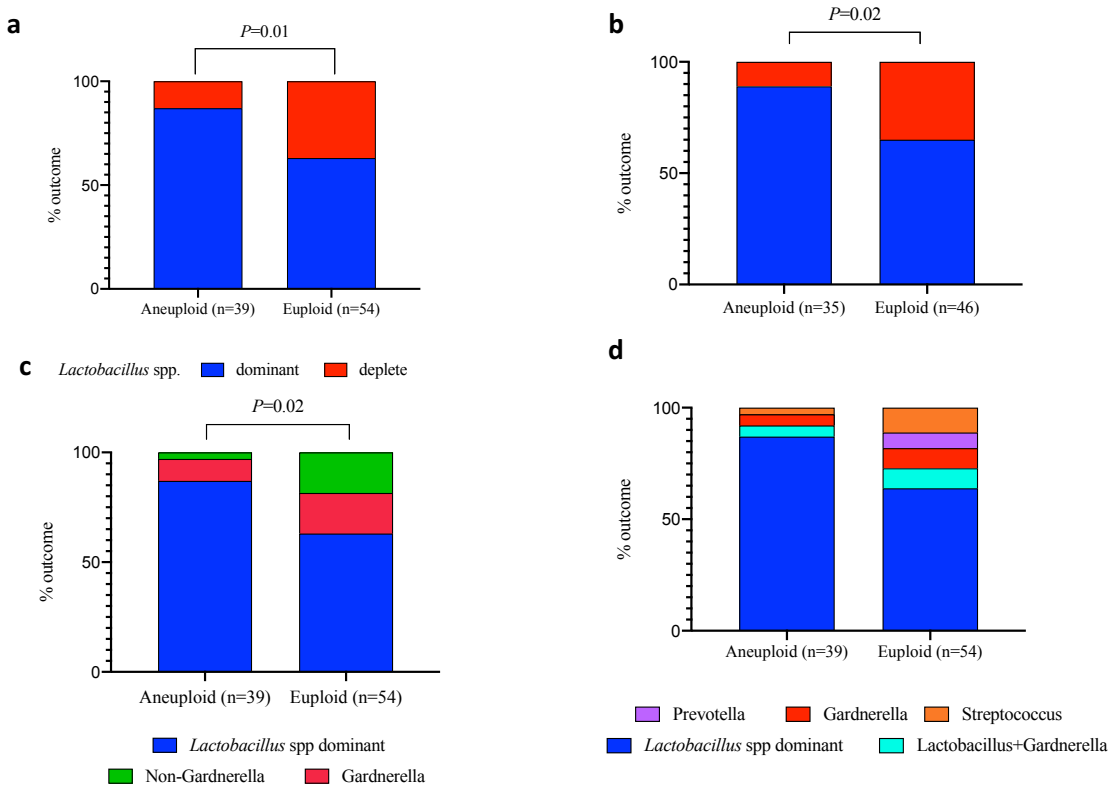

Figure 3.4 Miscarriage outcome according to vaginal microbial classification.

Stacked bar chart illustrating increased representation of *Lactobacillus spp.* depleted vaginal microbial communities in euploid miscarriages compared to aneuploid miscarriages (*P*=0.01, two tailed Fisher's exact test **a** and *P*=0.02 when bleeding score >1 removed, **b**). This difference was largely driven by non-Gardnerella-dominance of the vaginal niche (*P*=0.02, two-tailed Fisher's exact test **c**), and increased relative abundance of *Streptococcus* and *Prevotella* species in euploid miscarriages compared to aneuploid miscarriages (**d**).

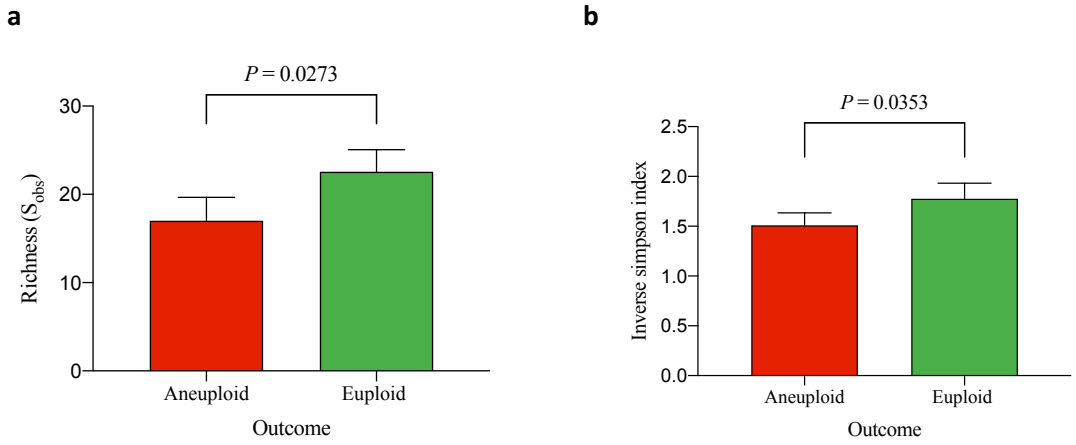

Figure 3.5 Species richness and diversity indices

Richness as determined by number of species observed (**a**) demonstrate a significant increase in euploid compared to aneuploid miscarriage (*P*=0.0273). Diversity, quantified by inverse Simpson index (**b**) demonstrate significantly increased diversity in euploid compared to aneuploid miscarriage (*P*=0.0353). Data represented as mean +/- standard error of mean. S_{obs} = species observed

Linear discriminant analysis Effect Size (LEfSe) analysis identified decreased levels of *Lactobacillus* spp. and increased levels of *Prevotella, Bacteriodia, Clostridia and Dialister* as characteristic features of euploid miscarriage compared to aneuploid miscarriage (Figure 3.6 a and b).

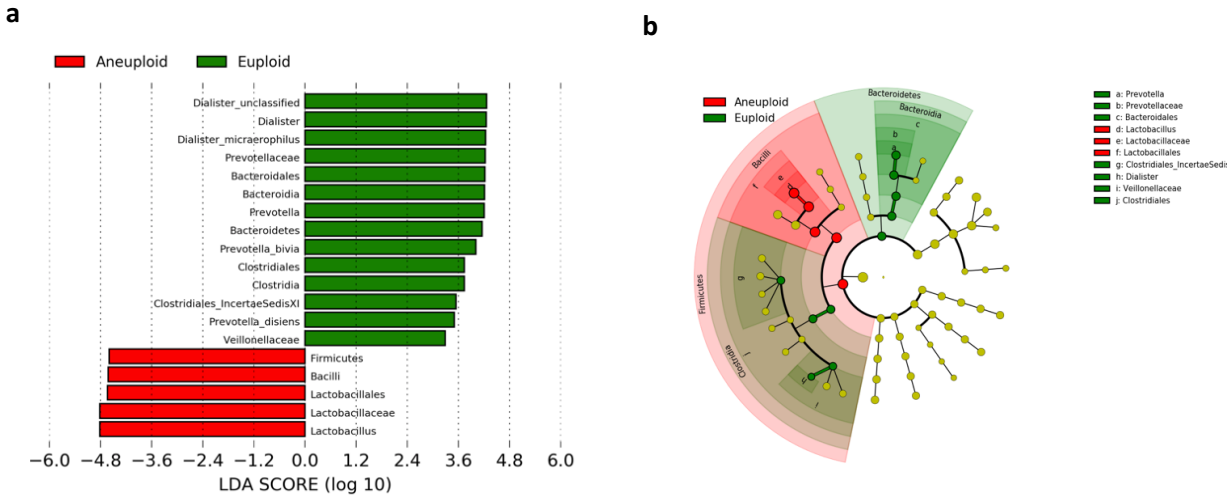

Figure 3.6 Identification of differentially abundant taxa between aneuploid and euploid miscarriages

Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to identify differentially abundant taxa in euploid compared to aneuploid miscarriage (**a**), which were presented at differing taxonomic levels using a cladogram (**b**).

3.3.5 Vaginal microbial composition in sporadic miscarriage and recurrent pregnancy loss

In our cohort 26 women had at least two previous miscarriages, prior to a further miscarriage in the index pregnancy and were therefore defined as having 'recurrent miscarriage' [3]. We separated these cases from the core analysis and found there was no difference in the proportion of *Lactobacillus* spp. deplete VMG or *Lactobacillus* spp. dominant VMG between euploid and aneuploid miscarriage (Figure 3.7b). Within the sporadic miscarriage and viable term pregnancy group the proportion of *Lactobacillus* spp. deplete VMG was greater in the euploid miscarriage group than either aneuploid miscarriage or viable pregnancy groups (*P*=0.02 and *P*=0.05 respectively, Figure 3.7a). The prevalence of Non-Gardnerella *Lactobacillus* spp. depleted VMG was also greater in the euploid miscarriage group than either aneuploid miscarriage or viable pregnancy groups (*P*=0.03 and *P*=0.04 respectively, Figure 3.7c) and was particularly associated with *Streptococcus* spp. dominated compositions (Figure 3.7d).

Similar results were obtained when re-analysis was performed on only those women where recurrent miscarriage was defined using the stricter criteria of three consecutive miscarriages with no live births (n=13, Figure3.9) [201]. LEfSe analysis identified increased relative abundance of *Prevotella* and *Streptococcus* spp. and reduced relative abundance of *Lactobacillus* spp. as being discriminatory for sporadic euploid miscarriage compared to viable term pregnancies (Figure 3.9e).

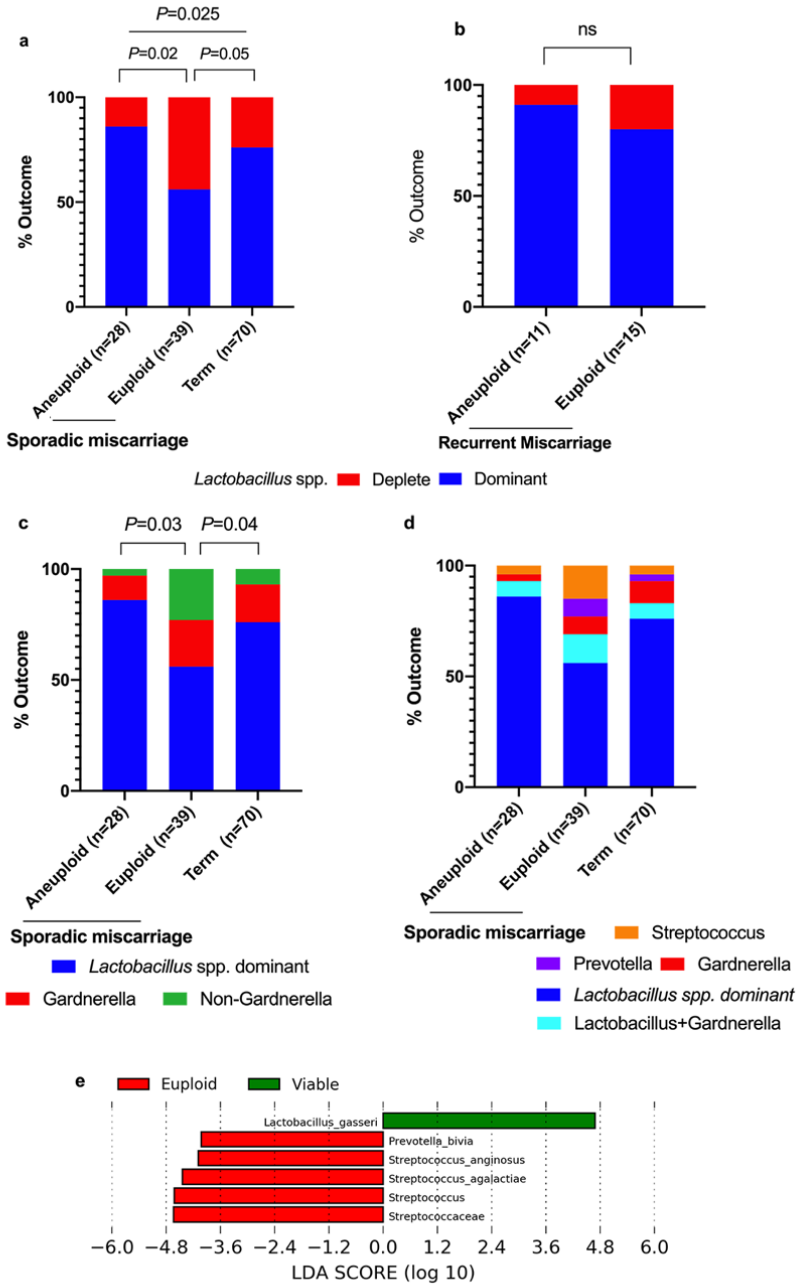

Figure 3.7 Clinical outcomes according to vaginal microbial composition in sporadic miscarriage.

The stacked bar charts in a-d use the microbial groupings that were defined by the heatmap in Figure 3.2. In Figure 3.7**a** increased *Lactobacillus spp.* depleted vaginal microbial communities were observed in sporadic euploid miscarriages (n=39) compared to sporadic aneuploid miscarriages (n=28), *P*=0.02 two-tailed Fisher's exact test and viable term pregnancies (n=70), *P*=0.05 two-tailed Fisher's exact test. No significant difference was seen between euploid (n=15) and aneuploid (n=11) miscarriages in the recurrent miscarriage group (**b**). A significantly increased prevalence of Non-Gardnerella vaginal bacterial communities was seen in euploid miscarriages compared to aneuploid miscarriage, *P*=0.03 and viable pregnancy *P*=0.04 (two-tailed Fisher's exact test, **c**). There was trend towards increased streptococcus and prevotella in euploid miscarriage compared to term pregnancy or aneuploid miscarriage but this did not reach significance (**d**). Differentially abundant taxa identified by LDA in euploid miscarriage compared to viable pregnancy are shown in (**e**). Data represented as percentages in a, b, c and d. LEfSe analysis (**e**) depicting particular vaginal bacterial taxa associated with euploid and viable pregnancy, confined to sporadic miscarriage.

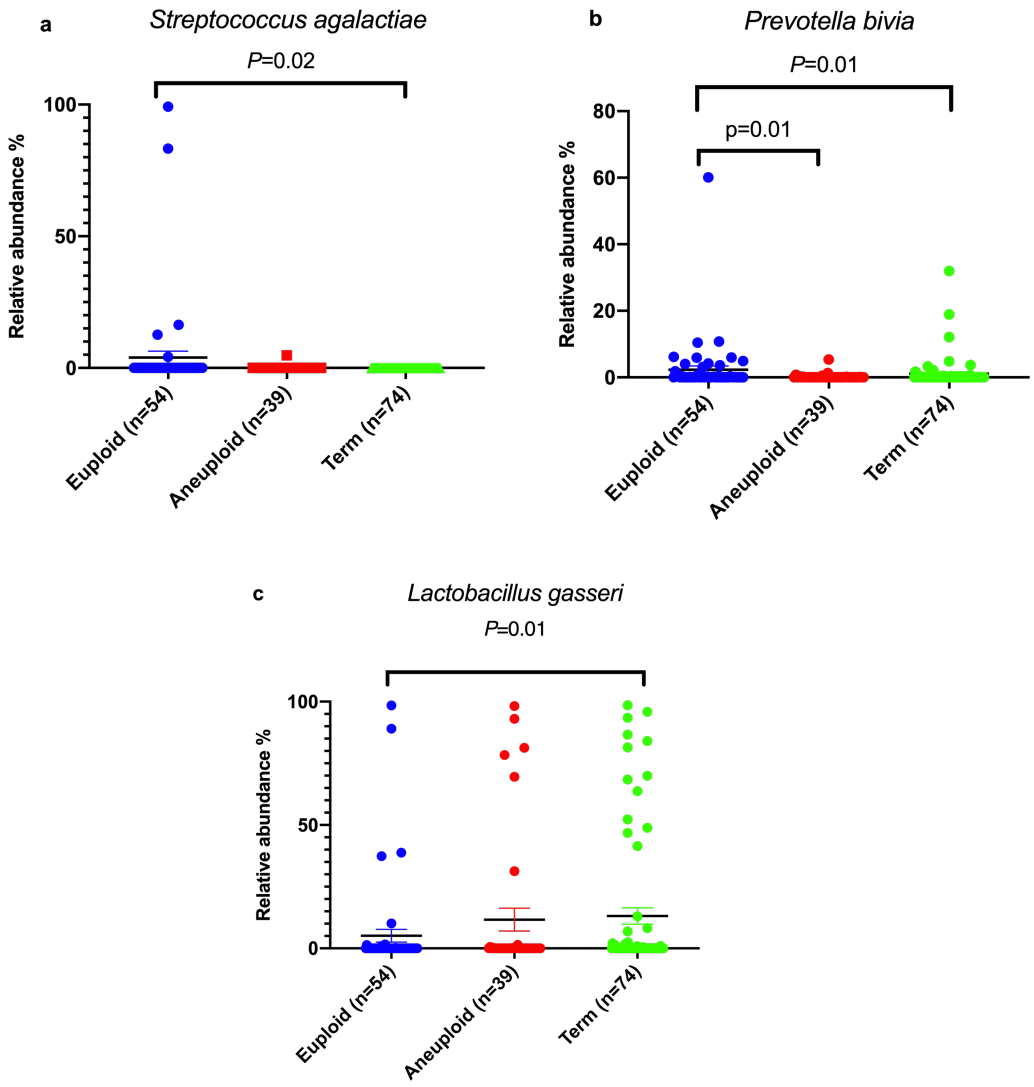

Figure 3.8 Relative abundance of particular taxa in relation to clinical outcomes

Data represented in a scatter dot plot with mean and standard error of the mean. Relative abundance counts for *Streptococcus agalactiae* and *Prevotella bivia* are significantly over represented in euploid compared to viable term pregnancy (*P*=0.02 and 0.01, a and b). *Lactobacillus gasseri* is significantly over represented in term pregnancy compared to euploid miscarriage (*P*=0.01, c).

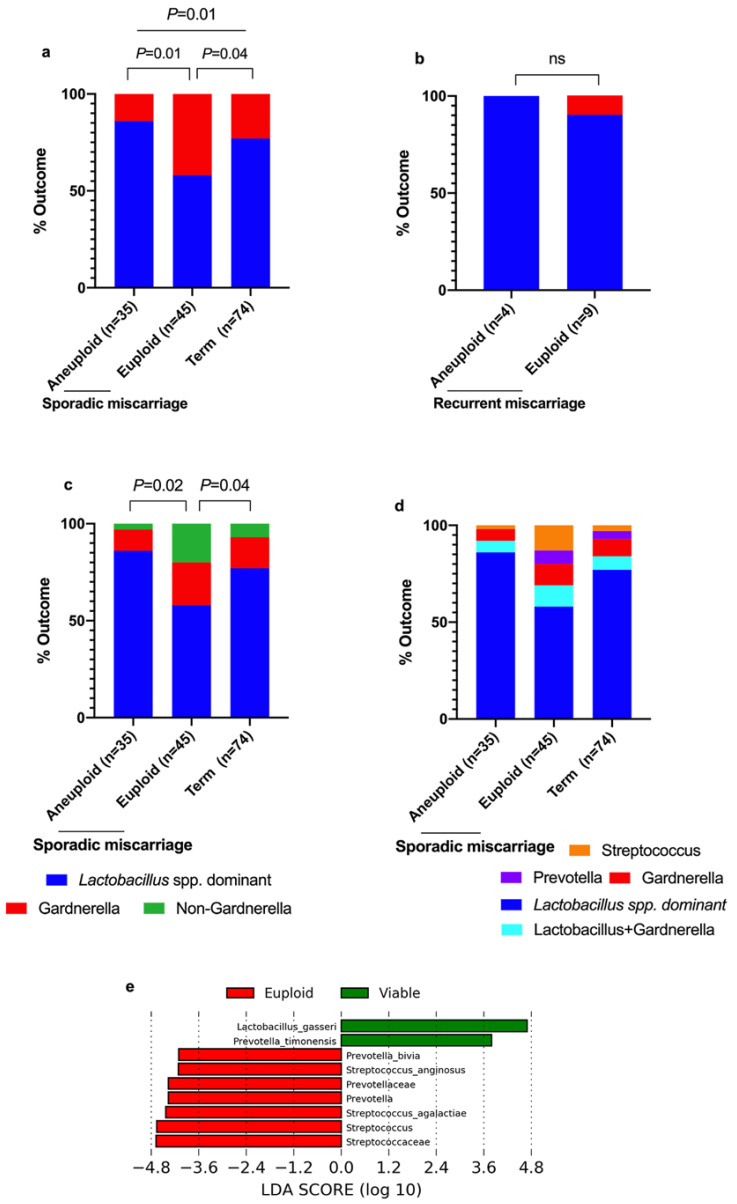

Figure 3.9 Clinical outcomes according to vaginal microbial composition in sporadic and recurrent miscarriage.

Patients with recurrent miscarriage (n=13) were excluded using the stricter criteria (≥3 miscarriages with no live birth). Increased *Lactobacillus spp.* depleted vaginal microbial communities were observed in sporadic euploid miscarriages compared to sporadic aneuploid miscarriages (*P*=0.01 two-tailed Fisher's exact test) and viable term pregnancies (*P*=0.04 two-tailed Fisher's exact test, **a**). No significant difference was seen between euploid and aneuploid miscarriages in the recurrent miscarriage group (**b**). A significantly increased prevalence of Non-Gardnerella vaginal bacterial communities was seen in euploid miscarriages compared to aneuploid miscarriage, (*P*=0.02 two-tailed Fisher's exact test) and viable pregnancy (*P*=0.04 two-tailed Fisher's exact test, **c**). Data represented as percentages in **a**, **b**, **c** and **d.** Differentially abundant taxa identified by LDA in euploid miscarriage compared to viable pregnancy (**e**).

3.3.6 Co-occurrence network analysis

Bayesian Analysis of Compositional Covariance (BAnOCC) was used to generate co-occurrence networks between vaginal microbiota at genera (Figure 3.10a) and species level taxonomy (Figure 3.9b) using the top 50 most abundant species identified in the whole dataset. This approach uses a Bayesian framework to analyse compositional covariance. High levels of co-occurrence were observed between bacterial vaginosis (BV) associated genera including Gardnerella, Atopobium and Prevotella. In contrast, Lactobacillus was negatively correlated with most other genera, especially Gardnerella, Sneathia, Atopobium and Megasphaera. When analysis was undertaken at the species level BV-associated taxa were again observed to positively correlate whereas *Lactobacillus* species, especially *L. crispatus*, tended to be negatively correlated with other bacterial taxa, including with other Lactobacilli. Strong co-occurrence between *Streptococcus vestibularis* and *Streptococcus pneumonia* was also observed and *Streptococcus urinalis* showed a high density of negative edges with BV-associated bacteria but a positive edge with *Streptococcus agalactiae* (Figure 3.10b).

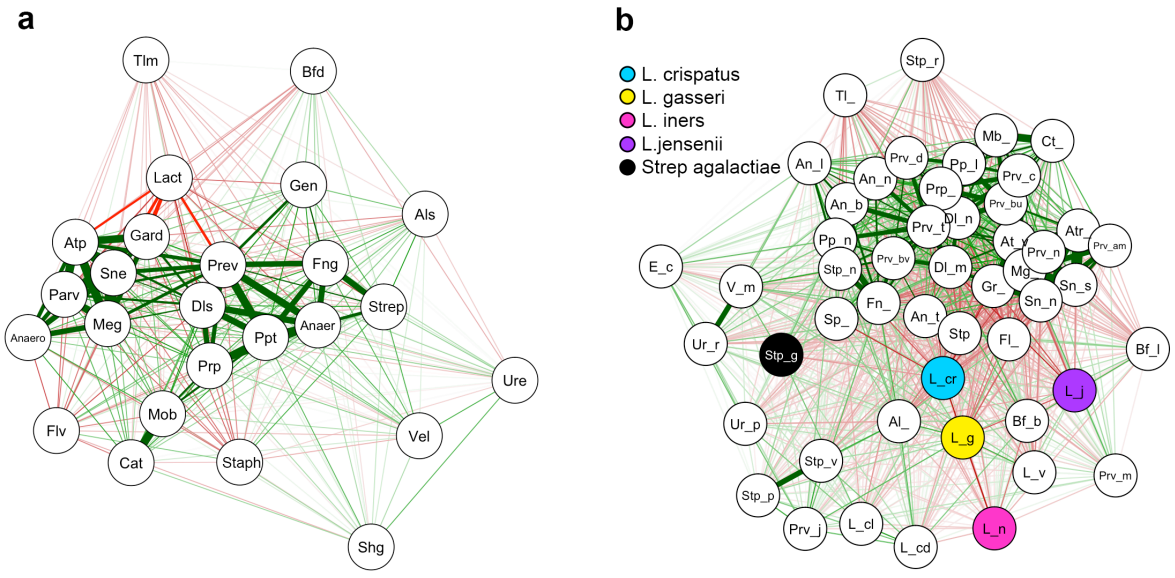

Figure 3.10 Co-occurrence network analysis of vaginal bacterial taxa in the overall patient cohort

High levels of co-occurrence were observed between BV associated genera, whereas Lactobacillus was negatively correlated with most other genera (**a**). At species level *L.crispatus* had the most number of negative edges with all other bacterial taxa (**b**). We ran the MCMC for 10000 generations and four chains and restricted subsequent analyses to edges with [r]>0.3. The red lines indicate negative correlations and the green lines indicate positive correlations and the lines are weighted according to the magnitude of the correlation.

3.3.7 Negative control samples

The negative control samples used in our study included extraction kit controls and operating theatre controls (n=5). Only one sample generated detectable reads (total read count =15), which had the following composition:

Species	Group	NC
Lactobacillus_iners	Otu00002	10
Lactobacillus_crispatus	Otu00001	4
Alloscardovia_omnicolens	Otu00032	1

All other control samples failed to amplify or did not generate any read data following sequencing. By comparison, the average read depth for experimental samples was 27,087 and the normalized read count was 1402.

3.3.8 DPP4 activity and recurrent miscarriage

Recurrent miscarriage is thought to be aetiologically different to sporadic miscarriage and recent studies have highlighted an association with abnormal endometrial function, caused by lack of mesenchymal stem cells and heightened cellular senescence during the midluteal implantation window. Recent work suggests that inhibition of the glycoprotein Dipeptidyl-peptidase 4 (DPP4) increases homing and engraftment of bone marrow derived cells to sites of tissue injury. DPP4 is ubiquitously expressed in many cell types but particularly endothelial and epithelial cells [202]. DPP4 may be therefore implicated in endometrial shedding and contribute to abnormal endometrial function in recurrent miscarriage [38, 40, 41]. We therefore set out to measure DPP4 activity in the cervicovaginal fluid of 89 patients at the time of miscarriage diagnosis (51 euploid miscarriages, 38 aneuploid) and 68 viable term pregnancies. In our cohort we had 23 patients with recurrent miscarriage defined as 3 pregnancy losses (including the current miscarriage in our study). There were no significant differences in DPP4 activity between euploid, aneuploid or viable term pregnancies (Figure 3.11 a, P= 0.16 Kruskal-Wallis test). Similarly, no difference in DPP4 activity was observed when comparing all miscarriages to viable term pregnancy (Figure 3.11 b, P=0.07, Mann Whitney U). There was a trend increase in DPP4 activity in RMC patients compared to sporadic

miscarriage patients (Figure 3.11c, *P*=0.5, Mann Whitney U). There was also a trend towards increase DPP4 activity observed in RMC euploid miscarriages compared to euploid sporadic miscarriages although not statistically significant (Figure 3.11d, *P*=0.4, Mann Whitney U).

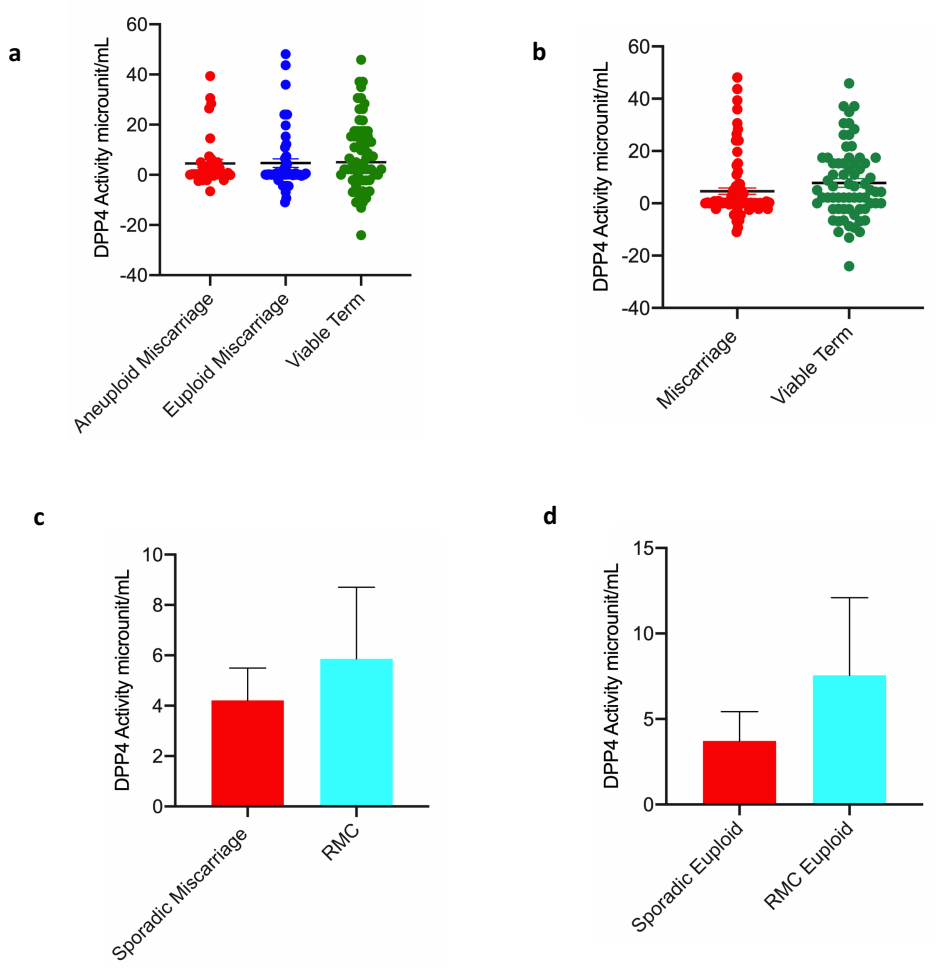

Figure 3.11 DPP4 activity in miscarriage and term pregnancy

Data represented in a scatter dot plot with mean and standard error of the mean in a and b (aneuploid miscarriage n=38, euploid miscarriage n=51, viable term n=68). Column bar graph demonstrated standard error of mean in c and d (Recurrent miscarriage n=23, sporadic euploid miscarriage n=38, RMC euploid n=13).

3.4 Discussion

The data presented in this chapter supports our primary hypothesis that euploid miscarriage is associated with *Lactobacillus* spp. depleted vaginal microbial communities compared to aneuploid miscarriage. This association was reflected in increased richness and diversity of bacterial communities detected in euploid miscarriages. A *Lactobacillus spp.* depleted vaginal microbiota characterised by increased Streptococci relative abundance was the most significant vaginal microbiota risk factor for sporadic euploid miscarriage. We also found that aneuploid miscarriages and healthy pregnancies had similar vaginal microbial compositions. These data support the notion that aneuploid and euploid miscarriages generally have different causal mechanisms. In general, aneuploid miscarriage is assumed to be due to a genetic intrinsic failure whereas a proportion of sporadic euploid miscarriage are due to host-vaginal microbe interactions.

Of the 200 patients recruited to this study, 119 eventually miscarried and 81 went on to have a viable pregnancy. The relatively high rate of miscarriage in this cohort was because most miscarriage patients (n=92) were recruited at the time of miscarriage diagnosis. Molecular cytogenetics was available for 93 miscarriage patients and results were reported after recruitment and sample collections, therefore eliminating selection bias. The proportion of aneuploid pregnancies in our miscarriage cohort is comparable with the aneuploidy rate reported in previous studies [203, 204]. In our cohort, there was a significantly higher maternal age in the aneuploidy miscarriage group, which is consistent with the known relationship between maternal age and the incidence of meiotic errors in embryos [205]. There was a significant difference in the gestational age at sampling between the viable pregnancies and miscarriages likely because women experiencing symptoms in a viable pregnancy tend to present at a later gestational age to the clinic. However, we ensured all patients were sampled in the first trimester to minimize the risk of over-classification of *Lactobacillus* spp. dominant microbiota in the term pregnancy group. Furthermore, our primary analysis only compared euploid with aneuploid miscarriage where there were no differences in gestational age at sampling. As expected, there was a significantly higher bleeding score in the miscarriage cohort compared to the viable term group [79]. The representation of different ethnic groups in our study population (64% White, 21% Asian and 12% Black and 1.2% Mixed) was comparable to the local background population (60% White,

18.5% Asian, 13% Black, 5% Mixed), which indicates a lack of bias in relation patient selection [206].

We used the VALENCIA algorithm to standardise the classification system but our results suggest that compositions observed in our cohort are not well-represented in the reference dataset used to train the VALENCIA algorithm. Therefore, the analysis was conducted using our defined community clusters.

Recurrent miscarriage is thought to be aetiologically different to sporadic miscarriage and recent studies have highlighted the association with abnormal endometrial function, caused by lack of mesenchymal stem cells and heightened cellular senescence during the midluteal implantation window [38, 40, 41]. Our data supports the relationship between miscarriage and vaginal microbiota composition principally in sporadic miscarriage. Although the numbers of recurrent miscarriages in our cohort were small (n=26) and the findings require confirmation in larger populations, this does reinforce the concept that different underlying causal mechanisms drive recurrent miscarriage.

There are limitations in this study to be considered. Firstly, this was a cross-sectional study with no longitudinal sampling performed. In non-pregnant women, vaginal microbial composition is dynamic and can be influence over time by the menstrual cycle, hormones and recent sexual activity [102, 207]. However, in pregnancy studies report a stable vaginal microbial composition, yet stability is not generally reached until the second trimester [208]. Our results are based on the assumption the vaginal microbial composition was stable throughout the first trimester yet increased patient sampling may reveal temporal changes in the microbiota across the early first trimester that are related to miscarriage risk. We have previously undertaken a prospective study design (Al-Memar et al [79]), that has shown first trimester miscarriage is indeed associated with a reduced prevalence of *Lactobacillus* spp. dominated vaginal microbiota. In our current study patients were recruited at diagnosis in consideration of the practicalities involved in obtaining trophoblast material for karyotyping, and the patients' need to undergo surgical management of miscarriage, rather than conservative or medical management, or manual evacuation. The surgical evacuation also needed to be undertaken at a time which enabled the study team to collect the necessary samples. For these reasons, recruitment occurred over a period of five years. Although our sample size is limited it is comparable to the only other study investigating VMB and

miscarriage [79]. Our study cohort is also unique in that it includes the molecular cytogenetic information of each miscarriage and a healthy control group.

In this study the further subdivision of the *Lactobacillus* spp. deplete cluster demonstrated that non-Gardnerella dominance of the vaginal niche significantly increased in euploid compared to aneuploid miscarriage. This was also observed when analyses focused on sporadic miscarriage. In particular, *Streptococcus* and *Prevotella* were found to be markers of euploid miscarriage compared to term pregnancies and aneuploid miscarriage. Increased relative abundance of *Prevotella* is associated with bacterial vaginosis (BV) and increased proinflammatory cytokines and Th17 related cytokines in the CVF. The risk of acquiring *Prevotella* in vaginal microbial communities may be linked to gene-environment interactions but once colonized there appears to be an enhanced capacity to drive inflammation [209]. Other studies have also linked *Prevotella,* and other taxa such as *Peptoniphilus, Streptococcus*, and *Dialister* with adverse pregnancy outcomes such as PPROM [85]. The presence of BV is associated with increased risk of first trimester miscarriage and colonization with group B Streptococcus (GBS) is shown to be an independent risk factor for chorioamnionitis [21, 210]. In our study we identified *Prevotella bivia*, *Streptococcus* and *Alphaproteobacteria* as specific bacterial markers of euploid miscarriage when compared to term pregnancy. We also identified *Dialister*, *Prevotella bivia* and *Clostridia* as specific taxa that were enriched in euploid miscarriage compared to aneuploidy miscarriage. *Dialister* has previously been described within vaginal samples and linked to poor health outcomes in women such as acquiring human papillomavirus infection and endometrial cancer [211, 212]. *Gardnerella vaginalis* was also observed as being an important feature in high-risk *Lactobacillus* spp. deplete compositions. Recent work has indicated the likely existence of different *Gardnerella vaginalis* clades with potentially varying degrees of pathogenicity [213]. However, the metaxonomics approach used in our study was not capable of differentiating these. Future investigations may shed light on whether Gardnerella *vaginalis* clades are responsible for the observed relationship with adverse outcomes. Nonetheless, Streptococci most strongly differentiated the vaginal microbiota of sporadic euploid miscarriage from those of viable pregnancies. Co-occurrence analysis revealed that whilst Streptococci often co-occur, they have a low co-occurrence with Lactobacilli. *L. crispatus*, has been shown to associate with vaginal bacterial stability and low levels of innate immune activation during pregnancy [91, 163]. The negative co-occurrence between *L. crispatus* and all other organisms suggests a possible therapeutic role for live biotherapeutics containing this organism. However, the

predominant role of Streptococci also suggests a potential benefit of targeted antibiotics, or a combination of both.

The potential mechanisms by which vaginal microbiota can be causally linked to miscarriage is explored in Chapter 4.

Conclusion

In conclusion, we demonstrate that vaginal microbiota depleted of *Lactobacillus* spp. and enriched for specific potentially pathogenic bacteria predispose pregnant women to euploid miscarriage. Whilst aneuploid miscarriage can be explained in terms of an intrinsic developmental defect, there is currently little evidence to explain the causative mechanisms underlying sporadic euploid miscarriage. There are currently no treatments to prevent this important clinical condition. The data presented here suggests that there is a group of women who would benefit from antibiotic or pre- or probiotic treatment to reduce the risk of miscarriage

CHAPTER 4. Cervical vaginal cytokines are altered in euploid miscarriage

Content from this chapter was published as:

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Grewal K, MacIntyre DA, Bennett PR. The reproductive tract microbiota in pregnancy. Biosci Rep. 2021 Sep 30;41(9): BSR20203908. DOI: 10.1042/BSR20203908. PMID: 34397086; PMCID: PMC8421591.

4 Cervical vaginal cytokines are altered in euploid miscarriage

4.1 Background

Successful pregnancy requires a dynamic and responsive immune system that can recognize and appropriately respond to harmful signals if required [149]. The immunological adaption of pregnancy occurs over the course of each trimester. Initially a pro-inflammatory environment is crucial to support implantation and placentation, then an anti-inflammatory shift supports fetal growth and finally a proinflammatory event promotes the onset of labour. The inability to maintain the correct balance of immunological and inflammatory response could alter pregnancy outcomes [150].

The inner cell mass in the human blastocyst gives rise to the embryo and the external layer (trophectoderm) forms the placenta. For successful implantation to occur, the trophoblast must breach the decidual lining and invade a receptive maternal decidua. This invasive process restructures the decidua and mimics the process of tissue injury and repair. Inflammation at the implantation site is characterised by increased Interleukin-6 (IL6), IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor (TNF) which are derived from endometrial stromal cells and infiltrating immune cells [150]. After implantation there is rapid fetal growth and development and the predominant state is a T_H2 type anti-inflammatory environment. It is thought that infection and the associated host immunological response can disrupt these carefully coordinated inflammatory-mediated events leading to poor implantation and/or trophoblast invasion and ultimately, miscarriage or preterm birth [41].

An important component of immunological response in early pregnancy is the ability of trophoblasts to sense and respond to pathogen associated molecular patterns (PAMPs) that are present in microorganisms [150]. Bacteria may gain access to the maternal-fetal interface by ascending infection or haematogenous spread leading lead to an inappropriate trophoblast cell response which may disrupt tolerance at the maternal-fetal interface and trigger pregnancy complications such as chorioamnionitis and preterm birth [154]. Studies have shown that when exposing both human and mice trophoblast cells to bacterial products an induction of IFN-β by the placenta modulates the maternal immune system and promotes tolerance [155].

An evolving concept is that an optimal microbiota also contributes to the immunological milieu. Depletion in vaginal *Lactobacillus* spp. is linked to adverse pregnancy outcomes, including preterm birth and preterm prelabour rupture of fetal membranes (PPROM) [111, 198, 199]. We have also shown that a *Lactobacillus* spp. depleted vaginal microbiome in early pregnancy is a risk factor for first trimester miscarriage [79]. As described in Chapter 3, we have also recently demonstrated that *Lactobacillus* spp. depletion associates with euploid sporadic miscarriage. Given that there exists a well-described positive correlation between vaginal communities deplete in *Lactobacillus* spp. and pro-inflammatory cytokines within the cervicovaginal fluid (CVF), it is feasible that an aberrant vaginal microbial environment may alter inflammatory mechanisms involved in establishment of early pregnancy [163, 164]. The objective of the work described in this chapter was to investigate the relationship between microbiota composition and specific cytokines implicated in early pregnancy including IL-1β, IL-6, IL-8, IL-10, TNF-α, IFN -γ, IL-4, IL-2 and IL-18. Further information relating to this is detailed in the Materials and Methods chapter 2.

4.2 Aims

1. To investigate the local immune response in relation to cytogenetically normal and abnormal miscarriage and in healthy controls.
2. To explore the local immune response in patients with a suboptimal vaginal microbial composition in the context of different miscarriage phenotypes and healthy term pregnancy.
3. To investigate the local immune response in the cervicovaginal environment prior to miscarriage.

4.3 Results

Two hundred women were recruited, of whom 119 eventually miscarried and 81 went on to a viable term pregnancy. Of the women who miscarried 92 women were recruited at first consultation, the time of miscarriage diagnosis. A live pregnancy was initially diagnosed in 108 cases of whom 27 subsequently went on to miscarry and 81 ultimately had a successful pregnancy and delivered at term. Molecular cytogenetic analysis was unavailable in 26 cases (in 24 cases because of inadequate numbers of villi and in two cases because of technical

failure). Of the 81 cases, which ultimately had a viable pregnancy, 8 swabs were unavailable for cytokine analysis. The final study cohort consisted of two patient groups, one miscarriage group (n=93) and one term pregnancy group (n=73), Chapter 3 Figure 3.1.

Table 4.1 Clinical and demographic characteristics of 166 patients included in cytokine study cohort

	Euploid miscarriage	Aneuploid miscarriage	Control (Term pregnancy)	p value
Number of women	54	39	73	
Maternal age, years	32	36	33	
Median (range)	(17-46)	(27-45)	(20-44)	†0.0044
BMI (kg/m²)	25.1	24.4	24.5	
Median (range)	(17.7-35)	(18.7-39.4)	(18-38.4)	†0.205
Smokers (%)	7	2	3	
	(13)	(5.1)	(3.7)	††0.189
Ethnicity (%)				
White	30 (56)	24 (62)	53 (73.4)	
Asian	15 (28)	11 (28)	10 (13.7)	†††0.1271
Black	7 (13)	4 (10)	10 (13.7)	
Mixed	2 (3)	0 (0)	0 (0)	
Previous miscarriage (%)				
0	30(55.6)	22 (56.4)	30 (41)	
1	9(16.7)	6 (15.3)	29 (40)	††††0.0084
2	9 (16.7)	7 (18)	10 (14)	
≥3	6 (11)	4 (10.3	4 (5)	
Gestational age group at sampling (%)				
5-8 weeks	14 (26)	14 (36)	7 (10)	
8-10 weeks	24 (44)	19 (49)	41 (56)	
10-14 weeks	16 (30)	6 (15)	25 (34)	†††0.0083
Bleeding score				
0	28	24	65	
1	13	11	8	
2	8	2	0	†††0.0002
3	3	2	0	
4	2	0	0	
Status at sampling				
Missed	40	35		
Incomplete	4	0		†††0.1
Viable	10	4		

†Kruskal-Wallis Test, ††Fisher's exact (two-tailed), ††† Chi-squared. ††††Chi-squared combining euploid and aneuploid miscarriages and comparing with viable term pregnancies

4.3.1 Detection of cytokines in the cervicovaginal fluid

Concentrations of cervicovaginal fluid immune analytes were determined using a custom Luminex Screen Assay (R&D Systems-Biotechne, MN, USA). This included IL-1β, IL-6, IL-8, IL-10 and TNF-α, all of which have been previously investigated in the context of endometrial function. We also included Interferon (IFN) -γ, IL-2 and IL-4 since we had seen alterations in these cytokines in a previous study linking vaginal microbiome to local inflammation [163] and IL-18 because of its role in host defence via induction of other pro-inflammatory cytokines and effector cells [172, 214]. Table 4.2 shows the analytes in relation to standard curve concentrations of the assay. Analytes that were detected outside the standard range of concentrations were calculated by extrapolation of the standard curve. The majority of analytes are within the standard range but IL-6,-8,-18and IL-1β have a maximum value that is beyond the standard range. For future work these analytes should be diluted to ensure they are within the reference range provided by the standards.

Table 4.2 Analyte detection compared to the standard range of the Luminex kit

Analyte	Standard Range		Analyte results		
	Min	**Max**	**Min**	**Median**	**Max**
TNF-α	9.19	2259.99	0.0001	2.14	47.53
IFN-γ	49.42	12329.75	0.0001	15.57	952.95
IL-2	57.29	10002.12	0.0001	3.18	73.53
IL-4	14.44	3378.25	0.0001	5.83	619.18
IL-10	4.48	1012.42	0.0001	1.2	49.38
IL-6	5.78	1399.99	0.0001	6.335	7687.25
IL-8	4.32	1060.22	12.29	1873.17	36891.5
IL-18	15.14	3790	0.0001	11.03	13148.47
IL-1β	19.71	4810.57	0.0001	159	11158.06
IL=Interleukin, IFN -Interferon, TNF=Tumour necrosis factor					

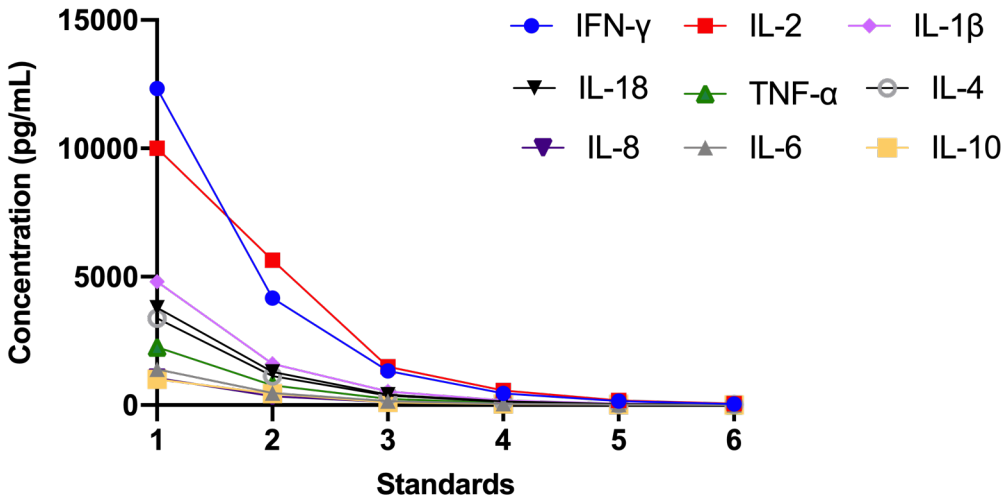

Figure 4.1 Standard curves generated from Luminex multiplex assay for standard concentrations of the nine analytes.

4.3.2 Cervicovaginal cytokines in relation to different pregnancy outcomes

We first explored the concentrations of all nine cytokines within the entire cohort in relation to different pregnancy outcomes. The anti-inflammatory cytokines (IL-4, IL2- and IL-10) were significantly higher in the viable term group compared to the euploid or aneuploid miscarriage group. In contrast, the pro-inflammatory cytokine IL-6 was significantly higher in the euploid and aneuploid miscarriage group compared to the viable term group. Overall, there was a trend towards viable term pregnancies having a significantly higher localised anti-inflammatory expression. The aneuploid and euploid miscarriages overall demonstrated proinflammatory expression (Figure 4.2).

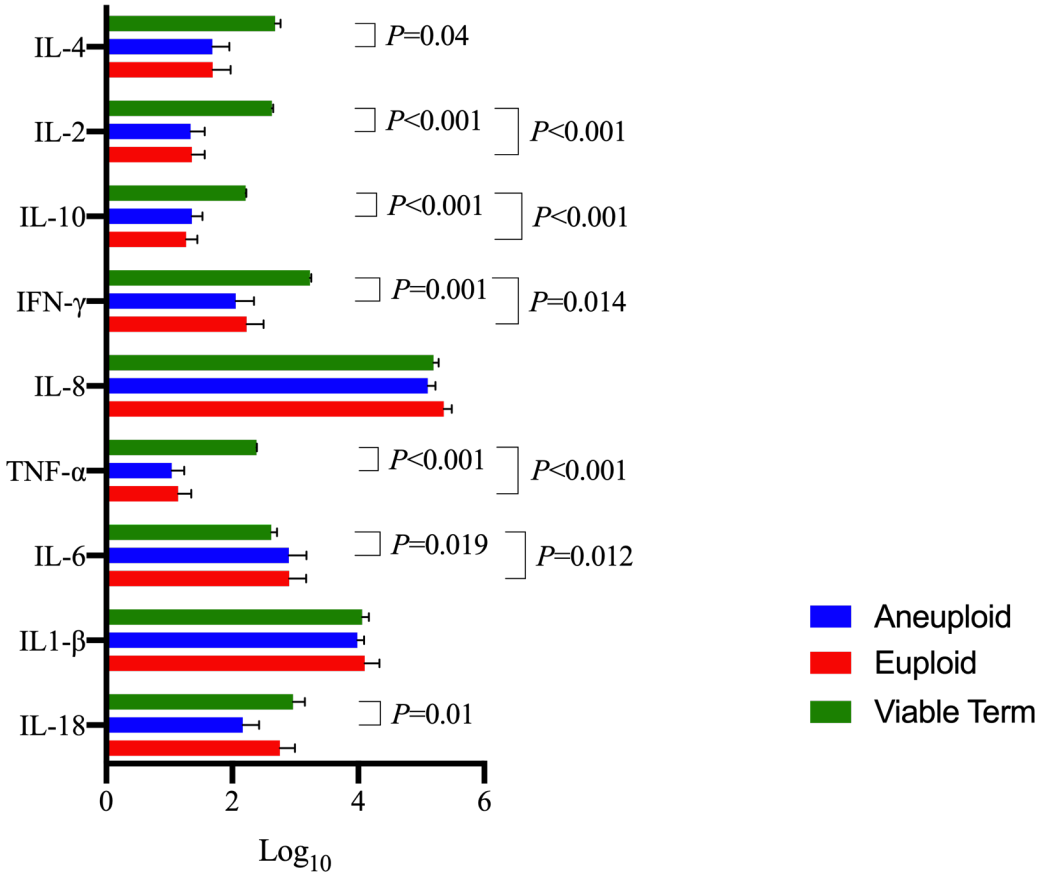

Figure 4.2 Cervicovaginal cytokine concentrations in relation to different pregnancy outcomes

Data represented as a clustered bar chart with mean +/- standard error of the mean for each cytokine by pregnancy outcome, n=166. P values, Mann-Whitney U test for viable term group versus aneuploid and euploid miscarriage.

4.3.3 Cervicovaginal cytokines in relation to different vaginal microbiome groups

To explore the relationship between the vaginal microbiota and cervicovaginal inflammatory markers, we compared the levels of nine cytokines between women with *Lactobacillus* spp. depleted or *Lactobacillus* spp. dominated VMG irrespective of pregnancy outcome. The patients were characterised as *Lactobacillus* spp. dominant or deplete based on the ward hierarchical clustering analysis shown in Chapter 3, Figure 3.2. There were significantly higher levels of TNF-α, IL-6, IL-1β (*P*=0.001, 0.03, 0.001 respectively) in the *Lactobacillus* spp. deplete cohort compared to *Lactobacillus* spp. dominant samples (Figure 4.3). Furthermore, the *Lactobacillus* spp. depleted non-Gardnerella VMG sub-group had significantly higher concentrations of Tumour Necrosis Factor (TNF)-α, Interleukin (IL)-6, IL-1β and IL-18 compared to *Lactobacillus* spp. dominated VMG (*P*=0.001,0.045,0.04,0.04 respectively, Figure 4.4).

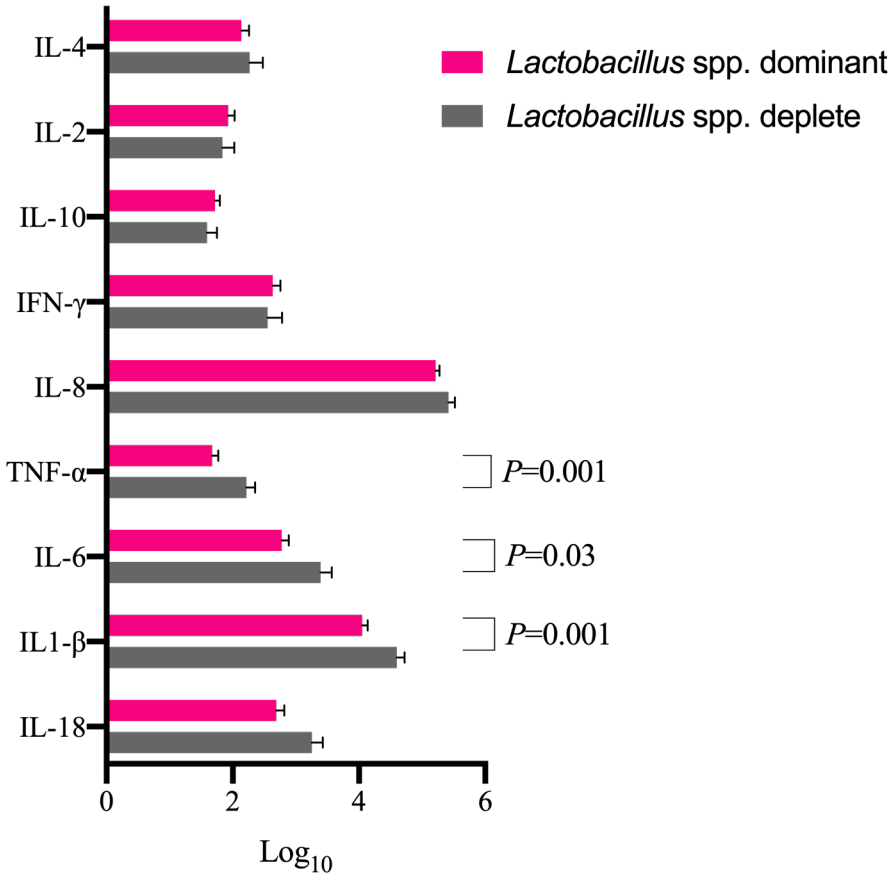

Figure 4.3 Cytokine expression according to vaginal microbiome grouping 1

Data represented as a clustered bar chart with mean +/- standard error of the mean for each cytokine by vaginal microbiome grouping 1, n=166. P values, Mann-Whitney U test for *Lactobacillus* spp. deplete versus *Lactobacillus* spp. dominant.

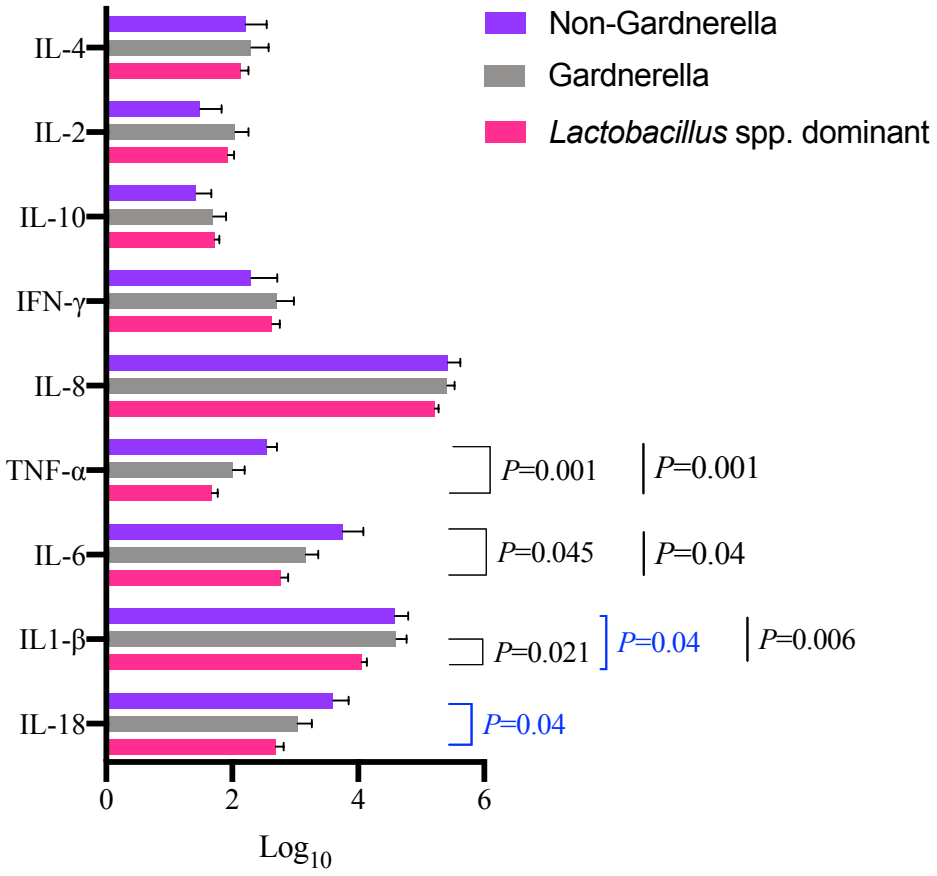

Figure 4.4 Cytokine expression according to vaginal microbiome subgrouping 1

Non-Gardnerella, *Lactobacillus spp.* depleted samples (16/166) had significantly increased vaginal levels of inflammatory cytokines TNF-α, IL-6, IL-1 β and IL-18 when compared to *Lactobacillus spp.* dominant samples (124/166). Gardnerella, *Lactobacillus spp.* depleted samples (26/166) had significantly increased IL-1 β when compared to *Lactobacillus spp.* dominant (black P values, Kruskal-Wallis test with *post hoc* Bonferroni correction and blue P values, Mann-Whitney U test for Non-Gardnerella versus *Lactobacillus spp.* dominant patients). Data represented as a clustered bar chart with mean +/- standard error of the mean for each cytokine.

4.3.4 Integrating the vaginal microbiome grouping with pregnancy outcome and cervical vaginal cytokine expression

The expression of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β was most elevated in the high diversity cohort compared to the *Lactobacillus* spp. dominant group. The mean fold change in cytokine expression from *Lactobacillus* spp. dominant to deplete was also more significantly observed for these cytokines (Figure 4.5).

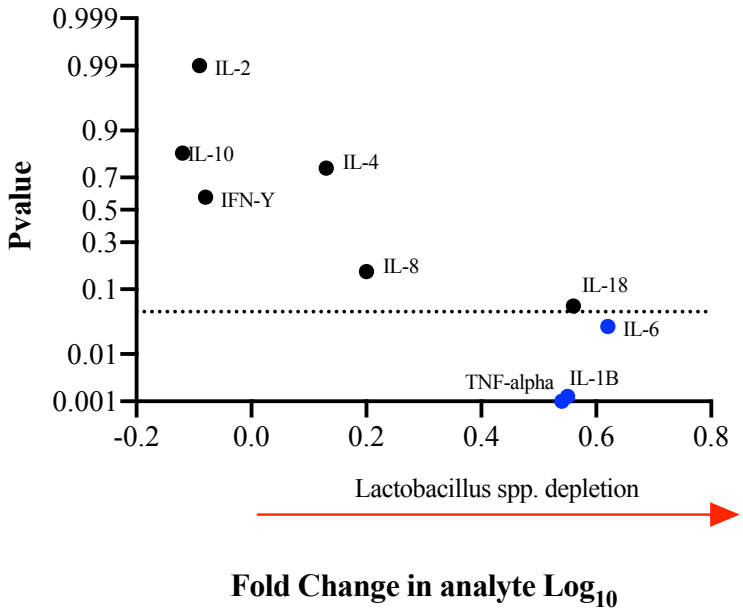

Figure 4.5 Mean fold change in analyte concentration between Lactobacillus spp. Dominant versus Lactobacillus spp. deplete

The mean cytokine concentration of the *Lactobacillus* spp. deplete group was subtracted from the mean cytokine concentration in the *Lactobacillus* spp. dominant group to give the mean fold change. The *p* values were calculated by using the Mann Whitney U test between *Lactobacillus* spp. deplete group and *Lactobacillus* spp. dominant group for each individual cytokine as seen in Figure 4.3.

Table 4.3 Mean fold change of analyte expression

Analyte	Mean fold change of analyte expression between Lactobacillus deplete and dominant	
	Fold Change	**P value**
TNF-α	0.54	0.001
IFN-γ	-0.08	0.58
IL-2	-0.09	0.99
IL-4	0.134	0.75
IL-10	-0.124	0.82
IL-6	0.616	0.03
IL-8	0.204	0.16
IL-18	0.563	0.06
IL-1β	0.545	0.001

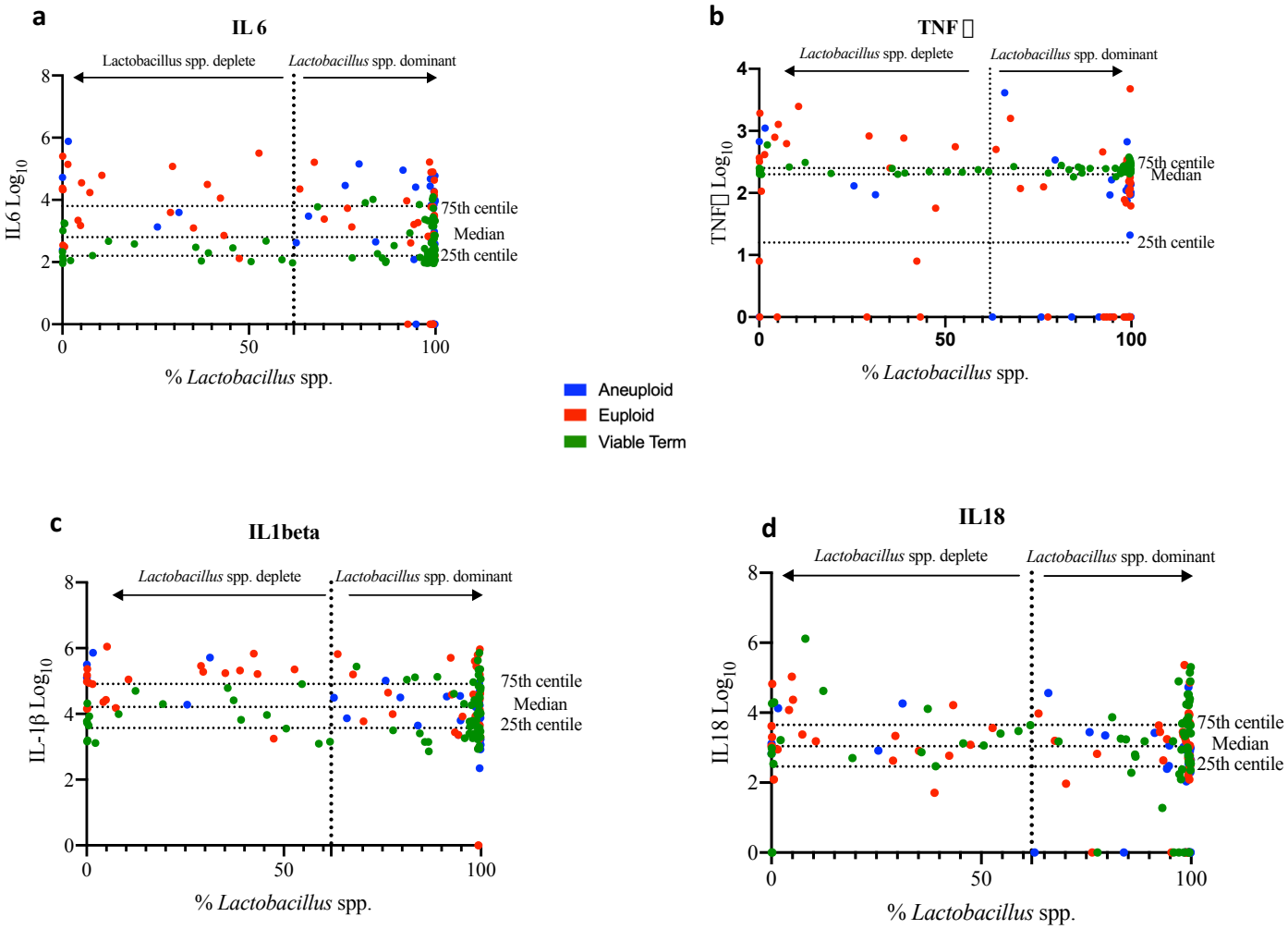

Figure 4.6 Cytokine expression according to vaginal microbiota and pregnancy outcome

Scatter plots representing the percentage of *Lactobacillus* spp. against the concentration of IL-6 (**a**), TNF- α (**b**), IL-1β (**c**), and IL-18 (**d**). Each individual case was given a *Lactobacillus* spp. percentage based on the relative abundance of that genera in the sample. The 25th, median and 75th centile levels of the cytokine concentrations were highlighted to show the spread of the data. The different colours represent different clinical outcomes as shown in the key.

Figure 4.6 demonstrates IL-6, IL1- β, TNF-α and IL-18 cytokine concentrations within the quartile ranges in the context of *Lactobacillus* spp. relative abundance for the pregnancy outcome groups. The pro inflammatory cytokines (IL-6, IL1- β and TNF-α) show a trend towards more euploid miscarriages within the higher cytokine concentrations with less *Lactobacillus* spp.

Table 4.4 Activation of cytokine expression in different microbiome groups

%	Code
<25	
25-49	
50-74	
>75	

a

Vaginal microbiome grouping 1	% activated IL1ß	IL6	IL8	TNFα	IL18	IFN γ	IL2	IL4	IL10
Lactobacillus spp. dominant (n=124)	19	21	23	20	23	23	23	25	21
Lactobacillus spp. deplete (n=42)	43	36	31	41	29	30	31	26	36
P value	**0.002**	0.055	0.3	**0.009**	0.5	0.3	0.3	0.88	0.055

b

Vaginal microbiome sub-grouping 1	% activated IL1ß	IL6	IL8	TNFα	IL18	IFN γ	IL2	IL4	IL10
Lactobacillus spp. dominant (n=124)	19	21	23	20	23	23	23	25	21
Gardnerella (26)	42	23	27	27	27	31	35	23	38
Non-Gardnerella (16)	44	56	38	63	31	31	25	31	31
P value	**0.007**	**0.009**	0.41	**<0.001**	0.76	0.6	0.4	0.83	0.14

c

Vaginal microbiome sub-grouping 2	% activated IL1ß	IL6	IL8	TNFα	IL18	IFN γ	IL2	IL4	IL10
Lactobacillus spp. dominant (n=124)	19	21	23	20	23	23	23	25	21
Gardnerella (14)	36	14	7	29	29	50	43	29	43
Lactobacillus+Gardnerella (n=12)	50	33	50	25	25	8	25	17	33
Streptococcus (n=9)	33	67	44	56	33	33	22	44	22
Prevotella (n=7)	57	43	29	71	29	29	29	14	43
P value	**0.018**	**0.02**	0.07	**0.007**	0.96	0.123	0.6	0.6	0.27

Activation was defined by cytokines expressed in the upper quartile. *P* values obtained by comparing activated versus non-activated cytokines using two-tailed Fisher's exact test (**a** and **b)** and chi-squared test (**c**).

High cytokine levels for TNF-α and IL-1β (defined as concentrations in the upper quartile) were more frequently observed in the *Lactobacillus* spp. depleted group (*P*=0.009 and 0.002 respectively, two-tailed Fisher's exact test, Table 4.4 a) particularly, non-Gardnerella dominated samples, which also had elevated IL6 levels (*P*=0.009, two-tailed Fisher's exact test, Table 4.4 b). Further subdivision of the VMG groups showed that increased levels of TNF-α and IL-1β were largely associated with *Prevotella* spp. dominance (*P*=0.007 and 0.018 respectively, Chi-Squared test) whereas IL6 was linked to *Streptococcus* dominance (*P*=0.02 Chi-squared test, Table 4.4 c).

4.3.5 Cervicovaginal cytokine expression in relation in patients with a suboptimal vaginal microbial composition.

The proportion of *Lactobacillus* spp. deplete vaginal microbiota in euploid, aneuploid and viable term pregnancy groups was 37%, 13% and 23% respectively. Given nearly a quarter of the pregnancies that went on to term had a suboptimal microbial composition, we decided to compare cytokine concentrations between euploid miscarriage and viable term pregnancies in only women with *Lactobacillus* spp. depletion. IL-1β, IL-6, IL-8 were significantly lower (*P*<0.001, *P*<0.001 and *P*=0.01, respectively) and IL-2 and IL-10 were significantly higher (*P*=0.004 and *P*<0.001 respectively) in viable pregnancies (Figure 4.7).

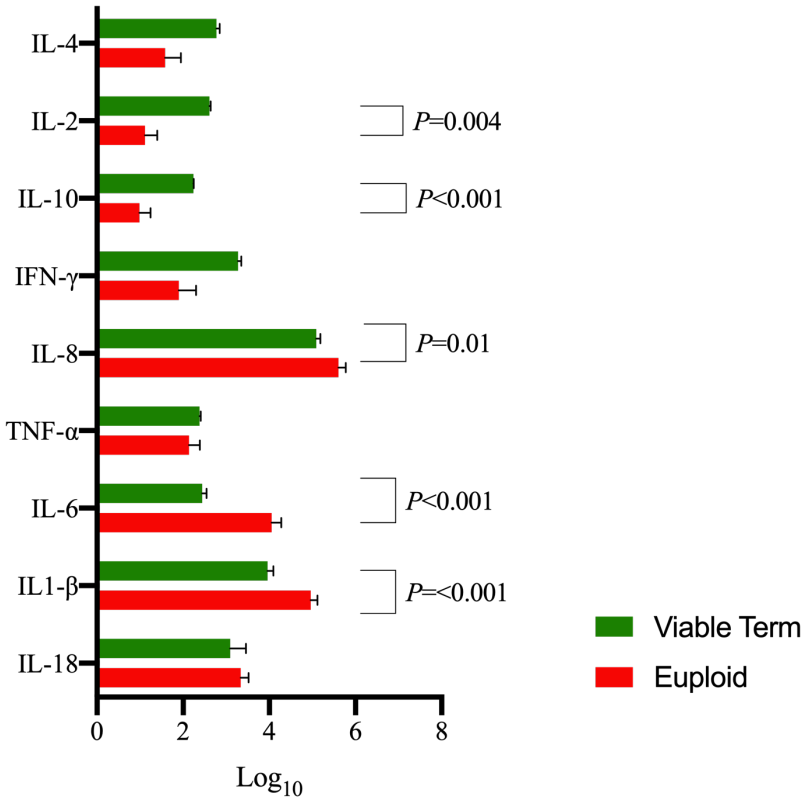

Figure 4.7 Cervico-vaginal cytokine levels according to pregnancy outcome in patients with Lactobacillus spp. depleted vaginal microbial composition.

N=37, euploid n=20 and viable term n=17. Data represented as clustered bar chart with mean +/- standard error of the mean for each cytokine according to clinical outcome. P values calculated using the Mann Whitney U test two tailed.

4.3.6 Cervicovaginal cytokine expression in patients sampled with a fetal heartbeat prior to miscarriage

To exclude the possibility that activation of inflammation in the miscarriage cases was due to the process of miscarriage, we compared CVF cytokine levels in a subset of patients sampled when the pregnancy was viable, which was defined as having a normal appearance on ultrasound and a normally beating fetal heart. Consistent with previous results, significantly increased levels of IL-1β, IL8 and IL-18 and reduced levels of IL2 and IL-10 were observed in pregnancies which subsequently miscarried compared to those which went on to viable term delivery (Figure 4.8).

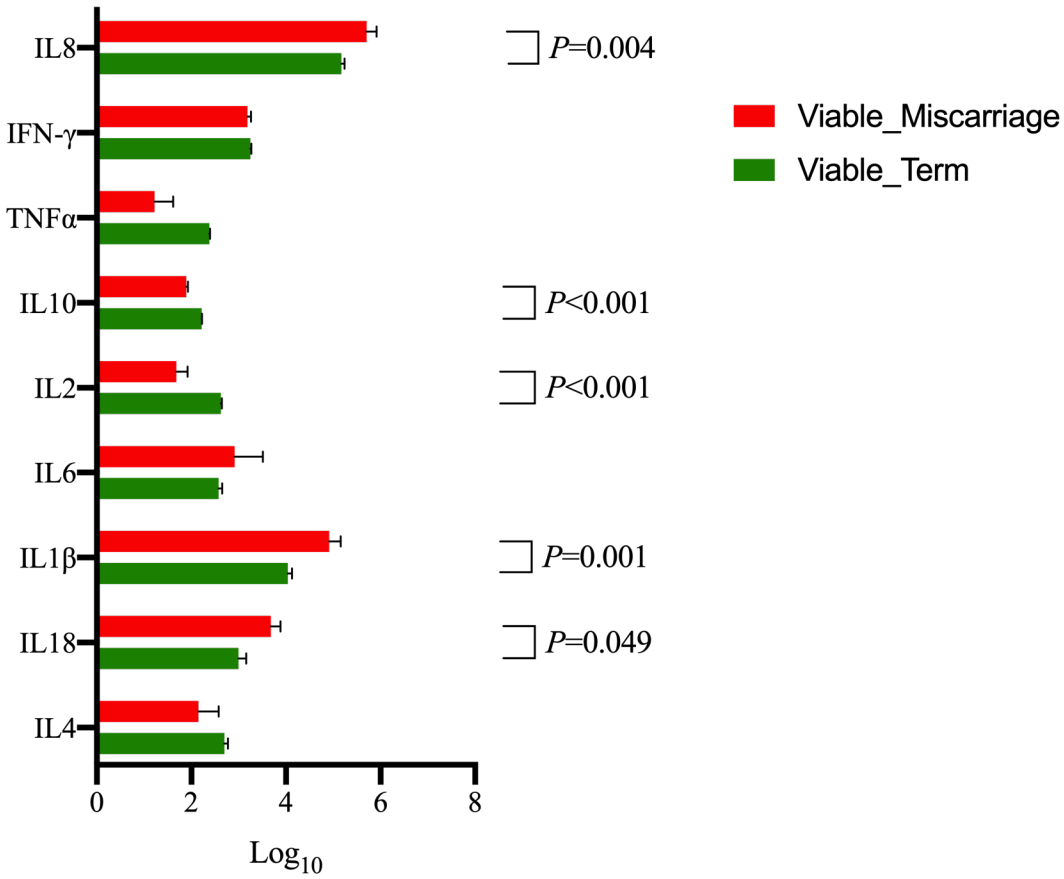

Figure 4.8 Cytokine expression in patients sampled when a viable pregnancy was seen but who subsequently miscarried or had a term pregnancy

N=87, N=14 sampled at viability who went on to miscarry and N=73 sampled at viability who delivered at term. Data represented as a clustered bar chart with mean +/- standard error of the mean. The Mann Whitney U test, two tailed was used to compare unpaired groups for each cytokine.

4.4 Discussion

In the previous chapter data was presented supporting the primary hypothesis of this thesis; that euploid miscarriage is associated with *Lactobacillus* spp. depleted vaginal microbial communities compared to aneuploid miscarriage. In this chapter, we show that high diversity vaginal microbial communities, in particular *Lactobacillus* spp. depleted non-Gardnerella subgroups are associated with higher concentrations of pro-inflammatory cytokines compared to *Lactobacillus* spp. dominated vaginal communities. Moreover, increased levels of TNF-α and IL-1β associated with *Prevotella* spp. dominance and IL6 was linked to *Streptococcus* dominance. In this cohort, *Lactobacillus spp.* depleted vaginal microbiota characterised by enrichment of these potential pathogens was the most significantly vaginal microbiota risk factor for euploid miscarriage and the principal driver of proinflammatory mediators. The vaginal microbiota is an important regulator of innate immune response in the reproductive tract [163]. A growing body of evidence has linked inflammation within the cervicovaginal niche to adverse pregnancy outcomes, particularly in relation to second trimester pregnancy loss and preterm birth [112, 172, 214-217]. Consistent with previous findings in pregnant [163] and non-pregnant women [218, 219], we found that proinflammatory cytokines were elevated in women with high diversity vaginal microbial composition reinforcing the view that *Lactobacillus* spp. depleted vaginal microbiota correlate with local inflammatory activation which during pregnancy, can associate with adverse pregnancy outcome [163].

While high-diversity microbiota was associated with inflammation overall in the studied cohort, it was notable that not all patients with a *Lactobacillus* spp. depleted vaginal microbiome had poor outcomes in this study. Nearly a quarter of these cases went on to have healthy pregnancies that delivered at term. Nonetheless, the local immune profile in these women was different when compared to those with a *Lactobacillus* spp. depleted vaginal microbiome that went on to miscarry. Women with a *Lactobacillus* spp. depleted vaginal microbiome who delivered at term had lower levels of proinflammatory cytokines in early pregnancy compared to those that had euploid miscarriage. There were also significantly increased levels of anti-inflammatory cytokines in *Lactobacillus* spp. depleted samples from viable term pregnancies compared to euploid miscarriage. Collectively these observations implicate both adverse vaginal microbiota composition and specific local host immune responses in early pregnancy with subsequent risk of miscarriage.

In our cohorts increased production of proinflammatory cytokines (IL-6, IL-1β, IL-18 and TNF-α) within the *Lactobacillus* spp. depleted VMGs was associated with dominance by *Prevotella* and *Streptococcus* species in women with euploid miscarriages. As reported in Chapter 3 (Section 3.3.6), these particular species rarely co-occurred with *L. crispatus*, which has been shown to associate with vaginal bacterial stability and low levels of innate immune activation during pregnancy [91, 163]. Therefore, while the underlying microbial structure may be the principal driver, interplay between the vaginal microbiota and is likely an important modulator of host immune response that determines the severity of the inflammatory response and the likelihood of miscarriage.

There are several potential mechanisms by which vaginal microbiota and host immune response could be causally linked to miscarriage. Higher levels of inflammatory cytokines are seen in decidual stromal cells from miscarriages versus normal pregnancy. It is postulated that this excessive inflammation could initiate pregnancy loss via stromal cell apoptosis [159, 160]. Levels of IL-6 and IL-8 in early pregnancy cervical mucus samples prior to miscarriage have also been reported in women who subsequently miscarried compared to those that had a live birth [161]. Inflammatory activation in the cervicovaginal niche is associated with damage to the cervical epithelial barrier [220] which could promote bacterial translocation [221]. Although studies of the endometrial microbiome are confounded by the difficulties of contamination and low biomass, the emerging evidence is that the lower uterine microbiome is distinct from but may be influenced by the vaginal microbiota [83, 222]. In this study, microbiota and cytokine measurements were limited to CVF, thus it is not possible to determine if our observations are reflective of microbiota-host interactions in the endometrial mucosa. Nonetheless, logistical and ethical considerations make direct sampling of the early pregnancy uterine environment difficult. However, the embryological origin of Mullerian duct fusion means that the upper one third of the vagina shares similarities with the endometrial epithelium and therefore a similar proinflammatory response to pathobionts such as *Prevotella and Streptococcus* species would be expected [223, 224].

Lactobacilli confer protection in the vagina by providing antimicrobial defence without initiating innate immune mediated inflammation [66]. Therefore, it is possible in cases of *Lactobacillus* spp. depletion that a proinflammatory environment alters successful implantation, which is a process of tissue injury and repair that is regulated by immune

mediators to allow the trophoblast to breach the decidual lining and invade the maternal decidua [154, 194]. Previous studies have also shown that altered levels of cytokines at the maternal fetal interface can trigger early pregnancy complications [157, 166, 170]. It is therefore plausible that an inflammatory response triggered by the vaginal microbiome could directly or indirectly contribute to dysregulation of the maternal decidua, promoting breakdown of the nascent maternal-fetal interface in early gestation [149].

To determine whether the presence of miscarriage tissue itself induced inflammation we explored the inflammatory mediators in patients who were sampled when a fetal heartbeat was seen but subsequently miscarried. We found a statistically significant increase in IL-1β, IL-18 and IL8 in those patients sampled at viability who eventually miscarried compared to those who had healthy pregnancies. A significantly increased expression of IL-10 was also seen in those that went on to have healthy term pregnancies. Similar patterns of increased inflammation in the miscarriage group and increase anti-inflammatory mediators in the viable group demonstrates that miscarriage itself is unlikely to be driving the microbiota-associated inflammatory response observed in this study. However, it is crucial that future work examines this in detail by collecting pre-miscarriage samples in larger cohorts to eliminate the possibility of miscarriage tissue induced inflammation.

Summary

In summary, the data presented in this Chapter provides evidence that vaginal microbiota depleted of *Lactobacillus* spp. combined with a heightened local inflammatory response, predispose pregnant women to euploid miscarriage. Although this may reflect intrinsic maternal immune response, it appears that the cytokine response is predominately driven by potentially pathogenic bacteria present in the vagina, which offers a therapeutic target for specific, directed intervention prior to conception or in early pregnancy. First trimester miscarriage occurs in 20% of pregnancies and is a major cause of physical and psychological pathology worldwide [50]. Whilst aneuploid miscarriage can be explained in terms of an intrinsic developmental defect, there is currently little evidence to explain the causative mechanisms underlying sporadic euploid miscarriage. There are currently no treatments to prevent this important clinical condition. The data presented here suggests that there is a group of women who would benefit from selective antibiotic, prebiotic and/or probiotic treatment to reduce the risk of miscarriage. Whilst further studies are needed to validate these findings and to understand what specifically triggers an inflammatory cascade in euploid

miscarriage, an important next step will be to explore which interventional regimes might change the vaginal microbiota and positively influence pregnancy outcome.

CHAPTER 5. The early pregnancy placental niche

5 The early pregnancy placental niche

5.1 Background

The existence of a viable microbial community within the placenta is a controversial topic that has generated a great deal of discussion in the scientific community. The long-held belief is that the lower reproductive tract is inhabited by microbes but the cervix is a sufficient barrier to the upper genital tract leading to a sterile fetal environment [225]. However, in recent years several studies have reported the existence of a unique placental microbiota, with particular microbial signatures being linked to adverse pregnancy events [125, 136, 140, 144]. However, given that there is an estimated 10^4 fewer bacteria in the upper reproductive tract compared to the vagina, studies of a putative upper reproductive tract microbiome are highly prone to contamination [226]. The inclusion of carefully matched environmental and kit extraction controls allow for the detection of such potential contaminants and questions regarding the plausibility of apparently detecting bacteria in the placenta that have otherwise only been identified in harsh environments such as soda lakes, oceans and industrial niches [112]. Indeed, several studies using carefully designed negative controls have failed to identify a microbiota within the placenta distinguishable from background sources of potential contamination [129-131]. A summary of potential background contaminants is presented in Table 5.1.

Table 5.1 Examples of potential background and laboratory contaminants

Water and soil associated contaminants [129]		PCR Mastermix contaminants [227]	Water-borne contaminants [228] and [229]
Acinetobacter	*Leifsonia*	*Escherichia/Shigella*	Pseudomonas
Alcaligenes	*Mesorhizobium*	*Pelomonas*	Stenotrophomonas
Bacillus	*Methylobacterium*	*Sphingomonas*	Xanthomonas
Bradyrhizobium	*Microbacterium*		Ralstonia
Herbaspirillum	*Novosphingobium*		Bacillus
Legionella	*Pseudomonas*		*Rhizobium/Agrobacterium*
Ralstonia	*Sphingomonas*		*Sphingomonas*
Stenotrophomonas	*Xanthomonas*		*Burkholderia*
Propionibacterium	Pseudomonas		

Many of the studies that support the concept of a placental microbiota have often solely used metataxanomics. Since the placenta has a role in removing offending organisms there is a risk such sensitive techniques are amplifying transient bacteria, rather than resident bacteria

[124]. Recent work combining bacterial culture with quantitative real time polymerase chain reaction, 16S rRNA sequencing approaches and metagenomics on 29 placental samples taken at caesarean delivery could not find a bacterial signal above background contamination [131]. Moreover, negative culture for microorganisms was reported in 28 out of 29 samples. The same group also investigated whether the amniotic fluid in mice (21 dams) has a microbiota using the same range of techniques. They found *Corynebacterium tuberculostearicum* was more abundant in the amniotic fluid but this was not culturable [225]. In the case of spontaneous preterm birth associated with chorioamnionitis organisms such *Mycoplasma* spp. and *Ureaplasma* spp. that have detected in the placenta [135, 136] however these findings appear to be more consistent with a transient colonisation event leading to infection rather than the detection of a "microbiome". The definition of the microbiome refers to an entire habitat and includes microorganisms, their genes and the surrounding environment. Since the lifespan of the placenta is no more than 9 months, there remains a question as to whether this provides sufficient time for any microbiota to develop a lasting, functionally relevant interaction with the host [52].

A recent systematic review of studies investigating placental microbial composition in healthy pregnancy included 57 studies whereby 33 where identified to be at high risk of bias and which lacked sufficient negative control samples. The review also reported high heterogeneity in the microbiological methods and the sampling process of the placental tissue. However, of the remaining studies with low/moderate bias and adequate negative controls there was a potentially low biomass placental microbiota in healthy pregnancies. Nonetheless, the review concluded that individual studies need to be interpreted with caution [230].

The limited evidence supporting the existence of a placental microbiota is further complicated by the consideration of where such microbiota may originate. Recent evidence suggests there is a continuum of microbial colonisation between the lower and upper reproductive tract [222]. Other work suggests haematogenous spread of microbes given reported similarities between oral and placental microbial communities [125]. In non-pregnant women, there is evidence that the endometrium may harbour a microbiome that is altered in different clinical outcomes [226]. To date, most studies have focused on investigations of term and preterm placentas collected vaginally or by caesarean section. Despite bacterial burden being highest in the first trimester, there exits very little data exploring the placental microbiota in early pregnancy [133].

5.2 Aims

- To determine whether a placental microbiome is detectable in early pregnancy above background contamination.
- To investigate if any early pregnancy commensal microbiota in the placenta are shared between vaginal microbial communities.
- To determine if a distinct placental microbial signature exists in the miscarriage patients which differs to the control group.

Sample collection:

The full details of the methods can be found in Chapter 2. In brief a vaginal swab was collected prior to the surgical management of miscarriage/termination of pregnancy procedure. In a subgroup of patients (n=3) we also collected a swab from the empty suction pot and of the residual material in the sample pot following the tissue evacuation (Figure 5.1). We then washed the placental tissue in normal saline with a view to removing the vaginal organisms potentially carried over in the sample collection process and stored the tissue at -80°C. DNA extraction was performed on 0.25 grams of tissue.

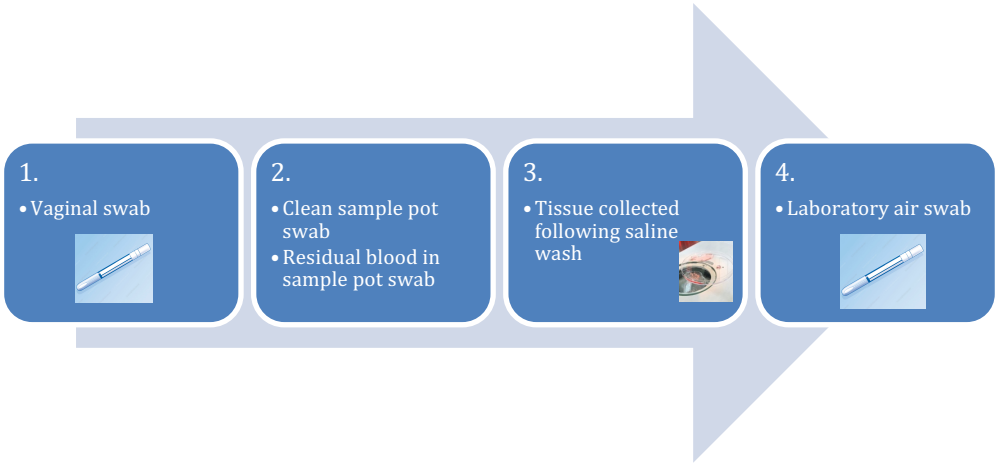

Figure 5.1 Workflow of samples collected

5.3 Results

One hundred patients were recruited for this study, 48 patients had healthy pregnancies with a fetal heartbeat but terminated the pregnancy under Clause C of the 1967 Abortion Act (as amended by the Human Fertilisation and Embryology Act 1990). This group acted as the control cohort. Fifty-one patients were recruited from the early pregnancy unit at the time of miscarriage diagnosis. Molecular cytogenetics was unavailable in 7 cases because of inadequate numbers of villi. Therefore, the total miscarriage cohort consisted of 44 patients; 17 aneuploid miscarriages and 27 euploid miscarriages. Table 5.2 displays the clinical and demographic characteristics of the 92 patients. Each patient had a matched vaginal swab and trophoblast tissue sample collected at the time of surgical management of miscarriage or surgical termination of pregnancy. Thus, a total of 184 patient samples and 19 negative control samples were analysed as part of this work.

5.3.1 Baseline microbial composition

In total 4,651,925 sequence reads were generated from the 184 patient samples with an average of 22,915 reads per sample. In comparison the average read count for the negative control samples was 20. Following removal of singletons and rare operational taxonomic units (OTUs), a total of 882 taxa were identified in the study cohort. To avoid sequencing bias, OTUs were randomly sub-sampled to the lowest read count of 4007 which maintained a minimum coverage of 98.9% (range 98.9%-100%) for all samples. Further analysis was restricted to the top 100 taxa which accounted for 97.8% of the total sequence reads in the dataset.

Table 5.2 Clinical and demographic characteristics of 93 patients included in study cohort

	Miscarriage		Control	p value
	Euploid	**Aneuploid**		
Number of women	27	17	48	
Maternal age, years	33	36	29	
Median (range)	(19-46)	(28-42)	(18-46)	†0.001
BMI (kg/m^2)	26	24	25	
Median (range)	(18-35)	(19-30)	(16-52)	†0.3158
Smokers (%)	3	0	15	
	(11)	(0)	(31)	††0.006
Ethnicity (%)				
White	18 (67)	8 (47)	32 (67)	
Asian	5 (19)	7 (41)	2 (4)	††0.2996
Black	2 (7)	2(12)	11 (22)	
Mixed	2 (7)	0 (0)	3 (6)	
Previous miscarriage (%)				
0	18(67)	11(65)	39 (81)	
1	4(15)	4 (24)	7 (15)	††0.2215
2	2 (7)	2 (11)	1 (2)	
≥3	3 (11)	0 (0)	1 (2)	
Gestational age group at sampling (%)				
5-8 weeks	8 (30)	8 (47)	7 (15)	
8-10 weeks	11 (40)	6 (35)	6 (12)	
10-14 weeks	8 (30)	3 (18)	29(60)	††0.02
>14 weeks	0 (0)	0 (0)	6 (13)	
Bleeding score				
0	9	10	39	
1	6	5	1	
2	5	1	7	††0.001
3	4	0	1	
4	3	1	0	
Status at sampling				
Missed	20	16	0	
Incomplete	7	1	0	
Viable	0	0	49	

† Kruskall-Wallis †† Chi squared Previous miscarriage 0 compared to >1, Ethnicity White versus black, asian and mixed combined, GA 5-8 versus >8, BS 0 versus >1 to ensure chi squared is appropriately used.

5.3.2 Cross sectional comparison of read counts for samples and contamination controls

Given that placental tissue potentially contains a very low microbial biomass, analysis was carried out to ensure that any reads detected in these samples were unlikely to originate from background environment or kit contamination. This included comparison of patient sample microbiota profiles with blank swabs, air swabs from the laboratory environment and air swabs collecting in the operating theatre. One of the negative control samples had an unusually high read count of *Lactobacillus crispatus*. This sample was extracted on the same day as other vaginal samples and it is thought that these results are likely due to cross contamination or mislabelling. Since the microbial composition of this sample was considerably different to all other negative controls, we removed it from further analyses. As shown in Figure 5.2, tissue and vaginal samples had a significantly higher read count compared to the blank controls (P<0.0001). However, tissue samples had a surprisingly high read count that was comparable to vaginal samples.

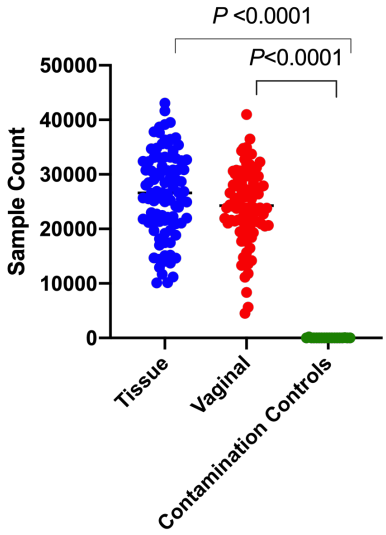

Figure 5.2 Sequencing read counts for samples and contamination controls

P-values obtained using Kruskall-Wallis initially and a post hoc Mann Whitney U.

5.3.3 Comparison of read counts for paired samples and contamination controls

Three patients had additional contamination control samples collected which included a swab of the sample pot residual material which included blood (Figure 5.3). Read counts were similar between tissue, vaginal and pot residue samples. Analysis of microbial composition for patients AT50 and AT51 indicated high compositional overlap between samples and the collection pot residue (Figure 5.3b). Despite washing the placental tissue prior to extraction, there remained a high relative abundance of *Lactobacillus iners* (86%) indicating cross contamination from the vaginal during collection. However, for patient AT52, the vaginal sample was dominated by *Alloscardovia omnicolens* and *Bifidobacterium bifidum*, which was also seen in the sample pot residual, whereas the tissue of the same patient and increased

richness and diversity and contained relatively reduced proportions of *Alloscardovia omnicolens* and *Bifidobacterium bifidum* (7% and 6% respectively).

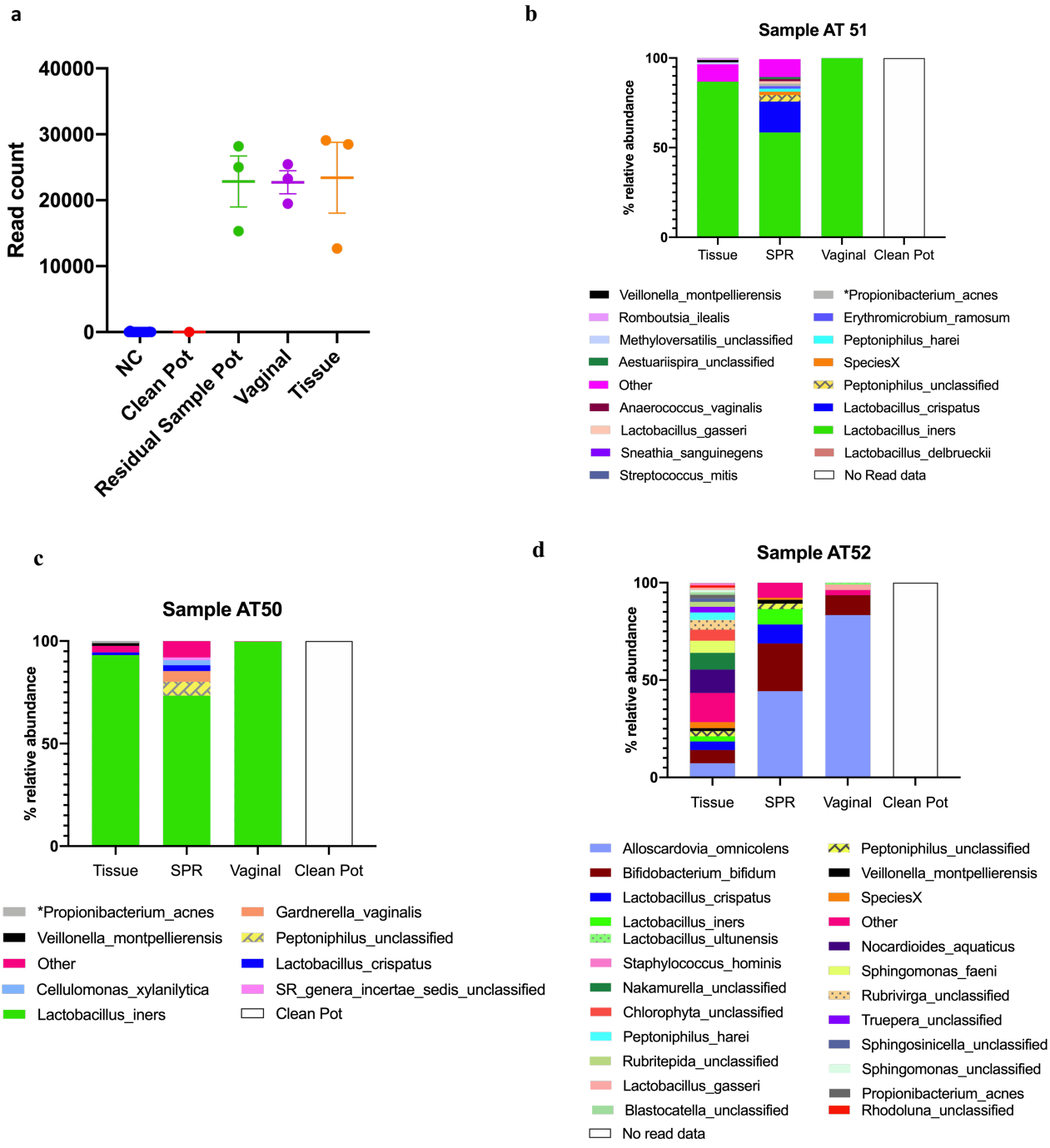

Figure 5.3 Microbial composition in paired samples and contamination controls.

The read count for each sample is shown in Figure 5.3a. The relative abundance for each sample is shown in b-d. Those with a relative abundance <1% were grouped into the other category. The * denotes those organisms that are known contaminants from the current literature.

We next calculated the microbial composition R^2 coevicient for all available paired samples (n=92) using G-test with Yates and two sided Fischer's test (Figure 5.4). A scatter plot of the resulting data shows a wide distribution with 26% (24/92) of samples having an R^2 value below 0.5 and 17% (16/92) between 0.5-0.9 with 57% (52/92) >0.9.

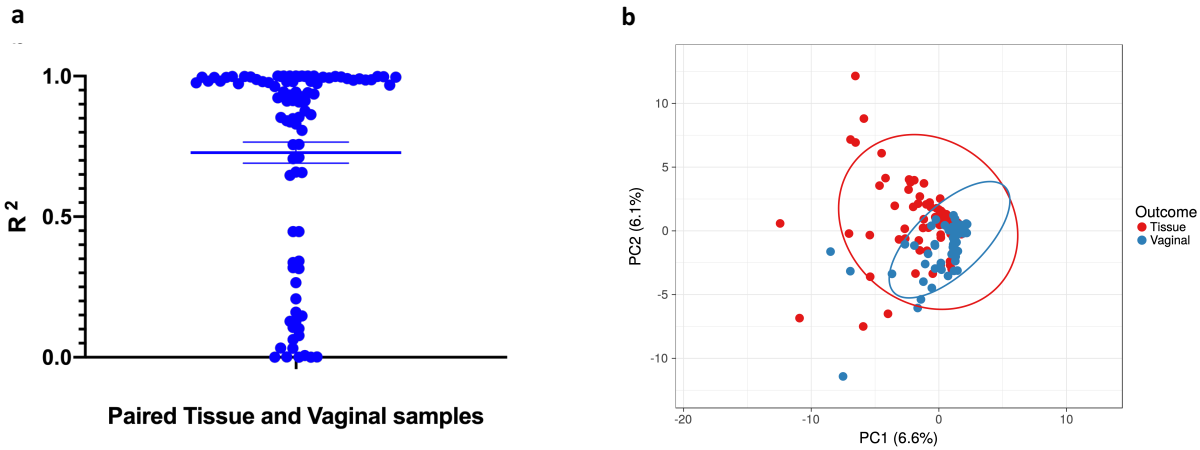

Figure 5.4 Correlation coefficient for paired tissue and vaginal samples (n=92 pairs) (a) and PCA plot for all tissue and vaginal samples (b).

A scatter plot for the R^2 value of paired tissue and vaginal samples is shown in a. The PCA plot (b) demonstrates that while a proportion of tissue samples have a similar composition to the vaginal cohort there is a group that cluster separately.

5.3.4 Microbial composition in samples with a low correlation coefficient

To further explore the differences in the microbial composition between placental tissue and vaginal samples, we restricted the next part of the analysis to only those samples that had a low correlation coefficient R^2 <0.5, N=24. We did not include the high R^2 cases in any further analysis as we were really unable to distinguish the between the vaginal and tissue signatures in those samples. As shown in Figure 5.5, Ward hierarchical clustering analysis confirmed differences in the microbial composition of vaginal and placental samples of these selected samples as indicated by a general clustering of tissue samples towards the righthand side of the heatmap. Initially this tissue sample cluster appeared to have a further subdivision which separated miscarriage control samples. However, further analysis of this data indicated the majority of the samples in this cluster were contaminants as shown by the asterisk in the heatmap and other species that initially appeared to enrich this cluster were low abundance. Therefore we did not identify a clear defining signature in this heatmap.

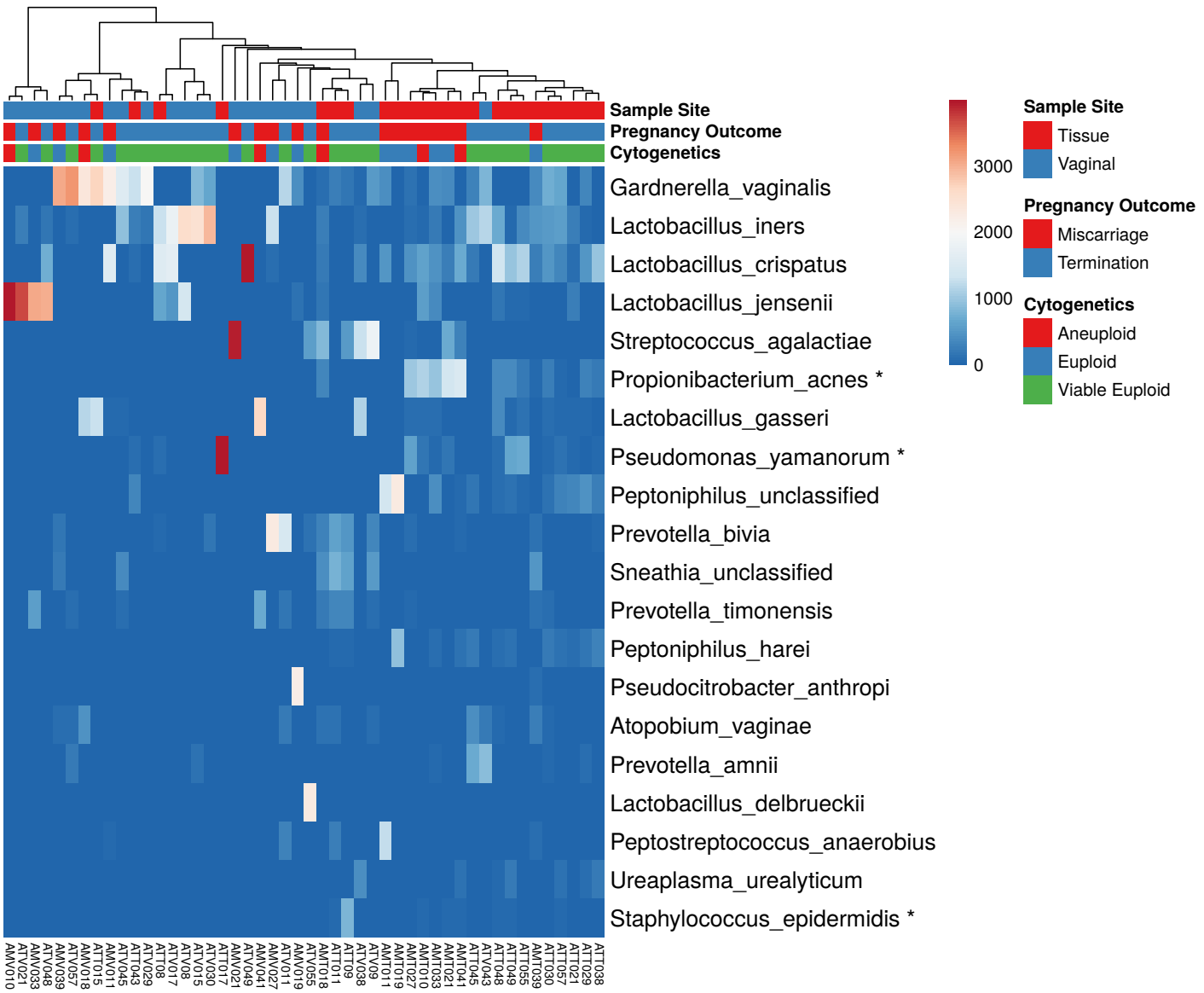

Figure 5.5 Heat map of the tissue and vaginal microbial composition of those with low correlation coefficient.

This heatmap uses Ward hierarchical clustering for the relative abundance at species level for the top 20 most prevalent bacterial species in the cohort of 48 individual samples (24 matched vaginal and trophoblast tissue samples). Each column represents a different sample not necessarily an individual patient. Only those cases with a R^2 value <0.5 were used in this heatmap to ensure key differences between the two body sites were demonstrated. The asterisk * indicates those organisms that are known contaminants from the current literature.

5.3.5 Placental microbial composition and reproductive outcome

The samples that clustered to the tissue group in Figure 5.5 are displayed as stacked bar charts for individual samples with paired tissue and vaginal microbiota data in Figure 5.6 and 5.7. Figure 5.6 shows the termination of pregnancy 'control' cohort and there are a small set of samples that have different composition within the tissue compared to the paired vaginal samples. Individual cases that have a suboptimal vaginal composition (d, e and g) do not appear to sustain these organisms in the paired tissue samples obtained. Figure 5.7 shows the miscarriage cases (7 euploid and 2 aneuploid) and a small proportion of euploid cases with a suboptimal vaginal composition broadly speaking did have a mixed pathogenic colonisation in the paired tissue sample. However, these are very few individual cases and it is difficult to draw conclusions from this data alone.

There were specific species that were associated with the healthy termination of pregnancy cohort compared to the euploid miscarriage. *Ureaplasma urealyticum* (P=0.026), *Megasphaera unclassified* (P=0.031) and *Veillonella montpelliernsis* (P=0.037) were significantly associated with the termination of pregnancy healthy viable cohort (Figure 5.8a). There was also a trend towards increased *Lactobacillus crispatus* and *Lactobacillus iners* in the termination of pregnancy cohort (Figure 5.8 b and c). There was a trend towards an increase in *Peptoniphilus unclassified, Methyloversatilis universalis, Streptococcus infantis* and *Propionibacterium acnes* in the euploid miscarriage cohort (Figure 5.8 d,e,f,g).

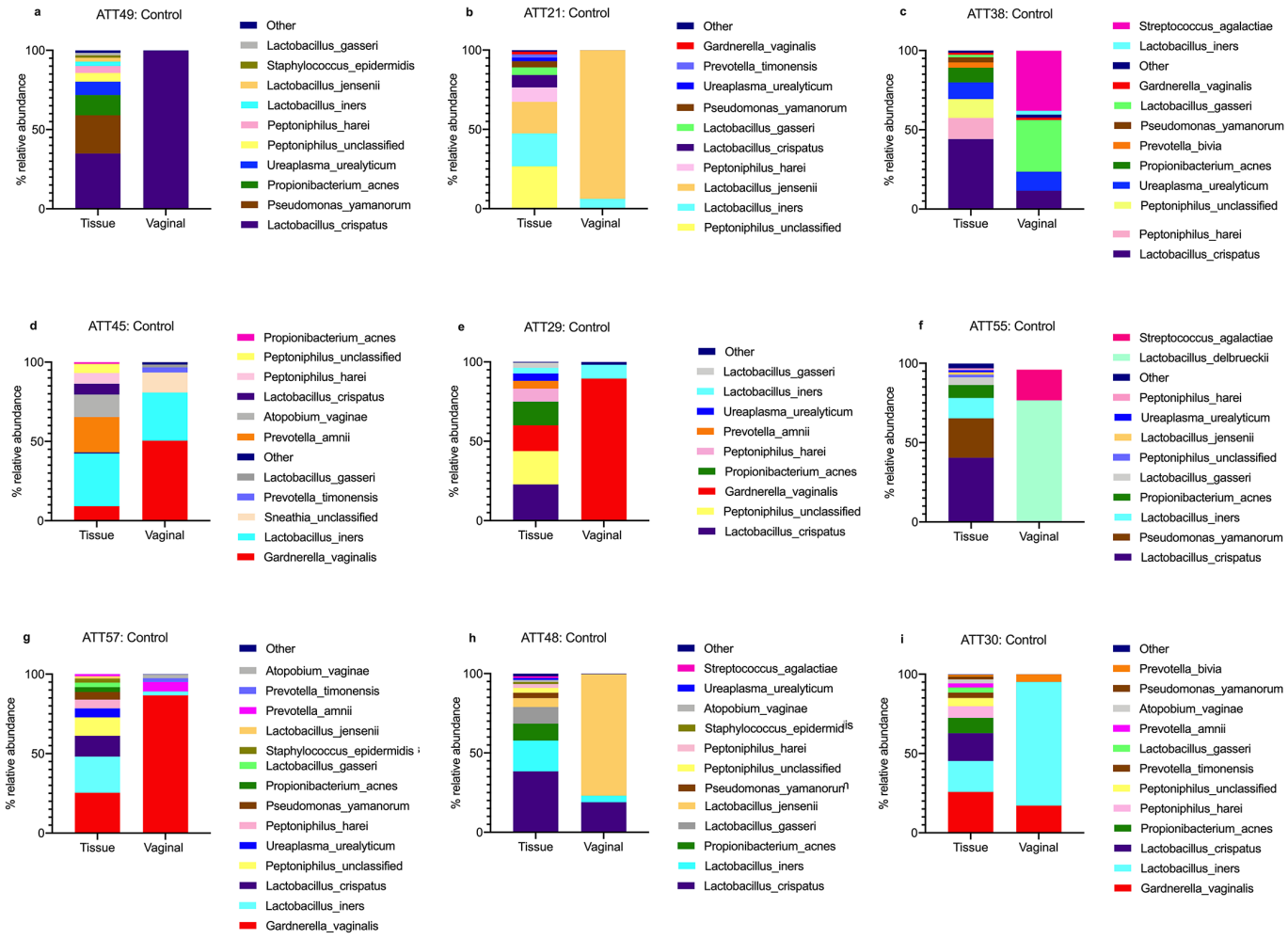

Figure 5.6 Paired tissue and vaginal microbial composition for termination of pregnancy patients

Stacked bar charts using the % relative abundance data for the top 20 bacterial species. Taxa comprising of <1% relative abundance were grouped into a category termed “other “.

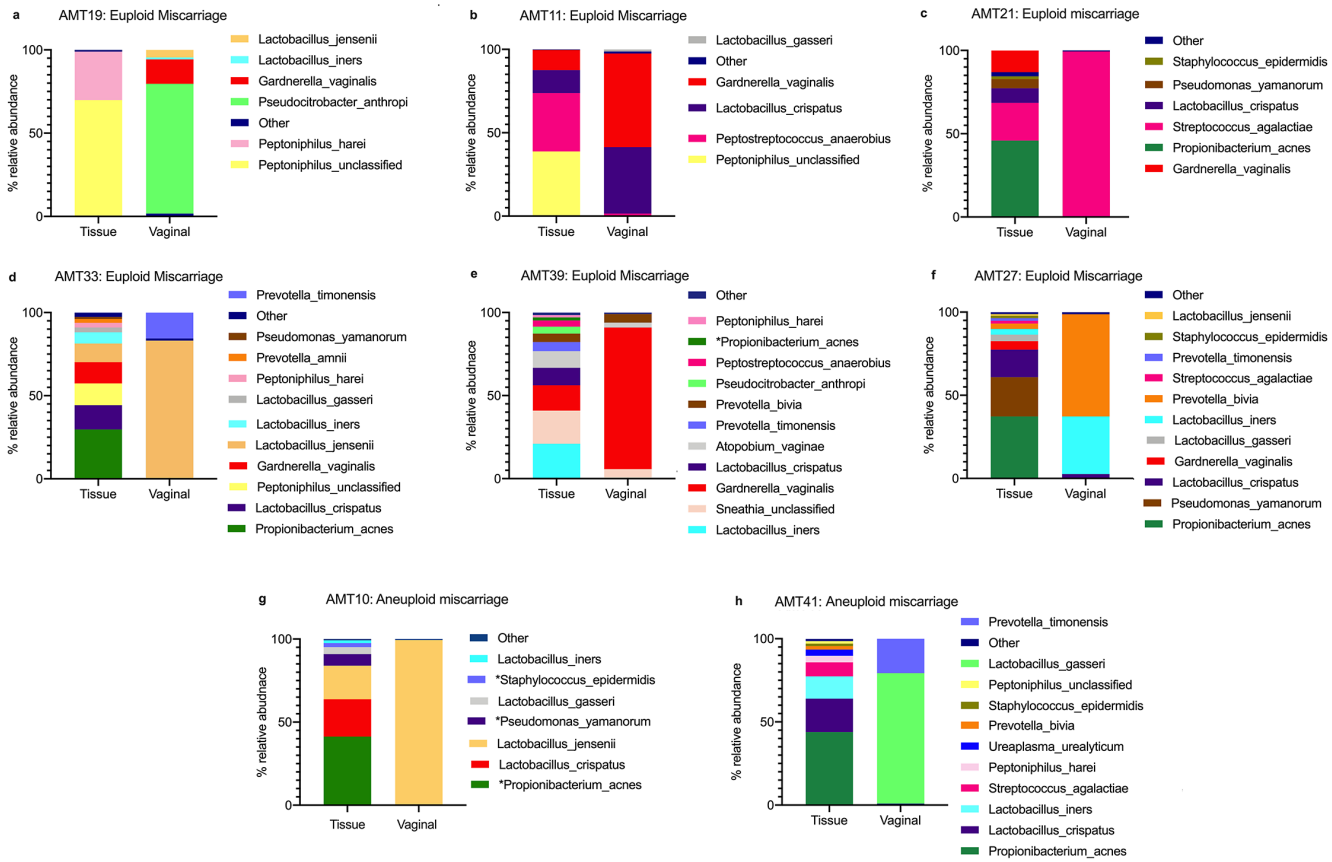

Figure 5.7 Paired Tissue and Vaginal microbial composition in miscarriage patients

Stacked bar charts using the % relative abundance data for the top 20 bacterial species. Those taxa comprising of <1% relative abundance are grouped in the other category.

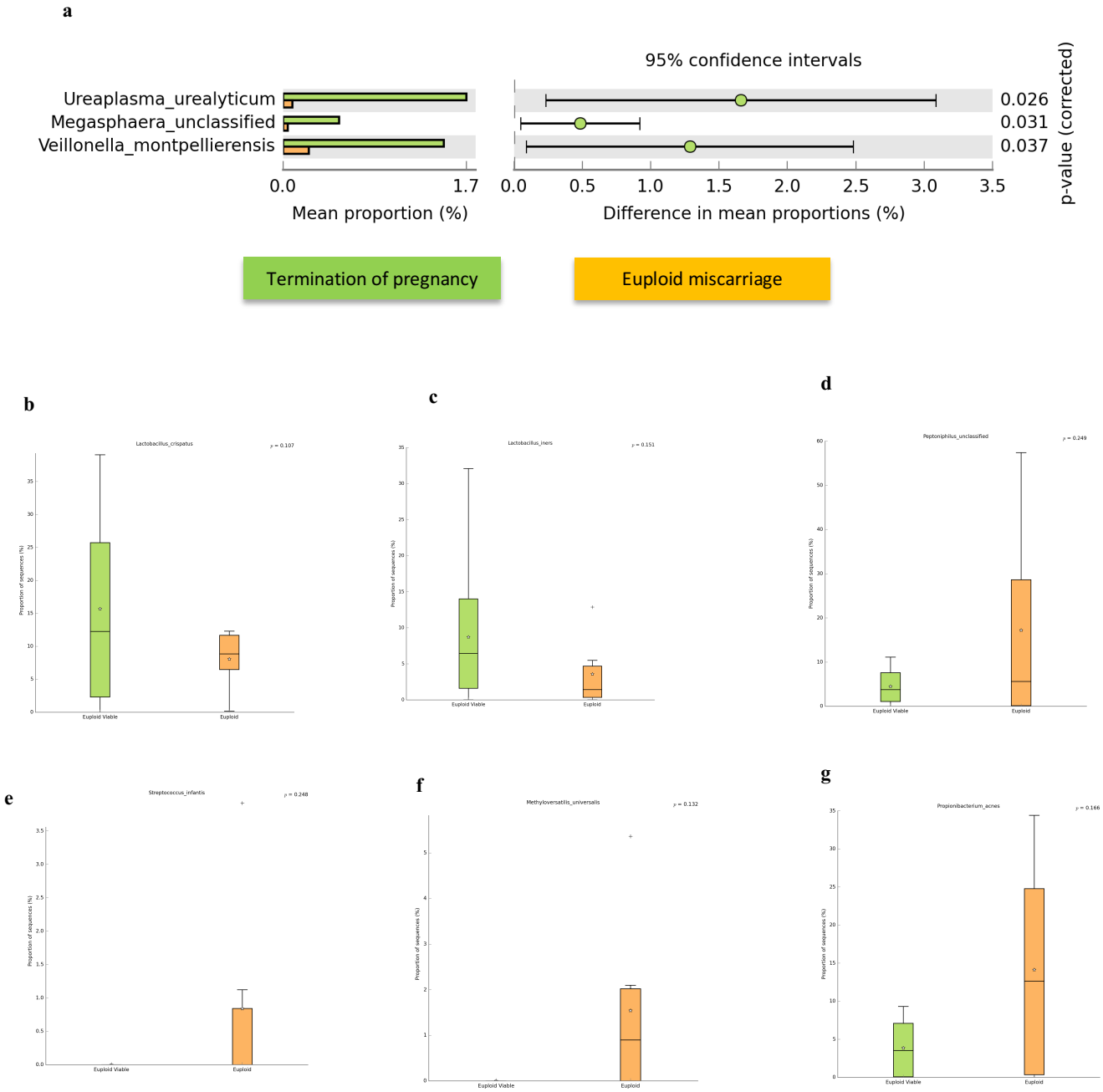

Figure 5.8 Significant species association with tissue samples of euploid miscarriage and viable termination of pregnancies.

Ureaplasma urealyticum, Megasphaera unclassified and *Veillonella montpelliernsis* were significantly more frequently associated with the termination of pregnancy healthy viable cohort compared to euploid miscarriage (a). There was a trend towards increased *Lactobacillus crispatus* and *Lactobacillus iners* in the termination of pregnancy cohort (b and c) although this did not reach significance. There was also a trend (although not statistically significant) towards an increase in *Peptoniphilus unclassified*, *Methyloversatilis universalis, Streptococcus infantis* and *Propionibacterium acnes* in the euploid miscarriage cohort (d,e,f,g). Corrected P-values were calculated using a two-sided Welch t-test.

5.4 Discussion

A criticism of the first papers in the field that purported to describe the discovery of a placental microbiome was the lack of adequate contamination controls. Molecular based methods used for examining microbiota composition are highly sensitive. 16S rRNA derived from the environment and present in extraction kits can be amplified in low microbial biomass samples leading to them erroneously being reported as members of the microbiota. In the context of placental research, studies that have incorporated the use of laboratory and reagent controls, as well as additional techniques to supplemental metataxonomic approaches have generally struggled to provide evidence for a functionally relevant placental microbiome. Regardless, such studies have been largely limited to placenta collected at the time of delivery. This chapter aimed to undertake a pilot study of the early pregnancy placental niche in a well characterised cohort of miscarriages, with known cytogenetic information and a control cohort, with matched vaginal microbiome samples.

The bacterial load of the early placental samples collected was significantly higher compared to background laboratory and operating theatre controls. However, these bacterial load data were substantial higher compared samples of placenta collected at the time of preterm or term delivery [132, 231]. This highlighted a potential risk of cross contamination of the placental samples with the vagina during collection. Unfortunately, collection of these sample types from patients undergoing surgical management of miscarriage and termination of pregnancy cases is only possible vaginally. Studies of the placental microbiome at later gestational ages can avoid this issue through sampling of tissues collected during caesarean section. Studies of the endometrial microbiome in fertility patients have also reported issues of carry-over from the high biomass vagina to the low-biomass endometrium during sampling. However, most of these studies have not collected matched vaginal samples and have therefore not been able to investigate this further [226].

Here, were tried to reduce the potential carry over of vaginal microbiota to the placental samples by washing the tissue in sterile saline after sample collection. Evaluation of the correlation coefficient between each paired sample and removed those with a high correlation coefficient to ensure the data analysed was purely within the tissue samples and minimised cross contamination.

We observed slightly different microbiota patterns between euploid miscarriage patients and termination of pregnancy patients. Those with euploid miscarriages that had a suboptimal vaginal microbial composition also had colonisation of the same organisms or similar pathobionts (*Sneathia spp., Gardnerella Vaginalis, Peptostreptoccocus spp., Streptococcus agalactiae*) in the matched tissue sample. However, in the control group those with adverse taxa in the vaginal samples did not have similar organisms in the matched tissue samples. These results must be interpreted with caution as they only represent small pairwise comparisons of individual cases and there is relatively low overall abundance of the particular taxa. However, it is possible that in euploid miscarriage certain adverse taxa colonise the vaginal niche and ascend into the tissue. However, in a control cohort these particular organisms stay within the vaginal niche and do not ascend into the tissue.

We investigated whether particular bacterial taxa in the tissue were associated with different pregnancy outcomes. *Ureaplasma urealyticum, Megasphaera unclassified* and *Veillonella montpelliernsis* were significantly associated with the control healthy viable cohort. Interestingly, these organisms have been linked to bacterial vaginosis and in some cases adverse pregnancy outcome [232-234]. However, the isolated presence of these organisms is not linked to pathogenicity. Nonetheless it is unusual to see such taxa in a supposed control population. This may be explained by certain demographic differences between the termination cohort and miscarriage cohort. The termination group consisted of unplanned pregnancies and higher smoking status, both have been shown to alter the vaginal microbial composition which could influence tissue composition [235, 236]. These cases are presumed to be healthy control cases but they were terminated within the first trimester therefore complications may have arisen at a later gestation. Nonetheless this is the best control cohort we had access to given the ethical concern of invasive tissue sampling in the first trimester of healthy pregnancies.

In a recent systematic review of placental microbiome studies the following genera were found at elective caesarean sections: *Prevotella*, *Lactobacillus, Streptococcus*, *Neisseria*, *Veillonella*, *Methylobacterium*, *Fusobacterium*, *Escherichia*, *Sneathia* and *Ralstonia.* Whereas the extraction blanks controls found *Propionibacterium* (~16 %), *Streptococcus* (~13 %), *Methylobacterium* (~12 %), *Prevotella* (~10 %), *Staphylococcus* (~5 %), *Tumebacillus* (~4 %) and *Corynebacterium* (~2 %) [230]. We have shown that there was a trend towards an increase in *Peptoniphilus unclassified*, *Methyloversatilis universalis, Streptococcus infantis* and

Propionibacterium acnes in the euploid miscarriage cohort. However, further investigation has questioned the significance of these particular taxa. *Methyloversatilis* and *Propionibacterium* have been shown to be likely laboratory contaminants [129] and *Streptoccocus infantis* is also likely to be a contaminant as it is found in the upper respiratory tract [237]. However, some new data suggests *Peptoniphilis* may be associated with bacterial vaginosis [238]. Recent work has focused on using a combination of methodologies to assist in detecting organisms within the placenta. Fluorescent in situ hybridization (FISH) is a tool used to visualise particular bacterial species within the placental tissue. A study recently designed FISH probes to detect Ralstonia insidiosa within the basal plate of human placental samples. Given the high prevalence of R. insidiosa in waterborne environmental bacterium it is often considered a contaminant originating from the DNA extraction kits often called the 'kitome'. However, this study demonstrated that R. insidiosa was visualised in human placental basal plate biopsies by FISH. Ex vivo work in the same study also found R. insidiosa was able to avoid inflammation and cell death and did not induce preterm birth in the mouse model [239]. Therefore, certain laboratory containments may require further investigation by other microbiological techniques before they are dismissed as being part of the 'Kitome'.

A plausible origin for the placental niche is the cervical- vaginal environment. Work by Chen et al has suggested that there is a continuum of organisms between the lower and upper reproductive tract [222]. Work has also shown that patients with bacterial vaginosis have a higher chance of presenting with an endometrial *Gardnerella vaginalis* biofilm. Vaginal contamination in this case was not considered an issue given the strong adherence of the biofilm to the endometrial tissue seen using FISH [240]. Given a large proportion of patients had a high correlation coefficient between the vaginal and tissue samples there is a possibility that the origin of taxa within the tissue is from ascending vaginal bacteria. However, we need corresponding FISH techniques to confirm visualisation within the placental tissue itself to confidently exclude cross contamination.

Strengths and Limitations

We have a unique cohort of patients with matched vaginal and tissue samples collected at the time of miscarriage diagnosis with known cytogenetic information. Our control cohort consisted of patients undergoing a termination of pregnancy under Clause C that had a fetal heart at a comparable gestation. The analysis is robust as all sample types were sequenced with the same 16S rRNA hyper variable regions and analysed using the same pipeline.

Interpretation of these results needs to be with caution as DNA contamination can confound the microbiome data. We accounted for this by comparing the tissue samples to the read count of the blank contamination controls. All of the tissue samples clustered above the blank amplicon concentration. We accounted for the potential cross contamination from the low biomass uterine cavity through the high biomass vagina by eliminating those patients with a correlation coefficient >0.5.

The main limitation of our work was the route of sample collection as it was difficult to decipher what was truly present within the tissue and what was carry over from the vagina. We did have a strategy to eliminate those potential contaminants but this restricted our sample size. There were also differences in the demographics between the miscarriage and termination cohorts. The termination group were more commonly sampled at 10-14 weeks whereas the miscarriage group were sampled at 8-10 weeks. This is most likely because patients presenting earlier in the pregnancy would likely be offered medical termination of pregnancy. We did not use quantitative PCR (qPCR) in this cohort of patients because there was a chance of vaginal cross contamination and therefore the read count would not have been useful. However, in future work it would be important to understand bacterial load in samples collected abdominally. In terms of data analysis, we used Operational taxonomic units (OTUs) which group together sequences that have >97% similarity. This is a well-recognised method but recent work has processed raw sequence reads using the DADA-2 package and used amplicon sequence variants (ASVs). This does not combine taxa and therefore allows for higher resolution of the dataset [241]. Although the consistent use of the V1-V2 primers allowed comparisons to be made across vaginal and tissue samples, other studies have shown that these primers could overlook certain organisms that are present in the placental tissue [143].

Future directions

The route of sample collection can be overcome by collecting excess chorionic villi samples from patients undergoing an abdominal chorionic villi sampling between 10-14weeks gestation. We currently have a collaboration set up with Professor Anna David at University College London to utilise excess tissue collected for clinical purposes. The main concern in this cohort is that a clinical diagnosis is the priority and the sample preparation needs to be carefully considered for the microbiome work. However, this could avoid the cross contamination from the vagina and help understand what is present within the tissue itself.

Quantitative PCR can then be performed to understand the copy numbers of particular bacterial taxa and FISH can be carried out to understand which organisms are penetrating the tissue itself. FISH could also be carried out on the current sample set to delineate whether organisms seen within the vagina are present within the tissue. This would help us understand whether cross contamination was a factor in the results.
Mechanistic work is also key to understanding if a low biomass microbiota in the placental niche interacts with the immune system and how such organisms are functionally relevant to pregnancy outcomes.

In conclusion the work in this chapter is a pilot study for future exploration of the early pregnancy placental niche. We have shown that there is a bacterial signal above contamination controls and a different microbial pattern is seen in matched vaginal and tissue samples from miscarriages and control patients. However, the process of sample collection needs to be addressed, a range of microbiological techniques should also be employed including qPCR, FISH and metagenomics to expand our knowledge on this interesting and controversial topic.

CHAPTER 6. The relationship between the rectal microbiota and early pregnancy outcomes

6 The relationship between the rectal microbiota and early pregnancy outcomes

6.1 Background

There is now substantial evidence that the gut microbiota plays an important role in various aspects of human host physiology including metabolism, nutrition and modulation of the immune system [242, 243]. The healthy gut microbiome consists of a diverse community of species from the following 4 phyla: Bacteriodetes, Firmicutes, Actinobacteria and Proteobacteria [242]. This is predominately made up by >90% Firmicutes and Bacteroidetes, with Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia to a lesser extent [244]. Gut microbiome homeostasis is associated with the Firmicutes/Bacteroidetes ratio (F/B ratio). A higher ratio is associated with pathological conditions and gut dysbiosis [245]. Gut microbiota derived metabolites such as short chain fatty acids and in particular butyrate, produced by a healthy gut microbiome contribute to intestinal integrity and immunological homeostasis [242]. Short chain fatty acids (SCFAs) lower the pH of the colon and support epithelial barrier integrity by stimulating mucus production and antimicrobial peptides. A gut microbiota dominated by Bacteroidetes, Bifidobacterium, Lactobacillus and deficient in Firmicutes and Prevotella express more SCFAs contributing to gut health [244, 246]. Therefore, an increase in the F/B ratio reduces the amount of SCFAs and leads to LPS induced inflammatory mediators being released. Additionally, microbially-derived metabolites can penetrate the epithelial barrier and enter the circulatory system where they can be detected by host immune cells and modulate immune cell function [243].

Gut dysbiosis can lead to immune dysregulation and disease processes such as inflammatory bowel disease (IBD). This dysbiosis is not commonly caused by a single pathogen, but rather several pathobionts which acquire pathogenic roles in a genetically predisposed individual as a result of environmental or dietary factors. Specific compositions have been found in IBD patients such as increases in Proteobacteria and a depletion in Bacteroidetes. A reduced number of Clostridiales, Ruminococcaceae, Faecalibacterium and Roseburia have also been shown in IBD patients. Many of these microorganisms such as Roseburia, Ruminococcaceae and Faecalibacterium are all butyrate producing organisms and involved in the metabolism of SCFA which are key in anti-inflammatory activity [247].

The role of the gut microbiota in pregnancy remains poorly elucidated. While several studies have found the bacterial communities in the gut remain stable throughout pregnancy [208, 248], a larger study exploring the changes in the gut microbiota of 91 pregnant women found an expansion in diversity, reduction in richness and increase in Proteobacteria and Actinobacteria from the first to third trimester. However, this study used a range of BMIs and varying gestational diabetes status, which impact on the microbiota. Furthermore, when the first trimester gut microbiota was mapped against 93 non-pregnant women from the human microbiome project dataset there was no difference in beta diversity [120].

Studies have demonstrated the crosstalk between the gut and vaginal microbiota by showing a correlation between species composition as well as strain identity [249, 250]. The role of vertical transmission has also been considered as studies have shown the mode of delivery effects the neonatal intestinal colonization. Babies born via caesarean section have lower numbers of Bifidobacteria and Bacteroides compared to vaginally born infants [251, 252]. However, studies have also reported no correlation between the Lactobacillus flora in the rectum and the vaginal flora in reproductively aged women and postmenopausal women [253]. Recent studies reporting that oral probiotics administered in early pregnancy do not modify the vaginal microbiota further brings into question the influence of the gut microbiota on the vaginal composition [114, 115].

Aside from proximity another link between the gut and vaginal microbial niche is via oestrogen metabolism. The gut microbiome is one of the principal regulators of circulating oestrogens through the secretion of the enzyme β-glucuronidase, which deconjugates oestrogen into its active form. As previously discussed in Chapter 3, oestrogen increases the availability of glycogen and its breakdown products in the cervicovaginal mucosa which provide key energy sources for *Lactobacillus* species. Therefore changes in the gut microbiome could mediate circulating levels of oestrogen and thereby influence the health of the local cervicovaginal environment [254].

Stool samples are considered the gold standard for sample type when undertaking studies of the gut microbiome, however, due to practical and logistical issues alternative methods such as rectal swab sampling has been explored. Although some studies have found differences in the taxa identified with such techniques there are also inter-individual differences [255]. Overall richness and evenness as well as community structure between rectal swab and stool samples from the same individuals is similar [256, 257]. Accordingly, rectal swabs are

considered a satisfactory proxy of the gastrointestinal environment, especially for examining the potential of seeding between the rectal and vaginal niches.

Data presented in Chapters 3 and 4 indicate that vaginal microbiota depleted of *Lactobacillus* spp. combined with a heightened local inflammatory response, predispose pregnant women to euploid miscarriage. Although this may be due to an intrinsic maternal immune response, it is likely driven by specific vaginal taxa. However, given that around a quarter of women with vaginal *Lactobacillus* spp. depletion continue to have healthy term pregnancies and an anti-inflammatory cytokine profile, an alternative pathway for immune modulation may be at play. Therefore, the work described in this Chapter set out to explore the cross talk between the rectal and vaginal niches as well as the localised immune profile.

6.2 Aims

1. To determine if the rectal microbiota correlates with paired vaginal samples.

2. To characterise the rectal microbial composition in chromosomally normal and abnormal miscarriages as well as healthy controls.

3. To investigate the link between cervicovaginal inflammation and gut dysbiosis.

6.3 Results

One hundred patients were recruited for this study, 49 patients had healthy pregnancies with a fetal heartbeat but terminated the pregnancy under Clause C of the 1967 Abortion Act (as amended by the Human Fertilisation and Embryology Act 1990). This group acted as the control cohort. Fifty-four patients were recruited from the early pregnancy unit at the time of miscarriage diagnosis. Chorionic villi were unavailable for cytogenetic analysis in 7 cases and therefore the final miscarriage cohort was 47 (18 aneuploid miscarriages and 29 euploid miscarriages). Table 6.1 displays the clinical and demographic characteristics of the 96 patients. Each patient had a matched vaginal swab and rectal swab sample collected at the time of surgical management of miscarriage or surgical termination of pregnancy. Therefore, the total number of samples analysed was 192 (49 control patients and 47 miscarriage patients with matched rectal and vaginal swabs.

Table 6.1 Clinical and demographic characteristics of 96 patients included in study cohort

	Miscarriage		Control	p value
	Euploid	Aneuploid		
Number of women	29	18	49	
Maternal age, years	33	36	29	
Median (range)	(19-46)	(28-42)	(18-46)	†0.0004
BMI (kg/m^2)	26	24	25	
Median (range)	(18-35)	(20-39)	(16-52)	†0.51
Smokers (%)	3	0	16	
	(10)	(0)	(33)	††0.003
Ethnicity (%)				
White	18 (62)	9 (50)	33 (67)	
Asian	7 (24)	7 (39)	2 (4)	††0.4
Black	2 (7)	2(11)	11 (22)	
Mixed	2 (7)	0 (0)	3 (6)	
Previous miscarriage (%)				
0	19(66)	11(61)	40 (82)	
1	4(14)	4 (22)	7 (14)	††0.1
2	2 (6)	3 (17)	1 (2)	
≥3	4 (14)	0 (0)	1 (2)	
Gestational age group at sampling (%)				
5-8 weeks	8 (28)	8 (44)	7 (14)	
8-10 weeks	12 (41)	6 (33)	6 (12)	
10-14 weeks	9 (31)	3 (22)	29(60)	††0.03
>14 weeks	0 (0)	0 (0)	7 (14)	
Bleeding score				
0	11	10	40	
1	6	6	1	
2	5	1	7	††0.0004
3	4	0	1	
4	3	1	0	
Status at sampling				
Missed	22	17	0	
Incomplete	7	1	0	
Viable	0	0	49	

Rectal swabs and paired vaginal samples † Kruskal-Wallis †† Chi squared
Previous miscarriage 0 compared to >1, Ethnicity White versus BAME, GA 5-8 versus >8, BS 0 versus >1.

Maternal age was significantly higher in the aneuploid miscarriage group (*P*=0.0004). Gestational age at sampling was categorized as 5-8, 8-10, or 10-14 weeks post last menstrual period (LMP). There were no significant differences in gestational age at the time of sampling between aneuploid and euploid miscarriage group, but the control pregnancy group were sampled significantly later (*P*=0.03). Bleeding scores were similar between euploid and aneuploid miscarriage groups and higher bleeding scores were more common in the

miscarriage groups compared to the control pregnancy group (*P*=0.0004). There were significantly more smokers in the control cohort (*P*=0.003). There were no significant differences in BMI or ethnicity between the groups.

6.3.1 Baseline vaginal and rectal microbiota composition

In total 4,186,264 sequence reads were obtained from 192 samples (96 vaginal swabs and 96 rectal swabs) with an average of 21,803 reads per sample. Following removal of singletons and rare operational taxonomic units (OTUs), a total of 881 taxa were identified. To avoid sequencing bias, OTUs were randomly sub-sampled to the lowest read count of 4007 which maintained a minimum coverage of 99% (range 96.8 %-100%) for all samples. Further analysis was restricted to the top 100 taxa which accounted for 93% of the total sequence reads in the dataset.

6.3.2 The relationship between the vaginal microbiota and paired rectal microbiota samples

The relationship between paired vaginal and rectal samples was analysed using principal component analysis (PCA). Figure 6.1 demonstrates different clusters relating to the sample site at phylum level (a) and genera level (b), illustrating key differences in the composition.

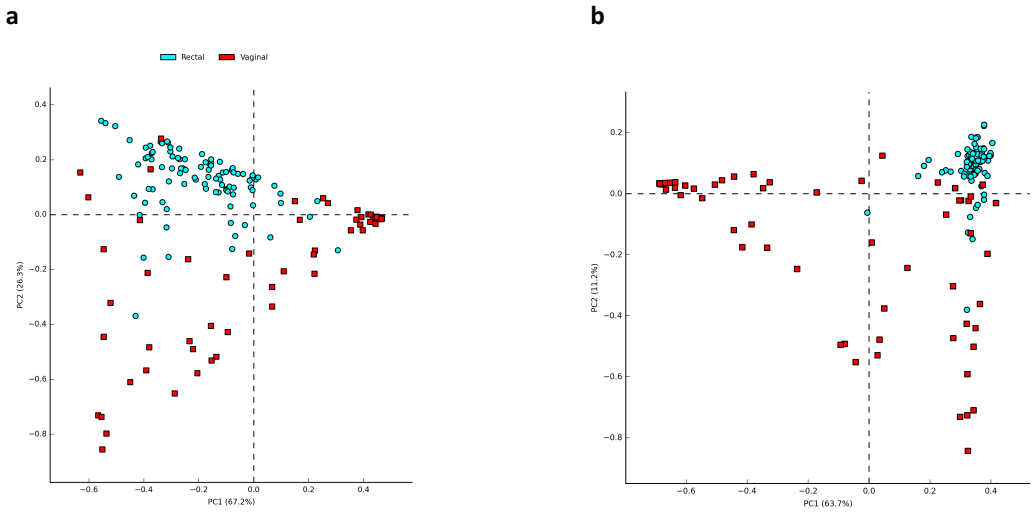

Figure 6.1 Sequence data at phylum (a) and genera (b) level in paired vaginal and rectal samples

Principal component analysis of the sequence data at phylum and genera level in paired rectal and vaginal samples. Vaginal shown as red and Rectal shown as blue

Significantly high diversity (a) and richness (b) metrics were observed in rectal samples compared to paired vaginal samples (Figure6.2).

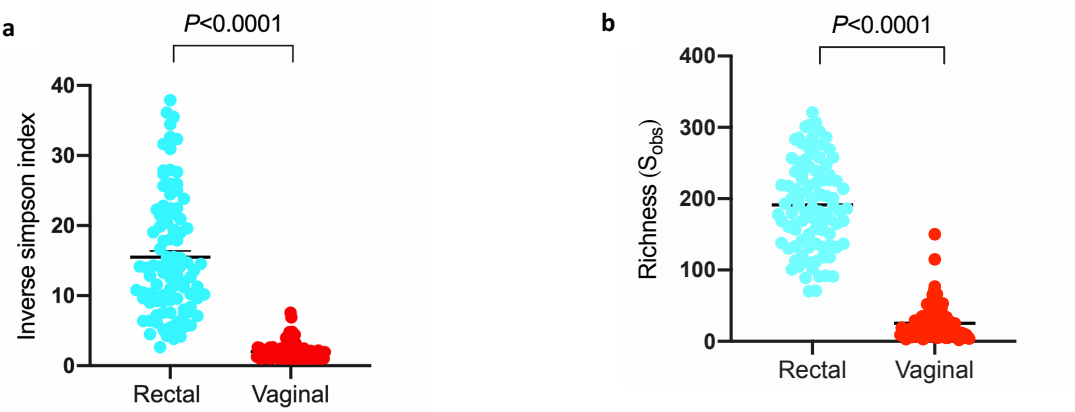

Figure 6.2 Diversity (a) and Richness (b) indices associated with different sample sites.

Dots in a and b are individual cases and data is presented as mean and standard error of the mean. The Mann-Whitney U test was used to compared two groups. S_{obs}: Species observed.

Correlation analysis of the paired rectal and vaginal samples at genera level also indicated major compositional differences as indicated by a low R^2 value of 0.003 (Figure 6.3).

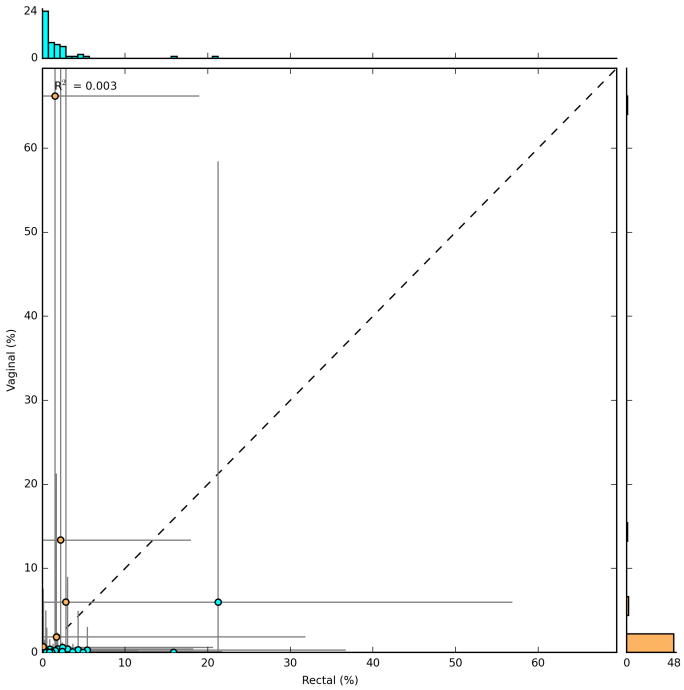

Figure 6.3 Correlation between rectal and vaginal sample composition at genera level

We used the 2-group comparison between composition at genera level and the correlation was 0.003 (Welch's t-test).

6.3.3 Gut microbiota composition in the context of pregnancy outcome

No significant difference in richness (*P*=0.46) or diversity (*P*=0.8) of the gut microbiota composition between the overall miscarriage cohort and the control cohort was observed (Figure 6.4 a and b). Likewise, principal component analysis also did not reveal any distinct clusters at genera or species level indicating overall compositional structure was similar between patient cohorts (Figure 6.4 c and d).

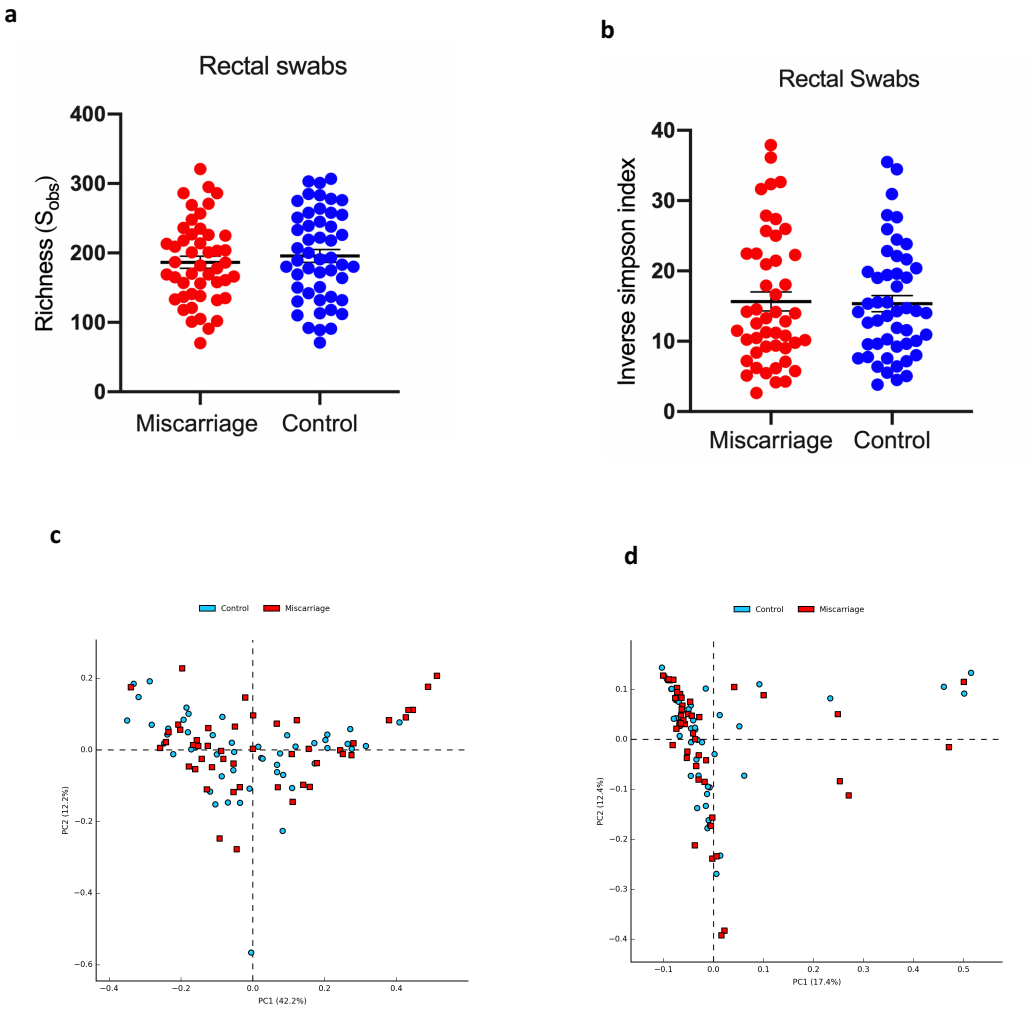

Figure 6.4 The gut microbiota in relation to healthy pregnancy and miscarriage

Richness (a) and Diversity (b) in miscarriage patient compared to controls are not significantly different (*P*=0.46, *P*= 0.86 respectively, Mann-Whitney U test). Principal component analysis comparing control and miscarriage patients is displayed at genera (c) and species (d) level. The individual dots in graphs a and b represent different patients and the data is shown as the mean and standard error of the mean.

We next separated the miscarriage cohort based on cytogenetic information. No differences in diversity or richness were observed between aneuploid or euploid miscarriages or viable euploid pregnancies (Figure 6.5 a and b).

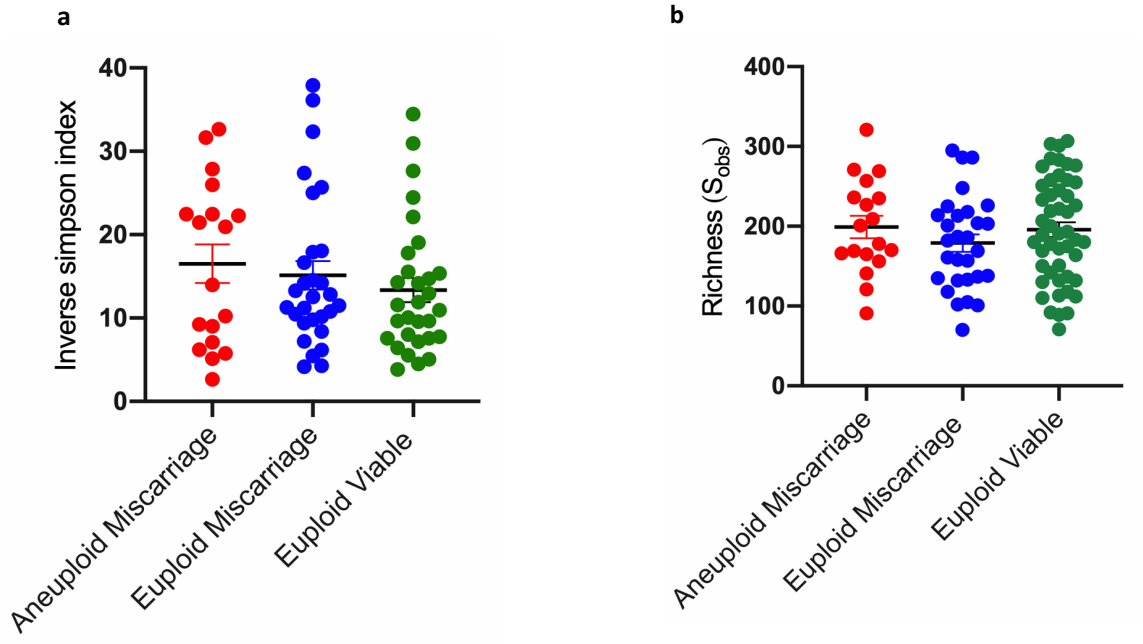

Figure 6.5 The gut diversity and richness indices for different reproductive outcomes

Diversity and richness are not significantly different in the different pregnancy groups, *P*=0.9 and *P*=0.46 using Kruskal-Wallis test in a and b respectively. The individual dots represent different patients and the graph shows the mean and standard error of the mean.

6.3.4 Univariate analyses of rectal taxa in the context of different pregnancy outcomes

Comparison of relative abundances of rectal species between different pregnancy outcome groups revealed a number of significant differences. *Anaerococcus vaginalis* (*P*=0.042), *Anaerosphaera* spp. (*P*=0.041), *Finegoldia magna (P*=0.009) and *Prevotella bivia* (*P*=0.017) were significantly more abundant in euploid miscarriages compared to aneuploid miscarriages. *Blautia luti* (*P*=0.029) and *Clostridium XIVa* spp. (*P*=0.039) were significantly more abundant in aneuploid miscarriages compared to euploid miscarriages (Figure 6.6 a)

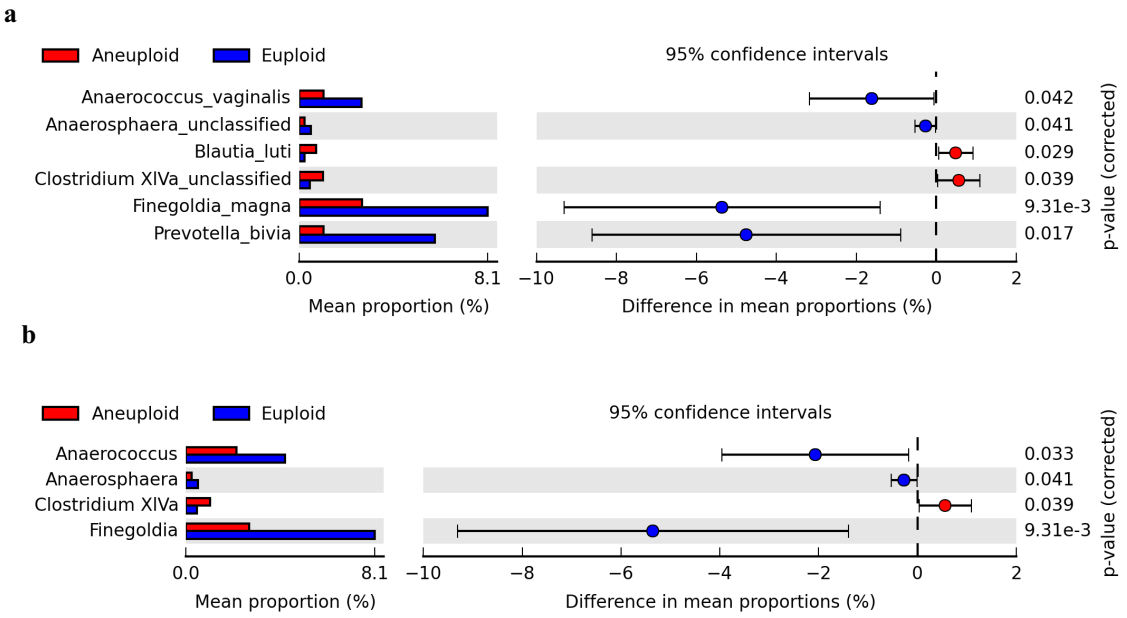

Figure 6.6 Significant rectal taxa associated with euploid and aneuploid miscarriage at species (a) and genera (b) level.

The following genera, Anaerococcus and Finegoldia were associated with euploid miscarriage compared to aneuploid miscarriage. Corrected *P* values were calculated using two-sided Welch's t-test.

We next compared the rectal microbiota in the euploid miscarriages to the control cohort (euploid viable). Relative abundance levels of *Blautia* spp. (*P*=0.02), *Holdemanella* spp. (*P*=0.007) and *Oscillibacter* spp. (*P*= 0.037) were significantly higher in the control cohort compared to the euploid miscarriages (Figure 6.7 a and b).

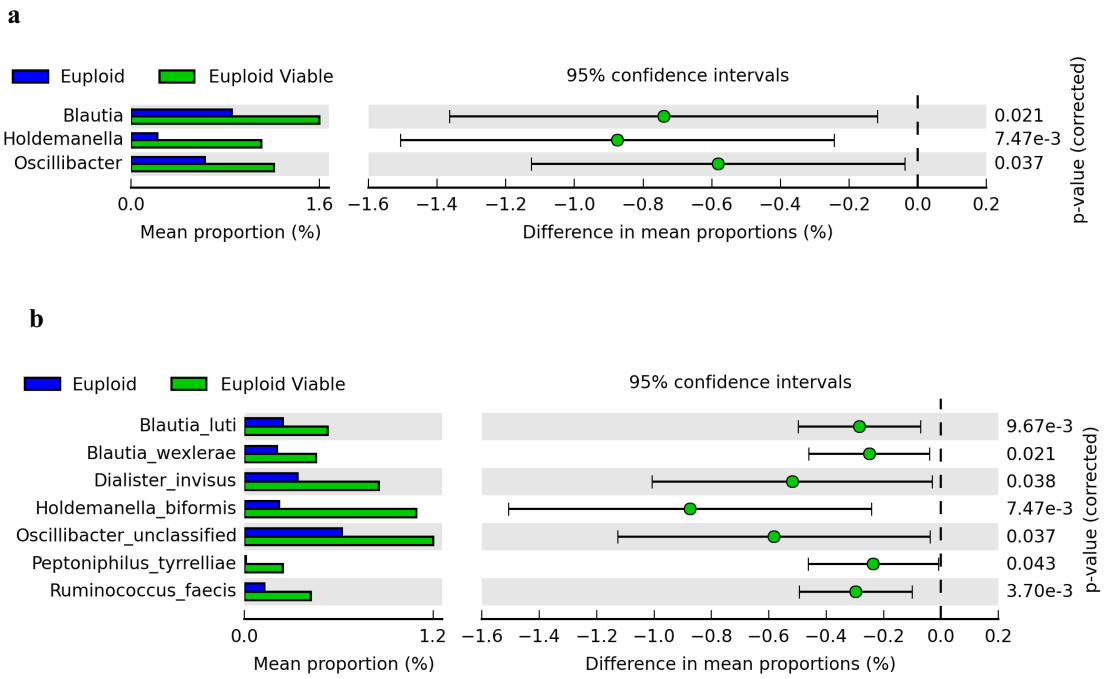

Figure 6.7 Significant taxa associated with different pregnancy outcomes at genera level (a) and species level (b).

Blautia spp., *Holdemanella* spp. and *Oscillibacter* spp. were associated with the control cohort compared to euploid miscarriages. Corrected P values calculated using two-sided Welch t-test.

6.3.5 Rectal dysbiosis and early pregnancy outcomes

A described in the introduction of this Chapter, studies exploring the relationship between the gut microbiota and health states often use the F/B ratio as a measure of gut dysbiosis. Comparison of F/B ratio between patients within the miscarriage group demonstrated that the euploid miscarriage cohort had a trend (P=0.05) towards higher F/B ratio (Figure 6.8).

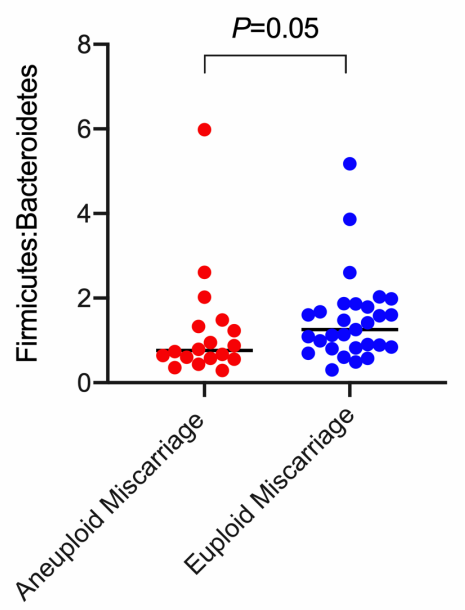

Figure 6.8 Firmicutes: Bacteroidetes ratio in relation to aneuploid and euploid miscarriage

There is a trend towards a significantly higher ratio in the euploid miscarriage compared to the aneuploid miscarriage (P=0.05, Mann-Whitney U test).

Given that the F/B ratio was higher in the euploid miscarriage group, we next used linear discriminatory Effect Size analysis (LEfSe) to determine the features that were differentially abundant between these cohorts (Figure 6.9). Finegoldia magna, Prevotella bivia, Dialister invisus and Peptoniphilus gorbachii were differentially abundant in the euploid miscarriage group. Roseburia, Blautia luti and Clostridium XIVa were differentially abundant in the aneuploid group.

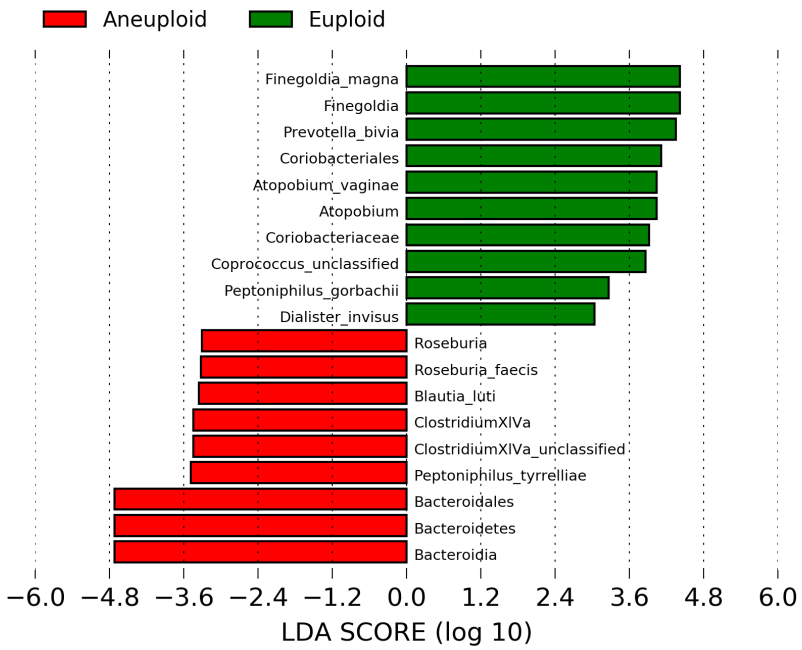

Figure 6.9 Identification of differentially abundant rectal taxa between aneuploid and euploid miscarriages

The linear discriminant analysis (LDA) effect size (LEfSe) method was used to identify differentially abundant rectal taxa in euploid compared to aneuploid miscarriage.

6.3.5.1 Exploration of smoking as a potential confounder

As presented in Table 6.1, the control cohort was enriched with smokers. Smoking has a well described impact on gut microbiota composition, therefore reanalysis of diversity and richness as well as differentially abundant specific taxa between key patient cohorts was performed after excluding smokers (N=16). There was no significant difference in diversity or richness observed between the euploid miscarriage group and control group (Figure 6.10 a and b). However, relative abundance of *Prevotella bivia* was found to be significantly associated with euploid miscarriage compared to the control group (Figure 6.10 c, *P*=0.013).

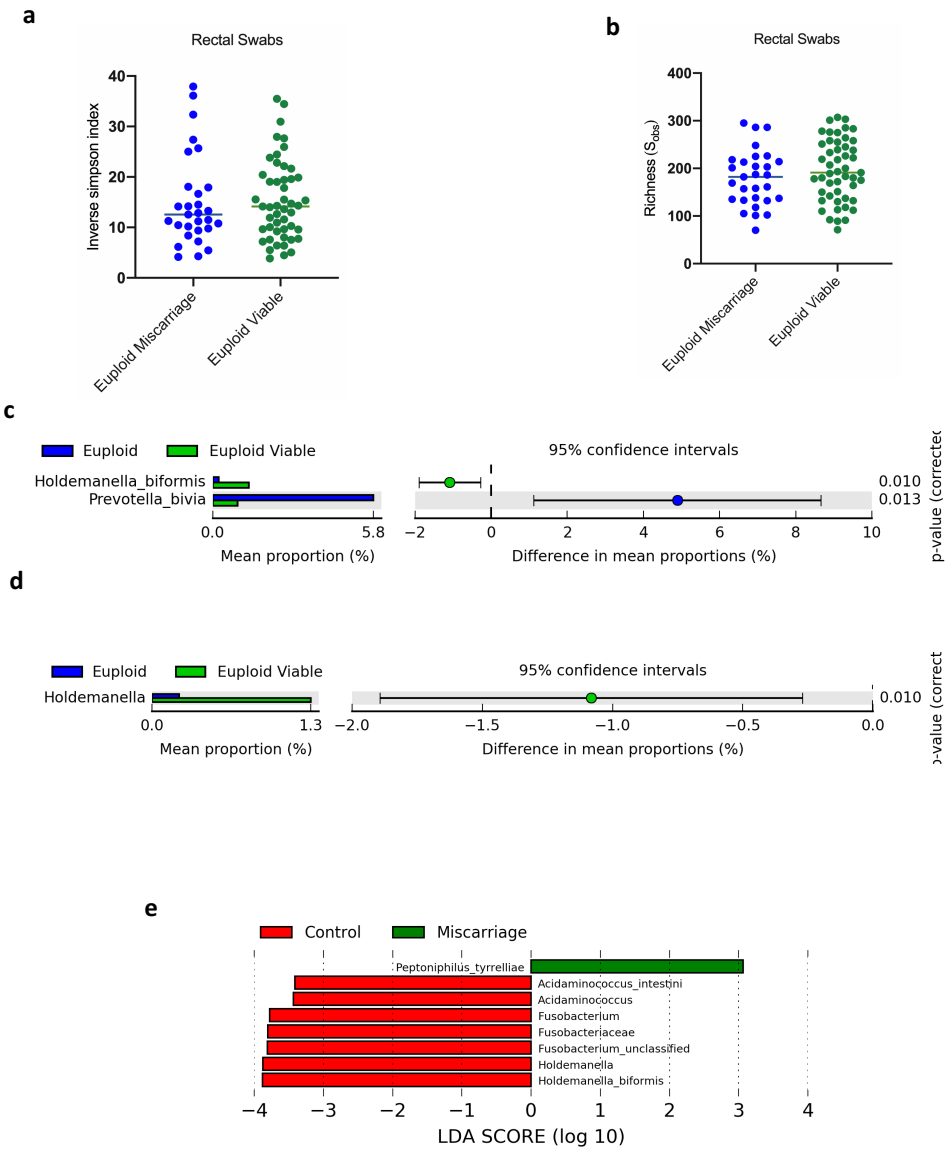

Figure 6.10 The rectal microbiota in the miscarriage and control population excluding all smokers.

Diversity and richness were not significantly different in the control versus euploid miscarriage group (a and b). The individual dots represent different patients and the graph shows the mean and standard error of the mean. *Prevotella bivia* was significantly increased in the euploid miscarriage group compared to the control euploid viable group (d). Corrected P values were calculated using two -sided Welch t-test. The linear discriminant analysis (LDA) effect size (LEfSe) method was used to identify differentially abundant taxa in miscarriage group compared to the control (e).

6.3.6 Rectal microbiota and high-risk vaginal microbiota

We next determined whether the rectal F/B ratio correlated with the vaginal microbiota composition in paired samples. Patients were classified as having a high-risk vaginal microbiome if they had <90% relative abundance of *Lactobacillus* species. This is a well described approach for stratifying patients on the basis of the vaginal microbial composition [241]. Initially all miscarriages and control patients were grouped and the rectal F/B ratio was plotted according to the vaginal microbiome classification (Figure 6.11a). No significant difference was observed in the F/B ratio between the high and low risk vaginal microbiome (Figure 6.11a). However, a sub-analysis of patients with high-risk vaginal microbiota types indicated significantly higher F/B rectal ratio in euploid miscarriages compared to the aneuploid miscarriage (Figure 6.11 c, *P*=0.02).

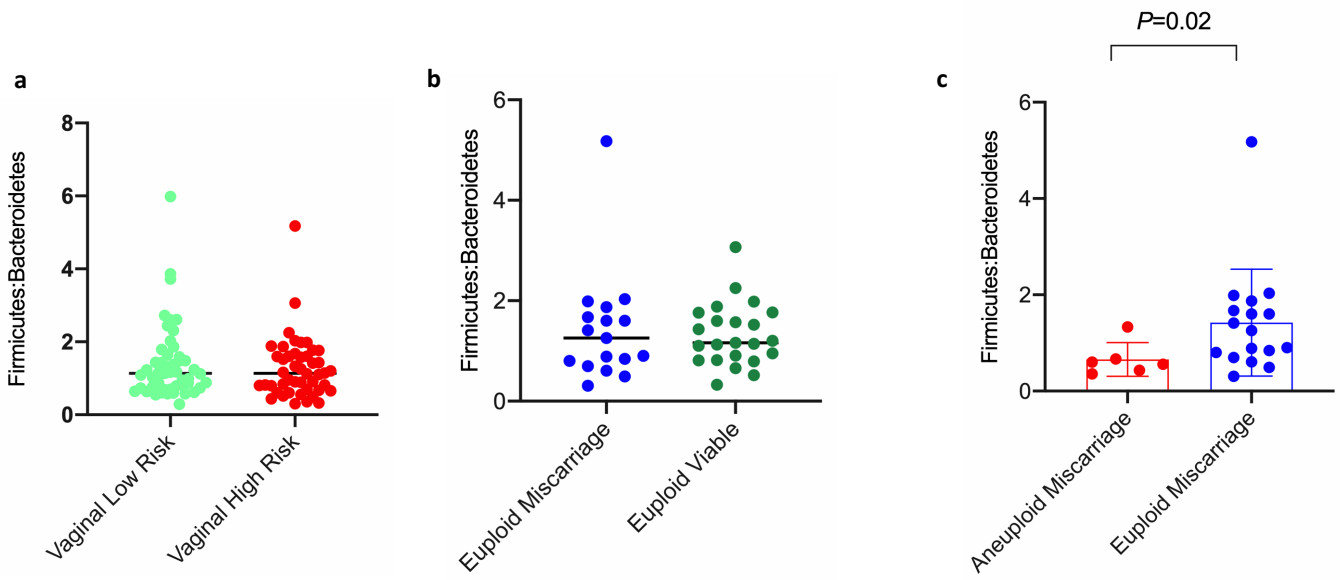

Figure 6.11 The rectal firmicutes/bacteroidetes ratio in relation to paired vaginal microbiome classification.

There was no significant difference between the rectal ratio in paired high and low risk vaginal microbiome samples in the combined control and miscarriage group (a, *P*=0.76, Mann-Whitney U test). There was also no significant difference in the rectal ratio between euploid miscarriage and control patients with a high-risk vaginal microbiome score (b, *P*=0.87, Mann-Whitney U test). There was a significantly higher rectal ratio in the euploid miscarriage cohort compared to the aneuploid miscarriage group in those patients with a high-risk vaginal microbiome score (c, *P*=0.02, Mann-Whitney-U test). Each individual dot represents a different patient and data is shown as mean +/- standard error of the mean.

Differential abundance analyses showed that *Dialister* spp. were enriched in the rectal microbiota of euploid miscarriages (Figure 6.12). Therefore, in patients with a suboptimal vaginal microbial composition similar taxa were also seen in the rectum of euploid but not aneuploid miscarriages. The question that remains is whether these organisms were seeded from the vagina to the rectum (or vice versa) or if they exist as separate entities.

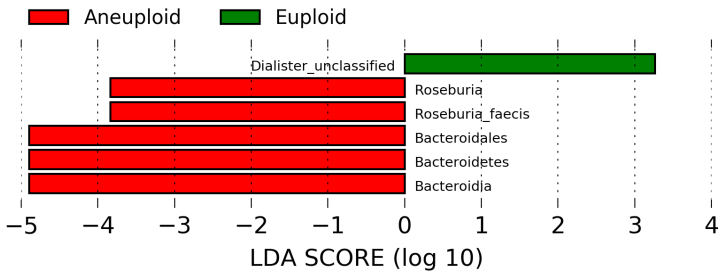

Figure 6.12 Differentially abundant rectal taxa between aneuploid and euploid miscarriage in those with a high-risk vaginal microbiome.

Histogram of the linear discriminant analysis score (LDA) for the features differentially abundant between aneuploid and euploid miscarriages in patients with a paired high-risk vaginal microbiome (Welch's t-test).

6.3.7 Rectal microbiota and cervicovaginal inflammation

To explore whether the rectal microbiota influences the host immune response to the vaginal microbiome, cervicovaginal cytokine concentrations were compared across patients with low and high rectal F/B ratio (Figure 6.13). High F/B ratio was defined as a cut off ≥ 1.6, based on 25th percentile of the data. Expression of anti-inflammatory cytokines (IL2, IL4 and IL10) were significantly higher in patients with a low rectal F/B ratio compared to the high F/B ratio. When similar analyses were performed on a subgroup of patients with *Lactobacillus* spp. dominant vaginal microbiome, no significant difference in the cytokine expression between high and low rectal F/B ratio was observed (Figure 6.14). However, in patients with a *Lactobacillus* spp. deplete vaginal microbiome a low F/B ratio was associated with significantly increased anti-inflammatory cytokine expression (IL4 and IL10) in the cervicovaginal fluid (*P*=0.01 and 0.04).

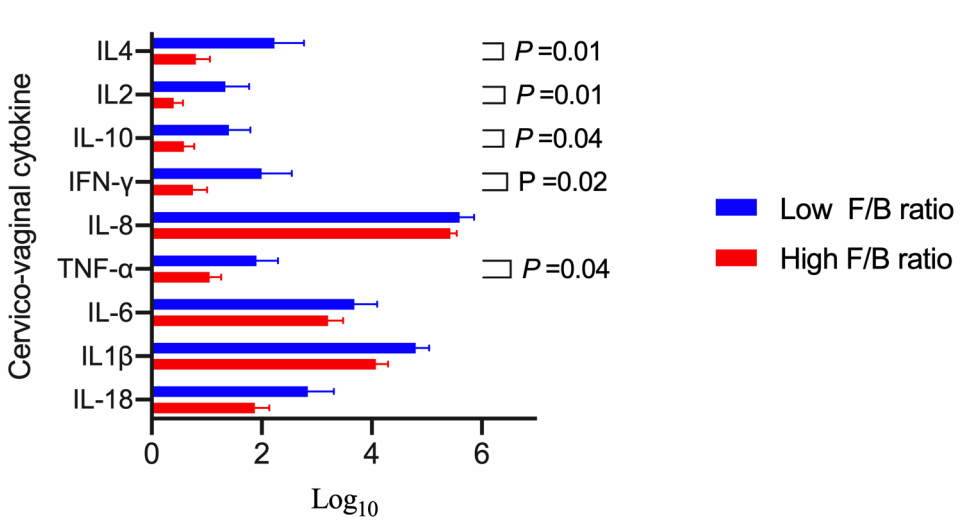

Figure 6.13 The Firmicutes/Bacteroidetes ratio and cervico-vaginal cytokine expression

The low rectal F/B ratio had a significantly higher expression of IL4, IL2, IL10, IFN-γ and TNF-α compared to the high F/B ratio in the cervicovaginal fluid (*P*=0.01, 0.01, 0.04, 0.02 and 0.04, multiple t-tests with discovery determined using two stage linear step up Benjamini, Krieger and Yekutieli with Q=1%).
High Firmicutes/Bacteroidetes ratio (F/B ratio) was defined as a cut off ≥ 1.6, based on 25th percentile of the data. Data represented as a clustered bar chart with mean +/- standard error of the mean for each cytokine. Low F/B ratio N= 13 and High F/B ratio N=34. P values, Mann-Whitney U test for Low F/B ratio versus High F/B ratio.

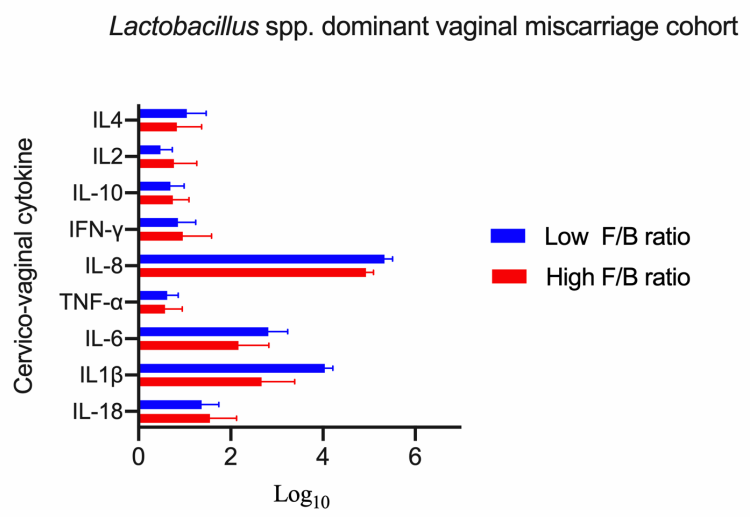

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Figure 6.14 The rectal Firmicutes/Bacteroidetes ratio and cervico-vaginal cytokine expression in patients with Lactobacillus spp. dominant vaginal composition

There was no significant difference between low and high F/B ratio in the cervicovaginal cytokine expression. High Firmicutes/Bacteroidetes ratio (F/B ratio) was defined as a cut off ≥ 1.6, based on 25th percentile of the data. Data represented as a clustered bar chart with mean +/- standard error of the mean for each cytokine. Low F/B ratio N= 17 and High F/B ratio N=7.

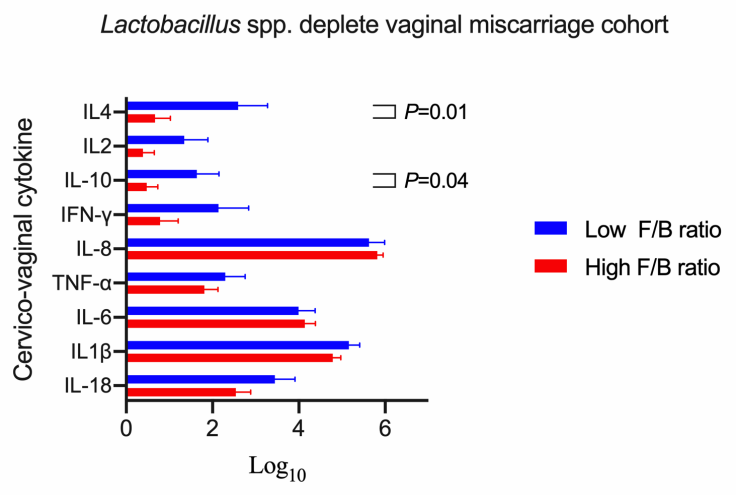

Figure 6.15 The rectal Firmicutes/Bacteroidetes ratio and cervico-vaginal cytokine expression in patients with Lactobacillus spp. deplete vaginal composition.

The low rectal F/B ratio had a significantly higher expression of IL4 and IL10 compared to the high F/B ratio in the cervicovaginal fluid (*P*=0.01 and 0.04, multiple t-tests with discovery determined using two stage linear step up Benjamini, Krieger and Yekutieli with Q=1%). High Firmicutes/Bacteroidetes ratio (F/B ratio) was defined as a cut off ≥ 1.6, based on 25th percentile of the data. Data represented as a clustered bar chart with mean +/- standard error of the mean for each cytokine. Low F/B ratio N= 9 and High F/B ratio N=14.

Correlation analysis (Pearson) was next perfromed between cervicovaginal cytokine levels and rectal diversity. A weak negative association was obseved between the npshannon index and IFN-γ, IL6 and TNF-α (Figure 6.16 a-c, *P*=0.05, 0.03, 0.04). This result indicates that higher levels of pro-inflammatory cytokines in the vagina may be associated with lower diversity in the rectum, which is a marker of adverse gut health.

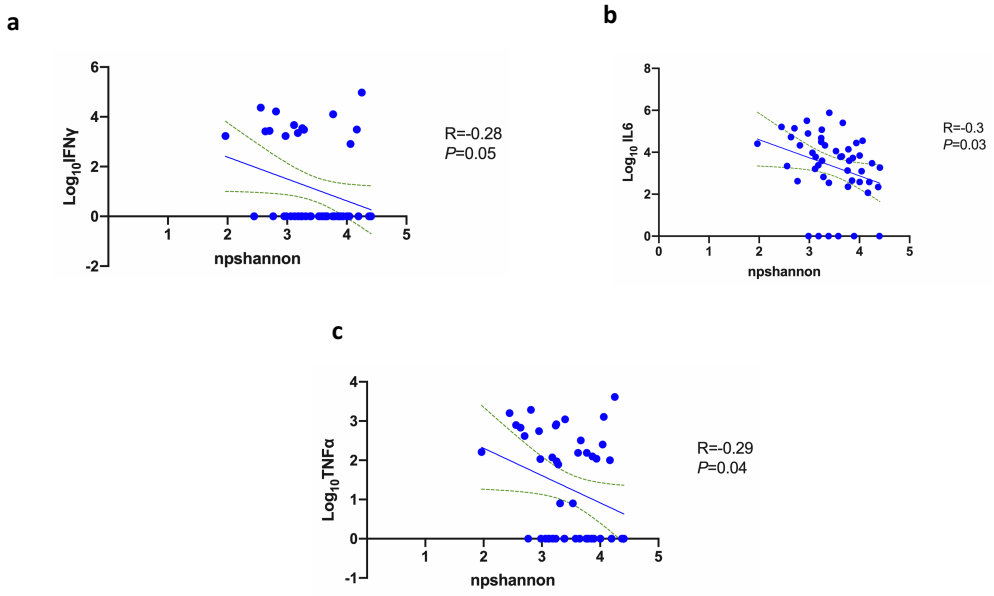

Figure 6.16 Cervicovaginal cytokines expression and rectal diversity

IFN- γ (a), IL-6 (b) and TNF-α (c) cervicovaginal cytokine concentration and rectal npshannon diversity indices. The greater the rectal diversity the lower the pro-inflammatory cervicovaginal cytokine concentrations.

6.4 Discussion

The data presented in this chapter supports existing knowledge that the rectum has significantly higher bacterial diversity and richness than vagina [258]. Analysis of paired samples indicated that compositionally, there is minimal co-occurrence of bacterial species between these niches. Across-patient cohort analyses failed to identify differences in the diversity and richness of the rectal microbiota between the overall miscarriage cohort and control group. Similarly, no difference was observed when the miscarriage cohort was sub-grouped according to euploid and aneuploid miscarriage. Given that smokers were over-represented in the control cohort and smoking is known to shape the gut microbiota, it was important to address this as a potential confounder. However, repetition of the analyses with smokers excluded made no difference to the key findings with richness and diversity indices for the rectal microbiota similar across all patient cohorts.

As diversity and richness indices only provide a broad overview of bacterial community ecology, we next explored whether specific species were associated with different reproductive outcomes. In the miscarriage cohort relative abundance of *Anaerococcus*

vaginalis, *Anaerosphaera* spp., *Finegoldia magna* and *Prevotella bivia* was higher in euploid compared to aneuploid miscarriage. Previous studies exploring the rectal microbiota in patients infected with HIV have reported an increase in *Prevotella* family members and enrichment for *Finegoldia magna* have been linked to microbial translocation and immune activation [259]. Interestingly, many of the differentially abundant taxa identified by LeFSe analysis in the aneuploid group are butyrate producing organisms which are known to be important for a healthy gut and the production of antimicrobial peptides (Roseburia, Clostridium XIVa and Bacteroidetes). In contrast, taxa from the phyla Firmicutes which are linked to inflammatory processes including Dialister, Peptoniphilus and Prevotella species were enriched in the euploid miscarriage group [260, 261].

The F/B ratio is a widely used measure of gut dysbiosis. Studies have shown that a higher ratio is often linked to disease pathology and poor outcome [245]. In this study, a trend towards a higher ratio in euploid miscarriage compared to aneuploid miscarriage was observed. However, there was no difference in the F/B ratio between high (<90% *Lactobacillus* spp.) and low (>90% *Lactobacillus* spp.) risk paired vaginal samples. When we focused on miscarriage patients with a high risk vaginal microbiome there was significantly higher F/B ratio in euploid miscarriage patients. We have previously shown that euploid miscarriage is associated with vaginal dysbiosis. Therefore, it is interesting that this particular cohort also appear to have an abnormal gut microbiota. Therefore, the original insult could occur in the gut and certain pathobionts seed into the vagina via the proximity of location or by haematogenous spread. Consistent with this *Prevotella* spp. and *Dialister* spp. were found in the vaginal microbiota and the rectal microbiota of euploid miscarriages. However, due to the limited genomic resolution of the metataxonomics approach used in this study, it is not possible to determine if these are the same strains nor the directionality of potential colonisation between the sites.

Another plausible interaction between the rectal and vaginal niche is via the oestrogen-gut microbiome axis. An adverse gut microbiota could influence the metabolism of oestrogen by preventing deconjugation into its active form by β-glucuronidase. The altered lower levels of systemic oestrogen could influence the local vaginal microbial composition through mediation of carbon substrates preferentially utilised by *Lactobacillus* species in the vagina [242]. This interaction needs to be explored in more detail by sampling systemic and local cervico-vaginal oestrogen concentration in the context of vaginal and rectal microbial compositions.

The gut microbiota has a crucial role in shaping host mucosal immunity and inhibiting inflammation. An adverse interaction between these two domains can lead to inflammatory disease processes [262]. Studies have found that certain organisms such as Clostridium IV and XIVa can promote the expansion of Treg cells [263]. Certain bacteria also express higher amounts of SCFA which promote the production of immunosuppressive cytokines (IL10) in T effector cells and overcomes oxidative stress in the gut [244, 245]. Interestingly, while SCFAs have an anti-inflammatory role in the gut, they induce the production of pro-inflammatory cytokines (IL1b, IL6 and IL8) and activation of innate immune cells in the female reproductive tract [244]. Some recent studies have begun to explore a potential link between the gut microbiota and immune response in the female reproductive tract [264, 265]. For example, alterations in the gut microbiota are associated with polycystic ovary syndrome and endometriosis through the potential disruption to circulating oestrogen levels [266]. Studies have also shown that oral and rectal administration of live bacterial vectors have altered the immune response at a local cervicovaginal level [264, 265, 267]. Consistent with these findings, we found that anti-inflammatory cytokines were significantly increased in women with low F/B ratio rectal samples. This relationship was limited to women with *Lactobacillus spp.* depleted vaginal microbiota. We have previously shown in Chapter 4 that an adverse vaginal microbiota coupled with a heightened inflammatory response predisposes to euploid miscarriage. However, a proportion of patients with a dysbiotic vaginal microbiome went on to have healthy term pregnancies and an anti-inflammatory immune profile. The results presented in this chapter indicated that a healthy gut microbiome (characterised by a low F/B ratio), may support an anti-inflammatory cervicovaginal immune profile even in the context of vaginal dysbiosis. This provides a possible explanation for why some patients with a suboptimal vaginal composition do not mount an inflammatory response and have healthy term pregnancies.

Strengths and limitations

The prospective study design and well characterised patient group contributed to the main strength of this study. This was a unique cohort of patients with paired rectal and vaginal samples as well as local cervicovaginal cytokine data from aneuploid and euploid miscarriages. We also had paired rectal and vaginal samples from a termination of pregnancy control cohort. We did not have cytokine data for the termination of pregnancy control group and therefore we were only able to explore local immune profiles in the miscarriage cohort. Another limitation is the cross-sectional sampling design. While this was practical, longitudinal

profiling of the patient cohorts would have provided insight into whether fluctuations in the microbial profiles of the rectum corresponded with alterations in the vagina and corresponding immune responses. We also did not collect data on diet and lifestyle both of which are likely to have had an impact on the gut flora [268, 269]. However, given the pilot nature of the study we were still able to shed light on certain taxa that were overrepresented in different reproductive outcomes. We collected rectal swabs to reflect the gut microbial communities and examine potential cross between the vagina. However, there remains the possibility that the rectal profile does not adequately represent the gut microbiome. The control cohort in this chapter also had key demographic differences to the miscarriage group. The enrichment of smokers in a cohort of unplanned pregnancies could influence the results. This group was used in this study because of the ability to obtain trophoblast samples for chapter 5. However, future work should focus on patients with low risk pregnancies that continue to term.

Conclusion

The data presented in this Chapter highlights substantial differences in the composition of the rectum and vagina. However, specific rectal taxa associated with euploid and aneuploid miscarriage were identified. Evidence for crosstalk between the vaginal microbiome and gut dysbiosis was observed for women with euploid miscarriages. Moreover, a healthy gut microbiome, characterised by a low F/B ratio, appears to dampen cervicovaginal immune response to *Lactobacillus* spp. deplete vaginal microbiota. These findings should shape future study design and highlight a focus on longitudinal, pre-conception and first trimester sample collection of rectal swabs, stool samples, vaginal swabs and matched cytokine data in those that miscarry and have healthy term pregnancies.

CHAPTER 7. Discussion

7 Discussion

7.1 Summary of hypotheses

Early miscarriage (loss before 12 weeks) occurs in 20% of pregnancies and at least 50% are due to aneuploidy. However, the mechanisms that drive euploid miscarriages are poorly understood. Recent work has implicated the reproductive tract microbiota in adverse pregnancy events and explored the interaction between the microbial communities and the immune response. However, there is paucity of data investigating the reproductive tract microbiome in early pregnancy loss. We hypothesised that aneuploid miscarriages would predominately be lost due to chromosomal errors, an intrinsic mechanism for pregnancy loss, whereas the vaginal microbiome, an extrinsic and potentially correctable mechanism, would be more strongly implicated in euploid miscarriage. We also hypothesised that vaginal microbiomes associated with euploid miscarriage would trigger localised inflammatory signals mechanistically implicated in miscarriage. To test these hypotheses, we characterised the vaginal microbiota and cervicovaginal host immune response in women with chromosomally normal and abnormal miscarriages as well as healthy pregnancies that delivered at term.

Subsequent work described in this thesis explored the possibility that microbial communities exist in the upper reproductive tract, including the placenta itself, in early pregnancy. We hypothesised that there would be a low biomass placental niche that was different to laboratory contamination controls. To test this, we undertook metataxonomic profiling of trophoblast tissue in miscarriage patients and compared the samples to trophoblast tissue collected from women undergoing a termination of pregnancy for psychological reasons to act as a control population.

Gut dysbiosis has been linked to reproductive health pathology involving the oestrogen-gut microbiome axis. We hypothesised that the gut microbiome differs in euploid compared to aneuploid miscarriages and pregnancy controls. We also hypothesised that there would be a crosstalk between the rectal and vaginal communities in adverse pregnancy outcomes. To address this, we compared microbiome profiles of rectal swabs and paired vaginal samples from miscarriage patients and healthy control patients undergoing a termination of pregnancy.

7.2 Summary of findings of each chapter

7.2.1 Euploid miscarriage is associated with *Lactobacillus* spp. depletion

The work in Chapter 3 is the first published data exploring the vaginal microbiome in chromosomally normal and abnormal miscarriage. The data showed that a significantly higher proportion of euploid miscarriage associated with *Lactobacillus* spp. depletion and increased richness and diversity compared to aneuploid miscarriage. Within the *Lactobacillus* spp. depletion group there was a significantly higher proportion of non-*Gardnerella* species enriched with *Streptococcus* spp. and *Prevotella* spp. in the euploid cohort. *Lactobacillus* spp. was increased in the aneuploid miscarriage group and *Prevotella* spp. and *Dialister* spp. was increased in the euploid group. We also found high levels of co-occurrence between BV associated bacteria and negative co-occurrence between *Lactobacillus* spp. and all other taxa. This highlighted the exclusionary behaviour of *Lactobacillus* spp. within the vaginal niche and therefore, prevention of pathogen colonisation. In summary, these results show that euploid miscarriage is associated with a suboptimal vaginal microbial composition and restoring the dominance with *Lactobacillus* spp. in a particular group of women could help positively influence their reproductive outcomes. However, longitudinal assessments of patients prior to conception in addition to their first trimester are required to determine the timing of causality. There is a considerable volume of work needed to enable us to understand the mechanism by which an adverse vaginal microbiota leads to miscarriage.

7.2.2 Cervical vaginal cytokines are altered in euploid miscarriage

In Chapter 4, the local immune response in euploid and aneuploid miscarriages and healthy pregnancies that delivered at term was studied and integrated with vaginal microbiota composition data. Increased levels of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) were observed in *Lactobacillus* spp. deplete patients compared to the dominant patient group. When patients were stratified into *Lactobacillus* spp. deplete group, the non-Gardnerella cohort (particularly *Streptococcus* spp. and *Prevotella* spp.) were found to have significantly increased proinflammatory cytokines. An additional key finding in this chapter was that there exists a subgroup of patients who harbour *Lactobacillus* spp. depletion but experience healthy pregnancy outcomes. Compared to euploid miscarriages with *Lactobacillus* spp. depletion who have significantly increased proinflammatory cytokines, healthy viable term pregnancies with *Lactobacillus* spp. depletion have a comparatively anti-inflammatory immune profile.

7.2.3 The early pregnancy placental niche

The work in this chapter explores the presence of microbial communities within the placental tissue of miscarriages and termination of pregnancy patients. We identified a signature within the tissue that was above the background contamination controls. However, we also found considerable overlap in the paired vaginal samples. The correlation coefficients in the paired samples had a wide distribution and we focused the analysis on those that had an R^2 value<0.5 to minimise the risk of cross contamination altering the results. In a small subset of individual cases, we identified a pattern that euploid miscarriage with an adverse vaginal microbiome also had similar pathobionts in the paired tissue. However, in the control cohort an adverse vaginal microbiome did not appear to ascend into the tissue, and one could argue there may be a degree of protection. Given the risk of cross contamination due to sample collection techniques these results need further exploration. Ideally abdominal sample collection and techniques such as FISH to understand which organisms penetrate the tissue will be important in future work.

7.2.4 The relationship between the rectal microbiota and early pregnancy outcomes

We integrated the rectal microbiota and paired vaginal microbiota data in euploid and aneuploid miscarriage as well as control patients. We found that the rectal and vaginal samples had a significantly different composition and diversity and richness indices. When we compared the diversity and richness of the rectal microbiota in miscarriage patients to control patients there was no significant difference. However, when we analysed specific species, we found that *Anaerococcus vaginalis*, *Anaerosphaera* spp., *Finegoldia magna* and *Prevotella bivia* were increased in euploid miscarriages compared to aneuploid miscarriages. *Blautia luti* and *Clostridium XIVa* spp. were significantly more abundant in aneuploid miscarriages compared to euploid miscarriages. There was a trend towards significantly increased gut dysbiosis (characterised by a high Firmicutes/ Bacteroidetes ratio) in euploid compared to aneuploid miscarriage. We also found that anti-inflammatory cervico-vaginal cytokines in miscarriage patients with *Lactobacillus spp.* depletion were significantly increased in women with low F/B ratio rectal samples. Future work is required to help understand how a healthy rectal microbiota can modulate the local cervico-vaginal immune response in miscarriage patients with an adverse vaginal microbiota.

7.3 Findings in the context of recent literature

Several studies have explored the relationship between the vaginal microbiota and adverse pregnancy events, but these have mainly focused on preterm birth and PPROM [23, 86, 111, 163, 270, 271]. The role of the vaginal microbiota in early pregnancy and miscarriage is an evolving field. Our work is the first and largest study to integrate cytogenetics, first trimester loss, cytokine data and microbiota data. Of the recent published studies linking vaginal dysbiosis to miscarriage, only one other study captures cytogenetic results and but did not collect longitudinal pre-conception or pre-miscarriage samples [272]. Consistent with the findings presented in this thesis, a number of other studies have reported increased relative abundance of vaginal *Atopobium spp., Prevotella spp., Streptococcus* spp., *Finegoldia spp., Dialister spp.* and *Staphylococcus spp.* in miscarriage [273-275]. Many of these organisms have also linked to pro-inflammatory cytokines, as reported in Chapter 4, but outside the context of miscarriage.

There is a paucity of data exploring the gut microbiota in relation to miscarriage. However, studies have shown that in contrast to the vaginal microbiota, in states of health, the gut microbiota is characterised by high diversity and richness. Consistent with the findings reported in Chapter 6, other studies have associated miscarriage with an increased Firmicutes/Bacteroidetes ratio [243, 276]. However, we extended this work by exploring the interaction between paired vaginal and rectal samples as well as the local vaginal immune response.

Appendix 8.5 details the current literature exploring the vaginal microbiota, gut microbiota and placental microbiota in relation to miscarriage.

7.4 Potential mechanisms exploring the interplay between the vaginal, rectal and placental niche in miscarriage

The findings presented here indicate that *Lactobacillus* spp. depletion, often characterised by vaginal enrichment of *Streptococcus* spp. and *Prevotella* spp. is associated with euploid miscarriage. Recent observational studies have also corroborated these findings more broadly with miscarriage [274, 277]. Previous work has also shown that Lactobacillus dominance is crucial for healthy ongoing term pregnancies [85]. Vaginal dysbiosis is often, but not always, linked to localised vaginal immune activation and increased expression of inflammatory mediators [163], which we have also shown in our work in Chapter 4. However, many studies investigating the vaginal microbiota in health and disease have shown that a proportion of healthy term pregnancies will harbour vaginal *Lactobacillus* spp. depletion [79]. Indeed, in our healthy term cohort nearly a quarter had *Lactobacillus* spp. depletion. Therefore, it is unlikely that a mechanism involving vaginal dysbiosis-driven inflammation is causal of all cases of euploid miscarriage. Consistent with this notion, we found that patients with *Lactobacillus* spp. depletion who experienced viable term pregnancies have a strong anti-inflammatory signature whereas those that have a euploid miscarriage have a pro-inflammatory signature. Furthermore, we have shown that specific organisms (*Streptococcus* spp. and *Prevotella* spp.) are most strongly associated with activation of inflammatory cytokines. These findings indicate individual patient level tolerance to a suboptimal vaginal environment. Alternatively, particular bacterial taxa that contribute to the *Lactobacillus* spp. depletion may breach the cervicovaginal barrier and promote bacterial translocation [220, 221] and trigger an inflammatory response and dysregulation of the nascent maternal-fetal interface in the first trimester. Therefore, while underlying microbial structure is an important component, its interplay with the host immune response is most likely the key factor in shaping risk of microbial-host driven euploid miscarriage. As shown in Chapter 5, placental tissue of control subjects does not harbour the adverse taxa that are present in matched vaginal samples. However, in euploid miscarriage those with a suboptimal vaginal microbiota colonise the paired placental samples with adverse taxa (*Peptostreptococcus* spp., *Streptococcus agalactiae* and *Peptoniphilus* spp.). Nonetheless one of the main limitations of this work was issues of cross contamination of placental tissue from the vaginal niche. Figure 7.1 summarises the potential mechanisms by which the vaginal microbiota could influence pregnancy outcome. Therefore, it is difficult to understand which bacterial taxa have penetrated the tissue and truly reflect the local uterine environment. In order to address this future studies will require alternative techniques such as FISH (Fluorescence in situ hybridization) to

interrogate the tissue and develop this concept. The mechanisms postulated here also need validation with in vitro and in vivo experiments to understand how certain organisms translocate into the uterine environment and the way they disrupt the maternal-fetal interface. Figure 7.1 summarises the mechanisms by which the vaginal microbiota could influence pregnancy outcome.

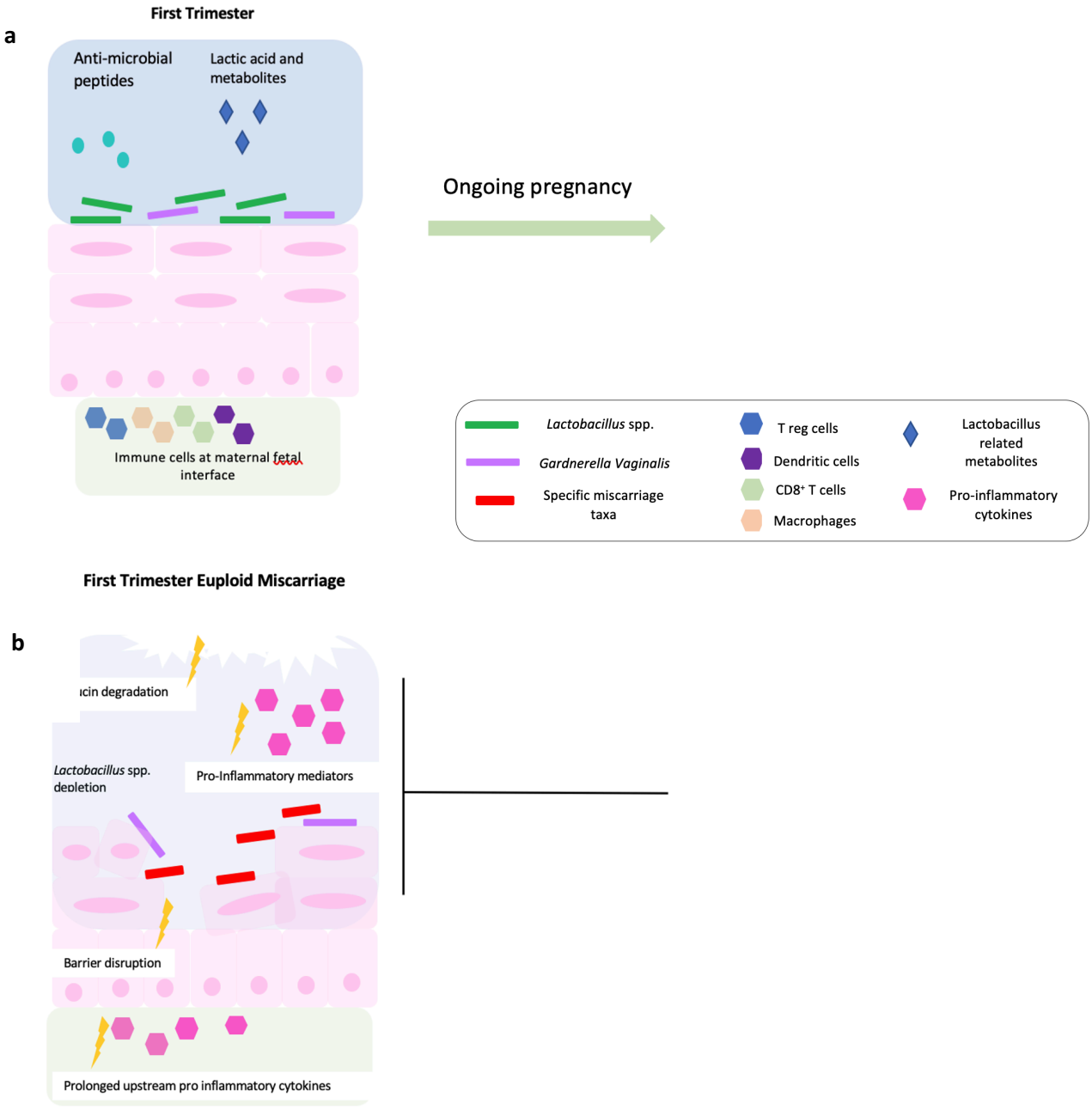

Figure 7.1 represents a potential model for immunological and physiological changes in host-microbe interactions in healthy term pregnancy (a) and first trimester miscarriage (b).

a. In the healthy first trimester state vaginal microbiota dominant in *Lactobacillus spp*. can help to maintain homeostasis by producing lactic acid and other antimicrobial compounds that deter pathogenic bacterial colonization. At the maternal-fetal interface innate and adaptive immune cells are present to orchestrate an important role in implantation. Inflammatory signals from the uterine stroma guide the invasion of the trophoblast into the endometrium for successful implantation. Throughout pregnancy, especially in the second and third trimester increasing oestradiol levels encourage glycogen deposition that enhances stability in the vaginal microbiota and continues a period of *Lactobacillus spp.* dominance.
b. Depletion in *Lactobacillus spp.* especially with the presence of specific miscarriage associated taxa can lead to localised inflammatory mediators and the breakdown in epithelial cells that could lead to bacterial translocation or initiation of upstream inflammatory mediators.
The carton images of implantation and immune regulation at the maternal-fetal interface are taken form Mor et al [194].

We explored the crosstalk between the vaginal and rectal microbiota in chapter 6. We showed that a dysbiotic gut is linked to vaginal dysbiosis in euploid miscarriage. The mechanism that may explain this relationship relates to certain gut taxa altering oestrogen metabolism and in turn influencing circulating levels of active oestrogen which can change the composition in the vagina. Furthermore, we also established that miscarriage patients with vaginal dysbiosis but a healthy gut profile (characterised by a lo F/B ratio) had increased expression of anti-inflammatory cytokines. This may help us understand why certain healthy term deliveries did not mount an immune response in the presence of vaginal dysbiosis in chapter 4. The interplay with the gut profile could be the determining feature that helps to minimise the activation of the immune response. Therefore, future work should focus on validating this interaction and consider cross fertilisation with other biological systems to deepen our understanding of poor reproductive outcomes.

7.5 Clinical impact

Our data and that of others highlights the vaginal microbiome as a modifiable risk-factor of adverse pregnancy outcomes. In the context of the work presented in this thesis, the ability to modulate vaginal microbiome composition with targeted antibiotics as well as pre- and probiotics offers a therapeutic strategy to optimise vaginal microbiome-host interactions and minimise risk associated with miscarriage. We have shown that euploid miscarriage is linked to *Lactobacillus* spp. depletion and the co-occurrence network analysis demonstrates that Lactobacillus does not co-exist with adverse bacterial taxa. It is feasible that a vaginal pessary live biotherapeutic which promotes *Lactobacillus* spp. dominance could positively impact the clinical course of a certain group of patients. The work presented in this thesis also highlights the potential cross talk between the gut and vaginal microbiota in early pregnancy. Oral probiotics designed to modulate the gut microbiota and its capacity to mediate important immune pathways may offer an additional avenue of investigation.

A major limitation of microbiome research to date has been the emphasis on association rather than causation. Pursuing mechanistic work is crucial to deepening our understanding of causation, but it is difficult given the heterogeneity of genetic and lifestyle factors. The Bradford Hill Criteria is a highly regarded tool in health research to aid causal inference [278]. If we consider the question, “does *Lactobacillus* spp. depletion in the vagina cause euploid miscarriage?”, we can apply the nine different criteria set out in the Bradford Hill Criteria. Table 7.1 details the criteria, indicates if each one is met from this work and what needs to be

done in the future to further our understanding. As summarised in the table, there is strength of association and consistency across studies when taking into consideration vaginal dysbiosis and first trimester loss. However, larger studies are required with an understanding of the cytogenetics of individual pregnancy loss to reinforce which miscarriages are influenced by the microbial composition. Temporality is a key feature which remains to be addressed with many studies, including the work described in this thesis, focusing on sample taken from patients at the time of miscarriage diagnosis. It is important that future studies focus on collection of pre-miscarriage samples, ideally taken longitudinally. In terms of specificity, exposure to vaginal dysbiosis has also been linked to PPROM, PTL and implantation failure as well as miscarriage. These all represent forms of adverse reproductive outcomes. This feeds into the concept of analogy as similar disease processes can be linked to dysbiosis. It is difficult to prove the biological gradient criteria as a dose response relationship is hard to establish in complex situations with genetic diversity and differing strains of bacteria causing a range of pathogenicity. In Chapter 4, we reported a trend towards more *Lactobacillus* spp. depletion leading to higher inflammation in euploid miscarriage but this was not consistent. Coherence and biological plausibility have been demonstrated from previous work highlighting bacterial translocation and damage to the cervical epithelial barrier [220, 279]. However, further work in this arena is necessary to further validate these studies and develop experimental models to strengthen our understanding of the link with miscarriage.

Table 7.1 Addressing the Bradford Hill criteria in the context of the work presented in this thesis

Bradford Hill Criteria	Explanation	Is the criteria met from this work?	Future work
Strength of Association	The larger the association the more likely causation exists. The measure is debatable but statistical significance contributes	Yes, statistical significance is present	Larger studies are still needed to validate this significance.
Consistency	Multiple studies with different populations confirming association	Yes, other work does illustrate that vaginal dysbiosis is linked to miscarriage	Larger studies need to consistently confirm this association. Also miscarriage work needs to focus on using cytogenetics to determine which cohorts are most influenced by the microbiota.
Temporality	Exposure must precede the onset of disease	No, we only had a small cohort where samples were collected pre-miscarriage. We did demonstrate altered immune response in those that subsequently miscarried.	Future study designs need to collect all samples before the diagnosis of miscarriage.
Specificity	Exposure causes one disease	No, other poor reproductive outcomes (PTL, PPROM and infertility) have been linked to vaginal dysbiosis.	Examining the different outcomes in more detail to determine if specific bacterial signatures trigger only miscarriage.
Biological gradient	Dose response relationship	Difficult to determine given the genetic and strain diversity	Future work should focus on metagenomics to highlight if specific clades trigger adverse outcome in a dose response manner.
Biological plausibility	Epidemiology and biology must interact	Yes, previous work in preterm birth has demonstrated correlation between genital inflammation, the vaginal microbiota and extra cellular matrix remodelling.	Further work needs to be carried out in miscarriage research to integrate mechanistic work.
Coherence	The story must make sense.	The argument is coherent	Mechanistic work is required
Experimental	Manipulation with invitro experiments can strengthen the cause	Limited invitro work currently exists in miscarriage research	Further *in vitro* work is needed
Analogy	Similar agent may cause a similar disease	We have seen associations and mechanistic work with preterm birth. The association with miscarriage may be an early continuation of the preterm birth disease process.	

7.6 Limitations

There are limitations of the work described in this thesis. Firstly, the study collected samples predominately at the time of miscarriage diagnosis. This has implications in understanding whether the miscarriage tissue itself alters the vaginal microbiota/immune profile or if these events precede the diagnosis. We have previously undertaken a prospective design [79] and shown that first trimester miscarriage is indeed associated with a reduced prevalence of *Lactobacillus* spp.-dominated vaginal microbiota. In our current study patients were recruited at diagnosis in consideration of the practicalities involved in obtaining trophoblast material for karyotyping, and the patients' need to undergo surgical management of miscarriage, rather than conservative or medical management, or manual evacuation. The surgical evacuation also needed to be undertaken at a time which enabled the study team to collect the necessary samples. For these reasons, recruitment occurred over a prolonged period of five years and at the time of miscarriage diagnosis. We are also lacking longitudinal samples which is important to consider as there may be temporal patterns of vaginal microbial composition that we have not recognised in this dataset. However, we standardised the timing of sample collection to the first trimester to ensure consistency across the cohorts.

We limited microbiota and cytokine measurements to the cervicovaginal fluid, therefore it is difficult to determine if our observations reflect microbiota host interactions in the endometrial mucosa. Direct sampling of the early pregnancy uterine environment in healthy pregnancy is logistically and ethically difficult [280]. However, the embryological origin of Mullerian duct fusion means that the upper one third of the vagina shares similarities with the endometrial epithelium and therefore a similar pro-inflammatory response to pathobionts such as *Prevotella* and *Streptococcus* species would be expected [223, 224].

Although we used vigorous negative control samples at different stages of the collection process in Chapter 5, we did not use mock community samples. It is now clear that DNA extraction methods, sequencing platforms and primer sequences can influence the results of high throughput DNA sequencing studies, particularly in the case of low biomass samples. The use of mock community samples permits tracking of extraction and environmental contaminants and the minimisation of sequencing bias [281]. This will be an important strategy to employ in future work.

Another possible limitation of our study relates to the use of normalisation of the sequencing data. Normalisation by rarefaction is widely used as a method to account for differences in

sequencing read depth between samples and to aid in comparisons of microbial diversity. However, it is increasingly recognised that without care this procedure can lead to unnecessary loss of data through exclusion of excess sequences and introduction of artificial variation. However, to address this, we constructed and analysed rarefaction curves where the number of counts per sample were plotted against the expected value of species to guide selection of depth for rarefaction. A Good's coverage value of >96% following rarefaction for all samples indicated adequacy of sub-sampling of the data.

The bacterial load was not estimated in the vaginal or placental samples. It would have been useful to determine the bacterial load in the vaginal samples of miscarriage patients to ascertain if there were any key differences between those with vaginal bleeding and those without. However, the sequencing depth was similar across the three groups with the average read count for Euploid, Aneuploid and Viable being 25,926, 25,257 and 28,899 respectively (p= 0.1196, Kruskall-Wallis). The placental bacterial load was not carried out as there was a concern about cross contamination from the vagina. Therefore, it would be more informative to perform quantitative PCR on placental samples collected abdominally (via chorionic villus sampling) to determine the true load of bacteria within the tissue itself.

The primer use was an important consideration for this thesis. We sampled vaginal, placenta and rectal niches but needed to compare each case alongside one another and therefore needed to use the same primer set and pipeline for analysis. The V1-3 hypervariable regions of the 16S rRNA gene is often targeted for the vaginal microbiota as it robustly classifies *Lactobacillus* spp. However, the universal forward primer widely used includes mismatches for *Gardnerella vaginalis* that can lead to underestimation of its relative abundance within samples. A mixed formulation to overcome this has been used in our study [92]. Studies of the placental microbiome examining all nine variable regions indicate that the V2 and V6 region generate the greatest total number reads [143]. However, similar studies of the gut microbiota have reported that V3-V6 regions are most efficient. Thus, it is possible that the primer set used in our study may have underestimate certain bacterial species in different sample types, however, it is important to note that an “ideal” primer set does not exist, and each has limitations.

7.7 Future Work

The work in this thesis has identified several areas that require further expansion and exploration. Larger cohorts of longitudinally sampled patients are required to detect fluctuations in the vaginal microbiota preceding the diagnosis of miscarriage. Ideally a study design would need to capture patients pre-conceptually and take serial samples enabling characterisation of the vaginal microbiota leading up to and beyond the pregnancy outcome. This would help establish causal inference of the microbial communities and local immune response on miscarriage.

In this thesis we have shown that specific bacterial taxa within *Lactobacillus* spp. deplete communities trigger inflammation and associate with euploid miscarriage. *Gardnerella vaginalis* was an important component in the high-risk *Lactobacillus* spp. deplete group in euploid miscarriage but was also present in viable healthy pregnancy. Current evidence has shown that certain clades within the *Gardnerella vaginalis* spp. have varying degrees of pathogenicity [282]. The capacity to distinguish these clades is key in determining the immunomodulating properties and how this contributes to disease [54]. Future work could assess taxonomic diversity in more detail using metagenomic approaches.

In terms of placental sample collection future work could minimise the chance of cross contamination by collecting samples abdominally. The excess villi obtained from chorionic villi sampling procedures can be used to determine which organisms are truly present within the tissue. Alternative techniques such as FISH (fluorescent DNA probes that allow specific signals to be detected using a fluorescent microscope [283]) would enable visualisation of microbes within the tissue samples and determination of their localisation and morphotypes.

Finally, future studies could focus on validating the interaction between the vaginal and rectal niche in the context of miscarriage risk by offering probiotics as part of interventional studies designed to reduce the risk of miscarriage.

7.8 Final Conclusions

This work has shown that *Lactobacillus* spp. depletion in the vagina alongside a heighted cervicovaginal immune response predispose pregnant women to euploid miscarriage. Our results show that this cytokine response is largely driven by a subset of specific vaginal bacterial pathogens. However, in a subset of women a suboptimal vaginal microbial

composition does not drive a pro-inflammatory response and these women tend to experience healthy term pregnancies. We have shown that in the context of an adverse vaginal microbiota a healthy gut can cause immune modulation. Therefore, it is likely these two niches interact and influence reproductive outcomes. The data presented here suggests that a group of women could benefit from antibiotic or pre/probiotic vaginal pessaries and oral probiotics to reduce the risk of miscarriage. Further studies are needed to validate these findings and understand the mechanisms that drive inflammation and breakdown at the maternal-fetal interface. An important next step is to conduct pre-conception clinical trials with different probiotic interventional regimes to determine if this alters reproductive outcome.

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CHAPTER 8. Appendix

Appendix 8.1 Co-occurrence network key

A key of code names (**a** genera and **b** species) for the co-occurrence network analysis of vaginal bacterial taxa in Figure 3.10 in chapter 3

a

Genus	**Code**
Timonensis	Tim
Bifidobacterium	Bfd
Lactobacillus	Lact
GeneraX	Gen
Atopobium	Atp
Gardnerella	Gard
Alloscardovia	Als
Sneathia	Sne
Prevotella	Prev
Finegoldia	Fng
Parvimonas	Parv
Anaerotruncus	Anaero
Megasphaera	Meg
Dialister	Dls
Anaerococcus	Anaer
Streptococcus	Strep
Peptoniphilus	Ppt
Porphyromonas	Prp
Mobiluncus	Mob
Flavobacterium	Flv
Catonella	Cat
Staphylococcus	Staph
Ureaplasma	Ure
Veillonella	Vel
Escherichia/Shigella	Shg

b

Species	Code
Alloscardovia_omnicolens	Al_
Anaerococcus_lactolyticus	AN_l
Anaerococcus_obesiensis	An_b
Anaerococcus_tetradius	An_t
Anaerococcus_unclassified	An_l
Anaerotruncus_unclassified	Atr_
Atopobium_vaginae	At_v
Bifidobacterium_breve	Bf_b
Bifidobacterium_longum	Bf_l
Catonella_unclassified	Ct_
Dialister_micraerophilus	Dl_m
Dialister_unclassified	Dl_n
Escherichia_coli	E_c
Finegoldia_magna	Fn_
Flavobacterium_unclassified	Fl_
Gardnerella_vaginalis	Gr_
Lactobacillus_acidophilus	L_cd
Lactobacillus_coleohominis	L_cl
Lactobacillus_crispatus	L_cr
Lactobacillus_gasseri	L_g
Lactobacillus_iners	L_n
Lactobacillus_psittaci	L_j
Lactobacillus_vaginalis	L_v
Megasphaera_unclassified	Mg_
Mobiluncus_mulieris	Mb_
Parvimonas_unclassified	Prv_n
Peptoniphilus_indolicus	Pp_n
Peptoniphilus_lacrimalis	Pp_l
Porphyromonas_uenonis	Prp_
Prevotella_amnii	Prv_am
Prevotella_bivia	Prv_bv
Prevotella_buccalis	Prv_bu
Prevotella_colorans	Prv_c
Prevotella_disiens	Prv_d
Prevotella_jejuni	Prv_j
Prevotella_melaninogenica	Prv_m
Prevotella_timonensis	Prv_t
Sneathia_sanguinegens	Sn_s
Sneathia_unclassified	Sn_n
SpeciesX	Sp_

Species	Code
Staphylococcus_epidermidis	Stp
Streptococcus_agalactiae	Stp_g
Streptococcus_anginosus	Stp_n
Streptococcus_pneumoniae	Stp_p
Streptococcus_urinalis	Stp_r
Streptococcus_vestibularis	Stp_v
Telmatospirillum_unclassified	Tl_
Ureaplasma_parvum	Ur_p
Ureaplasma_urealyticum	Ur_r
Veillonella_montpelliierensis	V_m

Appendix 8.2 Patient information leaflets

Imperial College Healthcare NHS
NHS Trust

Queen
Charlotte's & Chelsea Hospital
Du Cane Road
London
W12 0HS
Tel: 020 8383 1111
Fax: 020 8383 3588
www.imperial.nhs.uk

Participant's Information Sheet– 22nd November 2016

Information Sheet for Research Participants diagnosed with a miscarriage.

Study title:

A Cohort Study of the Early Pregnancy and Endometrial Microbiome (ASPIRE)

REC Number: 16/WA/0357

Principal Investigators:

Professor Phillip Bennett
Professor Tom Bourne
Dr David MacIntyre

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and members of hospital staff. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part in the study.
If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. If you decide not to take part or to withdraw at any other time without explanation, your future care will not be affected by your decision. Thank you for reading this.

Who we are
We are a group of scientists and doctors who are interested in finding out why people miscarry and in particular whether the presence of bacteria in the womb can be a cause of miscarriage.

What is the purpose of the study?

It has recently been realized that there are bacteria in almost all parts of the body. The term microbiome refers all the bacteria which are normally present at any body site. Sometimes if the wrong bacteria are present this might cause disease. Currently we do not know whether the types of bacteria in the uterus, vagina and gut might be linked to miscarriage.

Half of all miscarriages are caused by chromosomal abnormalities but the NHS does not offer testing for this until a patient's third miscarriage. Furthermore, the techniques used will fail in 50% of cases. We will also aim to find a cost effective way of testing the chromosomes in pregnancy. This will hopefully provide women with a greater understanding as to why they have miscarried.

This study is part of an educational project at Imperial College London.

Why have I been chosen?

We have asked you to participate as you attended the ultrasound department or early pregnancy unit in the first twenty weeks of your pregnancy, and were sadly diagnosed with a miscarriage. We appreciate this is a very difficult time but want to give you the opportunity to contribute to greater understanding of the process of early pregnancy complications. Additionally, you may have chosen to manage your miscarriage surgically.

Do I have to take part?

Your participation is voluntary. If you wish you be part of the study, we would like you to complete a consent form.
If you decide to take part, you are still free to withdraw at any time, without giving a reason. This will not affect the standard of care you receive in the future.
We can reassure you that all the information you give us and the results we obtain will be confidential and anonymous.

What do I have to do?

During your visit to the hospital we will ask you whether you would like to participate in the study. A member of the research group will explain the purpose of this study in detail, answer your questions and ask you to sign the necessary consent form.

What will happen to me if I take part?

We will ask you for a 20mls (4 teaspoons) sample of blood. If possible this will be taken at the same time as any preoperative blood test you require.
We will also ask you for a urine sample.

If you are managing your miscarriage surgically, we will aim for all other samples to be collected under general anaesthetic (while you are asleep) before or during your operation. We will take a swab from your mouth, vagina and back passage whilst you are asleep during the procedure. This is performed by gently passing a small cotton bud-type swab into these areas which you will not feel.
In some cases, we aim to collect a sample of placental tissue and tissue from the womb lining during the surgical procedure under direct vision with a small telescope. This is a procedure called an embryoscopy. We would still sensitively dispose of the tissue and collect a small amount to analyse for chromosomal abnormalities and changes in bacterial structure. If any chromosomal abnormalities are detected, we will write to you. This is a test that is not normally done until at least three miscarriages have occurred on the NHS, but will be offered routinely as part of this study.

What are the possible disadvantages and risks of taking part?

None of the tests will cause harm to you. The blood test is a routine simple blood test similar to one that you may have at the GP practice. Most of the swabs and tissue will be collected whilst you are asleep and so will not be uncomfortable. The main difference with this study is that in some cases we will use a telescope to visualise the inside of your womb and select the placental tissue for research. If you were having surgical management outside this study, it would be done without visualising the tissue. A suction curette would be introduced to the neck of your womb and the pregnancy tissue gently removed. The telescope test does not pose any different risks compared to the surgical procedure alone.

What are the possible benefits of taking part?

Taking part in the study will not modify your care, although it is possible that the direct visualization of the miscarriage will aid with the surgical procedure. We hope that women in the future will benefit from your participation and information we gain from this study. We hope that the findings of this research will help with the care of women suffering from miscarriage.

What will happen to the samples collected?

The samples will be anonymously stored in a locked laboratory and some samples will be analysed over the next 3 years. At the end of the study the samples will remain in the laboratory and may be used for other research projects looking at bacteria and miscarriage.

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential and anonymised. Your consent form will be stored securely on Imperial College premises. Your name and address will be removed from the information when it is shown to other medical staff outside the study.

The GP will be informed of your participation in the study.

What if a problem is detected?

If a problem is detected, we will refer you appropriately and the condition will be followed-up and managed according to standard practice and guidelines. The GP will also be informed.

What happens if I withdraw?

You can decide to withdraw at any time without explanation. If you do so, your future care will not be affected by your decision. The data that is already collected will be used in the study unless you ask us not to do so.

Who is organising the research?

This study is being organised by the staff of the Department of Obstetrics & Gynaecology at Imperial College NHS Trust and Imperial College London.

Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the Wales Research Ethics Committee 3.

What if something goes wrong?

Imperial College London holds insurance policies which apply to this study. If you experience serious and enduring harm or injury as a result of taking part in this study, you may be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.
If you are harmed due to someone's negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Investigators, Professors Phillip Bennett and Tom Bourne, whose contact details are shown below. The normal National Health Service complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial AHSC Joint Research Compliance Office. Telephone: 0203 313 5281

Support Groups

We appreciate this is a difficult time and you may want to access support groups available to you. There is a miscarriage support group that is run by the early pregnancy team at Queen Charlotte's hospital. This is also open to patients at St Mary's Hospital. Please ask a member of staff for details about the next available date or contact the team on 02033135131.

Transparency:
Imperial College London is the sponsor for this study based in the United Kingdom. We will be using information from you and/or your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. Imperial College London will keep identifiable information about you for 10 years. Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible. You can find out more about how we use your information. Principle investigator: Professor Phillip Bennett, p.bennett@imperial.ac.uk.Imperial College Healthcare NHS Trust will collect information from you and/or your medical records for this research study in accordance with our instructions. *Imperial College Healthcare NHS Trust will keep your name, date of birth, hospital number and contact details confidential and will not pass this information to Imperial College London. Imperial College Healthcare NHS Trust will use this information as needed, to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Certain individuals from Imperial College and regulatory organisations may look at your medical and research records to check the accuracy of the research study. Imperial College London will only receive information without any identifying information. The people who analyse the information will not be able to identify you and will not be able to find out your name, date of birth, hospital number and contact details.Imperial College Healthcare NHS Trust will keep identifiable information about you from this study for 10 years after the study has finished.* When you agree to take part in a research study, the information about your health and care may be provided to researchers running other research studies in this organisation and in other organisations. These organisations may be universities, NHS organisations or companies involved in health and care research in this country or abroad. Your information will only be used by organisations and researchers to conduct research in accordance with the UK Policy Framework for Health and Social Care Research**.**
This information will not identify you and will not be combined with other information in a way that could identify you. The information will only be used for the purpose of health and care research, and cannot be used to contact you or to affect your care. It will not be used to make decisions about future services available to you, such as insurance.

Contact for Further Information

If you want more information, before or after you return your form, you can phone
Professor Phillip Bennett on 020 7594 2176
Professor Tom Bourne on 0207 636 6765
Dr Karen Grewal on 0203 313 5131/0203 313 5859
Funding: Tommy's Charity

Imperial College Healthcare NHS
NHS Trust

ASPIRE Study Number: 16HH3593
ASPIRE Study Email:
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Participant's Information Sheet– 22nd November 2016

Information Sheet for Research Participants undergoing surgical termination of pregnancy.

Study title:

A Cohort Study of the Early Pregnancy and Endometrial Microbiome (ASPIRE)

REC Number: 16/WA/0357

Principal Investigators:
Professor Phillip Bennett
Professor Tom Bourne
Dr David MacIntyre

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and members of hospital staff. Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part in the study.
If you do decide to take part, please let us know beforehand if you have been involved in any other study during the last year. If you decide not to take part or to withdraw at any other time without explanation, your future care will not be affected by your decision. Thank you for reading this.

Who we are
We are a group of scientists and doctors who are interested in finding out why people miscarry and in particular whether the presence of bacteria in the womb can be a cause of miscarriage.

What is the purpose of the study?

It's recently been realized that there are bacteria in almost all parts of the body. The term microbiome refers all the bacteria which are normally present at any body site. Sometimes if the wrong bacteria are present this might cause disease. Currently we

don’t know whether the types of bacteria in the uterus, vagina and gut might be linked to miscarriage.

Half of all miscarriages are caused by chromosomal abnormalities but the NHS does not offer testing for this and the techniques used will fail in 50% of cases. We will also aim to find a new cost effective method for testing the chromosomes in a pregnancy. This will hopefully provide women with a greater understanding as to why they have miscarried.

This study is part of an educational project at Imperial College London.

Why have I been chosen?

We have asked you to participate as you are undergoing a surgical termination in the first twenty weeks of your pregnancy. We appreciate this is a very difficult time but want to give you the opportunity to contribute to greater understanding of the process of early pregnancy complications.

Do I have to take part?

Your participation is voluntary. If you wish you be part of the study, we would like you to complete a consent form.
If you decide to take part, you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive in the future.
We can reassure you that all the information you give us and the results we obtain will be confidential and anonymous.

What do I have to do?

During your visit to the hospital we will ask you whether you would like to participate in the study. A member of the research group will explain the purpose of this study in detail, answer your questions and ask you to sign the necessary consent form.

What will happen to me if I take part?

We will ask you for a 20mls (4 teaspoons) sample of blood. If possible we will collect this during the insertion of the line you need for the procedure.
We will also ask you for a urine sample.
All other samples we will aim to collect under general anaesthetic before or during your operation. We will take a swab from your mouth, vagina and back passage during the procedure. This is performed by gently passing a small cotton bud-type swab into these areas which you will not feel.
You may also be offered an embryoscopy procedure, which involves placing a small telescope into the womb to remove the tissue under direct vision. We will aim to collect a sample of placental tissue and tissue from the womb lining during this procedure. Those having the termination with local anaesthetic will have the samples collected whilst awake during the procedure.

What are the possible disadvantages and risks of taking part?

None of the tests will cause harm to you. The blood test is a routine simple blood test similar to one that you may have at the GP practice. The swabs and tissue will mostly be collected whilst you are asleep and so will not be uncomfortable. The main difference with this study is that in some cases we will use a telescope to visualise the inside of your womb and select the placental tissue for research. If you were having surgical management outside this study, it would be done with a suction curette being introduced into the neck of your womb and gently removing the tissue without visualising it directly. The telescope test does not pose any additional risks compared to the surgical procedure alone.

What are the possible benefits of taking part?

Taking part in the study will not modify your care, although it is possible that if embryoscopy is performed (camera look at the tissue) the direct visualization of the tissue will aid with the surgical procedure. We hope that women in the future will benefit from your participation and information we gain from this study. We hope that the findings of this research will help with the care of women suffering from miscarriage.

What will happen to the samples collected?

The samples will be anonymously stored in a locked laboratory and some samples will be analysed over the next 3 years. At the end of the study the samples will remain in the laboratory and may be used for other research projects looking at bacteria and miscarriage.

Will my taking part in this study be kept confidential?

All information that is collected about you during the course of the research will be kept strictly confidential and anonymised. Your consent form will be stored securely on Imperial College premises. Your name and address will be removed from the information when it is shown to other medical staff outside the study. Your GP will be informed of you taking part in the study.

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You can decide to withdraw at any time without explanation. If you do so, your future care will not be affected by your decision. The data that is already collected will be used in the study unless you ask us not to do so.

Who is organising the research?

This study is being organised by the staff of the Department of Obstetrics & Gynaecology at Imperial College NHS Trust and Imperial College London.

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Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.
You can find out more about how we use your information. Principle investigator: Professor Phillip Bennett, p.bennett@imperial.ac.uk.
Imperial College Healthcare NHS Trust will collect information from you and/or your medical records for this research study in accordance with our instructions.
Imperial College Healthcare NHS Trust will keep your name, date of birth, hospital number and contact details confidential and will not pass this information to Imperial College London. Imperial College Healthcare NHS Trust will use this information as needed, to contact you about the research study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Certain individuals from Imperial College and regulatory organisations may look at your medical and research records to check the accuracy of the research study. Imperial College London will only receive information without any identifying information. The people who analyse the information will not be able to identify you and will not be able to find out your name, date of birth, hospital number and contact details.

Imperial College Healthcare NHS Trust will keep identifiable information about you from this study for 10 years after the study has finished.
When you agree to take part in a research study, the information about your health and care may be provided to researchers running other research studies in this organisation and in other organisations. These organisations may be universities, NHS organisations or companies involved in health and care research in this country or abroad. Your information will only be used by organisations and researchers to conduct research in accordance with the UK Policy Framework for Health and Social Care Research**.**
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Contact for Further Information
If you want more information, before or after you return your form, you can phone
Professor Phillip Bennett on 020 7594 2176
Professor Tom Bourne on 0207 636 6765
Dr Karen Grewal on 0203 313 5131/0203 313 5859
Funding: Tommy's Charity
ASPIRE Study Number: 16HH3593
ASPIRE Study Email:
Early Pregnancy Assessment Unit,
Queen Charlottes and Chelsea Hospital,
2nd Floor,
Du Cane Road,
London W12 0NN
OR
Postal Address:
Women's Health Research Centre,
c/o Imperial College London,
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Du Cane Road, London W12 0NN
Office: 0203 313 5281
Fax: 020 3313 5284
Email: whrcenquiries@imperial.ac.uk

Appendix 8.3 Consent

Imperial College Healthcare NHS Trust

Imperial College London

ASPIRE

(A Cohort Study of the Early Pregnancy and Endometrial Microbiome)

Participant Consent Form Version 1

Date: October 2016 REC reference: 16/WA/0357

Principal Investigator:
Professor Philip Bennett PhD, FRCOG.
Professor Tom Bourne PhD, FRCOG.
Dr David MacIntyre PhD

Please initial each box

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that my clinical information and study data may be looked at by responsible individuals from the research staff, study sponsor, NHS Trust, or from regulatory authorities; I give permission for these individuals to have access to my records. ☐

4. I understand that my medical data will be collected for this study and may be used to help develop new research, and that data protection regulations will be observed and strict confidentiality maintained. ☐

5. I agree to take part in the above study and agree to have a blood test and urine sample collected. ☐

6. I agree to have a high vaginal swab during the procedure I am having. ☐

7. I agree to have a rectal swab during the procedure I am having. ☐

8. I agree to have an oral swab during the procedure I am having. ☐

9. I agree to tissue samples being taken during the procedure I am having. ☐

If applicable:

10. I agree to the visualisation of the tissue with embryoscopy during the procedure Yes ☐
No ☐

11. If required, I may be contacted via telephone, and/or via email address. Yes ☐
No ☐

12. I understand a copy of this signed consent form, containing my full name, will be sent to the Chief Investigator. ☐

13. I agree that the outcome of my pregnancy may be followed via the local hospital birth registry.
Yes ☐
No ☐

14. I give permission for my samples to be used for future ethically approved research projects.
Yes ☐
No ☐

15. I agree to my General Practitioner being informed of my participation in the study
Yes ☐
No ☐

Name of Participant _____ Date _____

Signature _____

Investigator taking consent _____ Date _____

Signature _____

Copy: Chief Investigator at ICHT Copy: Participant Copy: Hospital notes

8.4 ASPIRE Protocol

Imperial College Healthcare NHS Trust

Imperial College London

A Cohort Study of the Early Pregnancy and Endometrial Microbiome (ASPIRE)

26th October 2016

Main Sponsor	Imperial College London
Study coordination centre	IRDB Hammersmith Hospitals
REC/NRES Reference	16/LO/2004
Version Number	V4
Effective Date	
Author	Dr Karen Grewal
Protocol authorised by:	Date: October 2016

Study Management Group

Chief Investigator:

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Director of the Institute of Reproductive and Developmental Biology
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Study Management

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Queen Charlotte's & Chelsea Hospital
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Email: karen.grewal@imperial.nhs.uk

Study Coordination Centre

All clinical queries should be directed to Dr Karen Grewal (contact details as above).

Sponsor

Imperial College London is the main research sponsor for this study. For further information regarding the sponsorship conditions, please contact the Joint Research Compliance Office at:

Joint Research Compliance Office
Imperial College London and Imperial College Healthcare NHS Trust
Room 215, Level 2, Medical School Building
Norfolk Place
London, W2 1PG
Tel: 0207 594 1872
Fax: 020 7594 1792
http://www3.imperial.ac.uk/clinicalresearchgovernanceoffice

Funding

Funding for this study has been provided by the Tommy's Charity.

This protocol describes the study and provides information about procedures for entry. Problems relating to this study should be referred, in the first instance, to the Chief Investigator.

This study will adhere to the principles outlined in the NHS Research Governance Framework for Health and Social Care (2nd edition). It will be conducted in compliance with the protocol, the Data Protection Act and other regulatory requirements as appropriate.

Table of Contents

1.Introduction

Miscarriage is a devastating outcome for any couple and the underlying mechanism is not clearly understood. Such a lack of understanding is frustrating for both the patient and the clinician. Early miscarriage is defined as pregnancy loss before 12 weeks, this occurs in one in five pregnancies leading to grave psychological and physiological morbidity. Such a common occurrence also poses a significant health economic burden. Half of early miscarriages exhibit chromosomal abnormalities [8]. Late miscarriage occurs between 12-24 weeks and is less common but the majority are thought to be associated with histological chorioamnionitis [284]. Although certain pathogens have been associated with miscarriage the actual mechanism to induce the process is poorly understood. Such evidence is based on culturing specific organisms such as cytomegalovirus, varicella and listeria which neglects a whole host of bacteria which may also be implicated. The original immunological paradigm that dictates maternal suppression is required for a successful pregnancy, is slowly being contested. There is emerging evidence which examines the role of microbiota in reproduction. It is crucial that we understand the homeostasis between the maternal immune system, placenta and commensals. Only then can the complexity of the pathogenic interplay be clearly understood and targeted treatments be considered [151].

Microbiome in early pregnancy

The microbial cells in the human body accounts for 1-3% of the total body mass [285]. The Human Microbiome Project established by the National Institutes of Health in 2008 dictates the human body is inhabited by a large diverse microbiota. The ability to isolate such complex communities is possible with advanced DNA sequencing techniques. Recent evidence has focused on the crucial role of the oral, gut and vaginal microbiome in shaping the reproductive health throughout pregnancy. A change in the balance of these communities is associated with adverse pregnancy outcomes. In pregnancy there is a shift in the bacterial community structure of the vagina to a composition dominated by Lactobacillus. There have been reports of ethnic variation of vaginal microbiota which is a product of genetic, environmental, cultural and behavioural factors [93]. The genus lactobacillus comprises of >130 species, more than 20 are found in the vaginal flora. A study of non-pregnant North American women of a reproductive age found four out of the five community state types were dominated by Lactobacillus; L. Crispatus, Gasseri, Iners and Jensenii. It is thought the colonisation of such species protects the ecosystem from other pathogenic organisms by the production of lactic acid and providing competition [286, 287]. However, the question remains whether altered levels of bacteria predispose to infection or the change in microbiome alters the pH which fails to neutralize pathogens and subsequently decreases the immune response [288].

The original belief that human development occurred in a sterile environment has been dispelled with recent research which identified diverse placenta microbiomes. The work by Aagaard et al investigating placental specimens in a population based cohort study characterised a unique microbiome. The 16S ribosomal DNA-based and whole genome shotgun metagenomic studies were used and identified non-pathogenic commensal microbiota from Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes and Fusobacteria phyla. The study concluded that the placenta

harbours a low abundance but metabolically diverse microbiome [125]. This work has also shown the placenta has greatest similarity with the oral microbiome which infers a potential haematogenous spread [286].
Earlier cohort studies demonstrated that bacterial vaginosis increased the risk of first trimester miscarriage [21]. The evidence also suggested that early bacterial vaginosis was associated with late miscarriage and preterm delivery [289]. These studies pre-date the ability to further characterise the microbiota and were only dependent on gram stain and culture. Limited bacteria can be cultivated in the culture based methods and hence neglected the microbiome diversity. A change in the techniques used to identify such organisms has changed the understanding of this diversity [290]. Hence determining the normal vaginal microbiome and the fluctuations in disease states will provide clearer information on the cause and effect relationship [288].
Metagenomics includes various techniques such as fingerprinting using 16SRNA to cluster bacterial communities. Although this is inexpensive it is less specific. Other techniques such as targeted sequencing of hypervariable regions of 16S ribosome are more specific but reliant on bioinformatics. The key challenge with bioinformatics is to ensure alignment of data and correct subtraction of contaminating material. A key aspect is that such bacteria do not free float on the surface of tissues but have a three-dimensional biofilm, which adds an additional layer of complexity to the situation [285]. One must also consider with metagenomics that specific focus needs to be placed on the proportions of bacteria since presence of genetic material does not always imply significant concentrations (8).

The change in the microbiome in pregnancy is thought to also have an impact on neonatal microbiome. This demonstrates the possibility that the maternal microbiome holds the key to our entire future metabolism and immune system [291]. There is also evidence which links the gastrointestinal and reproductive microbiome. Studies have shown the rectum is a possible reservoir of colonisation for the vaginal microbiome [249]. There is also evidence that supports dietary supplementation in the third trimester that modulates the vaginal microbiome and cytokine secretion. This could potentially prevent such inflammatory cascades and prevent preterm birth [292].
There is recognition that the host human body and bacteria have an important relationship but the microbial environment in diseased states is not well understood [288]. If such imbalances are partly responsible for miscarriage this could lead to targeted therapeutic approach for subsequent pregnancies.
Hence this demonstrates the need to further study the microbiome in diseased states to develop a greater understanding of the interplay.

Metabolomic analysis in evaluating miscarriage
Spontaneous fetal aneuploidy is the most common cause for pregnancy loss in the first trimester. The frequency and type of such chromosomal abnormalities vary with gestational and maternal age. Chromosomal aneuploidy is present in 90% of specimens at 0-6week gestation, 50% of those losses between 8-11weeks and 30% of those losses between 14-16 weeks [293].

An understanding as to why a miscarriage occurred gives the patient insight into the process. This could help with the grief and psychological sequelae that surrounds this difficult pregnancy complication. However, given the current techniques available to evaluate such abnormalities it is not deemed cost effective to test every miscarriage. Karyotyping is often in excess of £100 per sample and relies on cell culture which presents many limitations. Mainly, culture failure, maternal contamination and the inability to store tissue in formalin [294]. An increasingly popular alternative method is comparative genomic hybridisation (CGH) which has a lower failure rate but considerably more expensive. It is able to detect abnormalities in the entire genome but balanced structural chromosomal rearrangements and female polyploidy cannot be identified [295]. Other non-culture based approaches are quantitative fluorescent-PCR (QF-PCR) and fluorescence in situ hybridization (FISH), which are less expensive but limited in the number of chromosomes that can be tested in one reaction. Multiplex ligation dependent probe amplification (MPLA) is a PCR based method that quantitatively assesses the changes in copies of DNA. Studies have shown that MPLA can be useful in detecting aberrations in first trimester losses. Although such methods are still expensive compared to cytogenetics and like CGH are unable to detect balanced chromosomal rearrangements and female polyploidy [296].

Metabolomics looks at the concentrations of all metabolites in a culture medium by an applied method in a given moment in time. These are the end products of the biological processes of the cells. The small molecules in biological samples serve as a good indicator of cellular activity [297].

A tissue's metabolome can be studied by Nuclear Magnetic Resonance (NMR) or Mass Spectroscopy (MR). NMR is highly reproducible, easily quantified and non-destructive whereas MS is more sensitive, yet time consuming and expensive [298]. Desorption electrospray ionization (DESI) is an ambient ionization technique used in mass spectrometry to allow real time characterization of human tissue by analysing the 'smoke' released from electrosurgical dissection. This was developed at Imperial college by Zoltan Takats and termed the 'iKnife'. The chemical characteristics of malignant tissues are specific to the histopathological subtypes and matched the postoperative diagnosis in 100% of cases. The relevance of such a tool is that such chromosomally abnormal placental tissue may also have a different metabolome from normal tissue that can be identified by DESI-MS. Hence this could provide a cost effective way of determining which miscarriages were caused by chromosomal abnormalities [299]. Furthermore, recent small pilot studies have investigated the use of cell free fetal DNA in miscarriage. Evidence suggests cffDNA remains in maternal plasma when the placenta remains in situ. However, further larger studies need to be conducted to investigated whether aneuploidy can be reliably detected. (23)

2. Study Rationale and Hypothesis

We hypothesise that different vaginal, oral and gut microbiome structures increase the risk or protect against miscarriage.

Furthermore, we also hypothesise chromosomally abnormal placental tissue will have a different metabolome from normal tissue which can be detected by NMR, MS or DESI-MS. The significance of this is that it could provide a cost-effective way to determine which miscarriages had chromosomal abnormalities.

Study Objectives

To determine the normal placental and decidual microbiome in the first trimester.
To determine if there is a normal endometrial microbiome and if women with recurrent miscarriage have an abnormal microbiome.
To understand if miscarriage is associated with an abnormal placental microbiome.
To ascertain if nuclear magnetic resonance or mass spectroscopy can predict placental karyotype compared to other quantitative methods.

Methodology and Design

3.1 Study Centre

The study will be conducted at Queen Charlotte's and Chelsea Hospital (QCCH) at Imperial College Healthcare NHS Trusts (Primary Centre of the study). Part of the study will also be conducted at St Mary's Hospital (SMH).

There will be:
A named individual (Clinical Research Fellow) in the relevant Study centre responsible for co-ordinating the project locally, and reporting directly to the chief investigators.
Regular inspection by the study co-ordinator, under the supervision of the chief investigator, will be carried out ensuring that both the service (literature, communication, documentation, supervision and logistics) and facilities (equipment and space) are of high standards.
This study will form part of an MD thesis.

3.2 Design

This is a prospective, cohort observational study.

3.3 Duration

The study will be conducted over a 5-year period.

3.4 Inclusion Criteria

Patient undergoing a termination of pregnancy<20 weeks
Patient at point of diagnosis of miscarriage <20weeks (with part or all the pregnancy in situ)
Patients aged between 18-45 attending the recurrent miscarriage clinic who are undergoing endometrial biopsy, early pregnancy or miscarriage.
Patients aged between 18-45 in General Gynaecology Clinic requiring an endometrial biopsy in the outpatient hysteroscopy clinic.

3.5 Exclusion Criteria

Younger than 18 years presenting with miscarriage or TOP

Younger than 18 years presenting to general gynaecology clinic or recurrent miscarriage clinic.
A termination or a miscarriage greater than 20weeks.
Women who in the opinion of the researcher by virtue of language or learning impairment would be unable to give fully informed consent to the study.

3.6 Withdrawal Criteria
Patients may withdraw from the study at any stage and all data captured in relation to their participation may be destroyed at their request.

3.7 Consent and Information leaflet
(see supporting documents)
All participating patients will be consented prior to enrolment, and will receive a full explanatory information leaflet.

3.8 Method of study
Study population and sampling method:

Women will be recruited via open advertisements (study poster) in local GP surgeries, local hospitals, and the university where the study is being conducted (Imperial College).
We will recruit patients from the following groups;

Group 1
Patient at the point of the diagnosis of miscarriage, or with retained products requiring surgical, medical or conservative management in Early Pregnancy Unit at Hammersmith Hospital.
Group 2
Women undergoing surgical termination of pregnancy at SMH or QCCH.
Group 3
Patients attending the Recurrent miscarriage clinic at SMH undergoing an endometrial biopsy, early pregnancy or miscarriage.
Group 4
Patients undergoing an outpatient hysteroscopy and endometrial biopsy for abnormal bleeding in the General Gynaecology clinic in Hammersmith Hospital.

If potential participants have seen this advertising and are interested in taking part in the study, they can contact the study team either by email or telephone, details of which will be provided in the advertisements. Once they have made contact, potential participants will be interviewed during an initial telephone consultation by the clinical research fellow or research midwife to ensure they meet the inclusion and exclusion criteria. If eligible, a patient information sheet will be emailed or posted to them, and they will be invited for a first study visit if they agree to participate or if they prefer, they may visit the department for further information and to obtain the leaflet.

First study visit for those patients who responded to advertising:

The first study visit will take place at Queen Charlotte's & Chelsea Hospital, if recruited from EPU or General Gynaecology OPD. Those recruited from the recurrent miscarriage or unplanned pregnancy clinic will have the visit at St Mary's Hospital. At the first visit, the research midwife or clinical research fellow will:

Explain the outline of study and what is expected from participant.
Provide patient with information leaflet.
Reconfirm that the participant is eligible for the study. If so, written consent will be obtained for participation in the study.
Complete a questionnaire regarding demographic details, past medical, gynaecological and fertility history, and recent drug history with the participant.
Provide each participant with unique study identity code. Personal data linking to each unique study code will be kept in paper form in a confidential file, which is locked in a secure filing cabinet in the Early Pregnancy Units at QCCH and St Mary's.
If a patient is willing to participate, consent will be obtained.

Information gathered:

Symptoms
As part of routine clinical management, patients will be asked to complete a questionnaire. This is a standard clerking proforma used which includes routine demographic data and information regarding previous obstetric, medical and gynaecological history, and questions regarding symptoms.

Ultrasound
- As part of routine clinical management, patients in groups one and two will have 2D ultrasound and where possible 3D crystal vue imaging will be undertaken. There will be uniform measurements of the crown-rump length, yolk sac and gestational sac. If the case is for termination of pregnancy then the above criteria will be measured with a visible fetal heart to help identify those pregnancies that would have had a normal outcome.

c. Biomarkers-metabolomics
- During this initial visit, patients will be asked to provide a 20mls sample of venous blood during their visit for metabolomics biomarker studies, HCG level, full blood count and cell free DNA. This will be necessary to act as a comparative tool for the NMR and MS studies on the placenta tissue.
- We will also ask the patient for a urine sample. Urine samples will be stored in a -80 C freezer and will be used for metabolomics studies.

Microbiome studies. Samples will be taken under general anaesthesia during surgical management of miscarriage and termination of pregnancy. Those undergoing medical

or conservative management of miscarriage as well as outpatient hysteroscopy will have the swabs taken while awake during one of the routine visits to the clinic.
A High-vaginal swab will be taken for microbiome and metabolomics studies. This will involve gently parting the labia, and inserting a small swab, the size of a cotton bud, into the high portion of the vagina. The samples will be collected, labelled with anonymous patient identifier and stored according to our protocol. This will be logged into the database and in our laboratory log book. Those patients who require misoprostol prior to the procedure will have the swab taken before insertion of the vaginal tablet.
An oral swab will be taken after intubation by gently inserting a swab the size of a cotton bud in the mouth.
A rectal swab will be taken by gently inserting a swab the size of a cotton bud into the rectum while the patient is asleep during the surgical procedure and unable to feel the discomfort.
Hysteroscopic visualization of the placental and decidual tissue will be performed at the time of surgical management of miscarriage and termination using a Bettocchi integrated operative hysteroscope. The tissue will be removed under direct vision using the biopsy grasping forceps through the hysteroscope. The endometrial biopsy will be taken routinely as part of the hysteroscopy for the recurrent miscarriage and general gynaecology patients. The placental and decidual tissue will undergo microbiome studies as well as metabolomics (NMR, MS, DESI, QF-PCR, MPLA and CGH). The endometrial tissue will undergo purely microbiome studies.

See appendix 11.a for a flowchart of the patient pathway, which highlights the routine standard care and additional tests that will be performed as part of this study.

Missed Study Visits:
There will be limited extra appointments for the study. Hence, if the patient agrees to participate the samples required will be opportunistically collected in the visits they would have had as part of their management. However, there will be a pre-operative clinic running prior to the date of surgery to reconfirm consent and collect blood and urine if necessary.

3.9 Study Closure
We aim to recruit 600 patients in total over a 5-year period.
300 in Group 1 and 100 each in Groups 2,3 and 4.

3.10 Documentation and Communication
Clinical data, patient demographics will be recorded and analysed prospectively on a structured proforma and on a computer software programme. Data will be stored for a minimum of 3 years after completion of study. If the karyotyping of miscarriage trophoblast tissue leads to an abnormal result the patient will be informed of this via email, phone or post according to the patient's preference.

Study Outcome Measures

4.1 Primary outcome measures

To determine if the risk of miscarriage is linked to the structure of the vaginal, oral or gut microbiome.

4.2 Secondary outcome measures

To assess how the structure of oral, vaginal, gut microbiome change and interact during the first trimester of normal pregnancy.

To identify if there is a normal endometrial microbiome

To determine the effect of aneuploidy on the metabolome.

Study Population and Sample size

Formal power analysis was not possible in this type of study, as there is no data available about the effect of aneuploidy and the metabolome or the significance of microbiome and miscarriage. However, in similar studies where metabolomic differences have been used to predict microbe/epithelial interactions, significant differences have been seen with less than 100 subjects.

5.1 Statistical and Data Analysis

We will observe frequencies and associations with the difference in microbiome of the termination placental tissue compared to the miscarriage tissue. We shall also observe frequencies in what constitutes the normal endometrial microbiome and if this is different to the microbiome of those with recurrent miscarriage. The microbiome can be targeted by in situ hybridization using 16S rRNA probes or direct sequencing of the hypervariable regions of 16S RNA genes from DNA extracted from the tissue.

The predictive value of Nuclear Magnetic Resonance Spectroscopy or Mass spectroscopy or DESI for placental karyotype will be carried out by linear logistic regression analysis. A p value of <0.05 will indicate significance.

Study supervision and Monitoring

RISK ASSESSMENT - This study will not involve any risk to the participants.

If there is a bacterial culture on the vaginal swab at any stage during the study, the patient will be managed accordingly in concordance with local guidance and follow up arrangements will be made. The patient will still be followed up for the purposes of the study.

Central and local supervision: the chief principal investigator at Imperial College London will be responsible for the protocol, quality control, interim analyses of the data, advice on progress and final analysis and reporting of the study.

Regulatory Issues

7.1 Ethical approval

The Chief Investigator has obtained approval from the HRA and Research Ethics Committee. The study must also receive organisational readiness from each participating NHS Trust before accepting participants into the study. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964 and later revisions.

7.2 Consent

Consent to enter the study will be sought from each participant only after a full explanation has been given, an information leaflet offered and time allowed for consideration. Signed participant consent will be obtained. The right of the participant to refuse to participate without giving reasons will be respected.

After the participant has entered the study the clinician remains free to deviate from the outlined pathway at any stage if he/she feels it is in the participant's best interest, but the reasons for doing so will be recorded. In these cases, the participants remain within the study for the purposes of follow-up and data analysis. All participants are free to withdraw at any time from the protocol treatment without giving reasons and without prejudicing further treatment.

7.3 Confidentiality

The Chief Investigator will preserve the confidentiality of participants taking part in the study and is registered under the Data Protection Act. The GP will also be aware of the patient participation in the study.

7.4 Indemnity

Imperial College London holds negligent harm and non-negligent harm insurance policies which apply to this study.

7.5 Sponsor

Imperial College London will act as the main sponsor for this study.

7.6 Adverse events

There are no risks to the patients from this study.

Definitions

Adverse Event (AE): Any untoward medical occurrence in a patient or clinical study subject.

Serious Adverse Event (SAE): Any untoward and unexpected medical occurrence or effect that:

Results in death

Is life-threatening – *refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe*

Medical judgement will be exercised in deciding whether an AE is serious in other situations. Important AEs that are not immediately life-threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, will also be considered serious.

Reporting Procedures
All adverse events will be reported. Depending on the nature of the event the reporting procedures below will be followed. Any questions concerning adverse event reporting will be directed to the Chief Investigator in the first instance.

Non-serious AEs
All such events, whether expected or not, will be recorded.

Serious AEs
An SAE form will be completed and faxed to the Chief Investigator within 24 hours. However, relapse and death and hospitalisations for elective treatment of a pre-existing condition do not need reporting as SAEs.
All SAEs will be reported to the Chief Investigator who will determine if the event was:
Related- resulted from the administration of any of the research procedures; and
Unexpected- an event that is not listed in the protocol as an expected occurrence
Reports of related and unexpected SAEs will be submitted within 15 days of the Chief Investigator becoming aware of the event, using the NRES SAE form for non-IMP studies. The Chief Investigator will also notify the Sponsor of all SAEs.
Local investigators will report any SAEs as required by their Local Research Ethics Committee, Sponsor and/or Research & Development Office.

Contact details for reporting adverse events:

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Women's Health Research Centre,
c/o Imperial College London, IRDB, Ground Floor,
Du Cane Road, London W12 0NN
Office: 0203 313-5281 Fax: 020 3313 5284
Email: whrcenquiries@imperial.ac.uk

7.7 Audits and Inspections

The study may be subject to inspection and audit by Imperial College London under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition). Direct access to source data/documents as requested will be permitted.

Study Management

The day-to-day management of the trial will be co-ordinated through the Early Pregnancy Unit at Queen Charlotte's and Chelsea Hospital by Dr Karen Grewal.

Publication Policy

All publications and presentations relating to the study will be authorised by the Study Management Group. The investigators will therefore be responsible for publication of the data. As such they are co-authors in all resulting clinically relevant papers, to which they made significant contributions. Co-authors will be included according to their contribution in the study and depending on the journal's publication guidelines.

Appendix 8.5 Current literature examining the vaginal, placental and rectal microbiota in relation to miscarriage

Vaginal Microbiota and first trimester miscarriage		
Study	Summary of findings	Main Study characteristics
Grewal et al. 2022 [280] Presented in Chapter 3 and 4	-*Lactobacillus* spp. depletion is significantly higher in euploid compared to aneuploid miscarriage (*P*=0.01). -*Lactobacillus* spp. depleted vaginal microbiota associated with proinflammatory cytokines (IL-6 and IL-1β and IL-8) in euploid miscarriage versus viable term pregnancy.	**Sequencing technique and experiments performed:** **-**16S rRNA metataxonomics (V1-2) was used and the following analytes were measured IL2, IL4, IL6, IL8, IL-1β, IL10, IL18, IFN-γ, and TNF-α. **Cohort demographics: 9**3 first trimester miscarriages (39 aneuploid and 54 euploid) 74 term pregnancies
Al-Memar et al. 2019 [79]	-Reduced prevalence of *Lactobacillus* spp. dominance (*P*=0.005), higher diversity and richness in the vaginal microbiota of first trimester loss compared to viable controls.	**Sequencing technique and experiments performed:** **-** 16S rRNA metataxonomics (V1-2) **Cohort demographics:** -64 first trimester miscarriage (cytogenetics unknown) -83 term pregnancies
Zhao et al. 2021[300]	-RMC had lower richness than healthy women. -Metformin combined with aspirin treatment significantly increased the relative abundance of vaginal *Lactobacillus* spp.	**Sequencing technique and experiments performed:** **-**16S rRNA metataxonomics (V3-4) **Cohort demographics:** **-**43 non pregnant patients with a history of RMC (≥ 3 miscarriages) taking metformin. -65 non pregnant patients with a history of RMC no medication -18 Healthy women who previously had live births without a history of miscarriage.
Caliskan et al. 2022 [273]	-Lactobacillus spp. decreased in women with a history of recurrent miscarriage. -*Megasphaera* spp., *Veilonella* spp.,*Dialister* spp. and *Peptostreptococcus* spp. were significantly higher in women with recurrent miscarriage compared to healthy women (*P* <0.05).	**Sequencing technique and experiments performed:** -Real time polymerase chain reaction vaginal and cervical canal specimens **Cohort demographics:** **-**25 non pregnant women with history of RMC (≥ 3 miscarriages) -25 healthy volunteers (non-pregnant)
Xu et al. 2020 [301]	-The miscarriage group had increased diversity and decreased *Lactobacillus* spp. compared to control group (*P*<0.001) -Increase in IL-2 and decrease in IL-10 in the miscarriage group compared to control.	**Sequencing technique and experiments performed:** **-**16S rRNA metataxonomics (V4) and IL2 and IL10 analysed using Elisa kit **Cohort demographics:** -25 embryonic miscarriage (missed miscarriage, cytogenetics unknown) -25 healthy pregnant women
Zhang et al. 2019 [274]	-*Atopobium spp., Prevotella spp. and Streptococcus* spp. were more abundant in the RMC group. -*Lactobacillus* spp. and *Gardnerella* spp. were more abundant in the control group.	**Sequencing technique and experiments performed:** **-**16S rRNA metataxonomics (V3-4) **Cohort demographics:** -10 non pregnant women with a history of RMC (≥ 3 miscarriages) -10 healthy non pregnant controls

Chen et al. 2022 [277]	-Higher species richness in the miscarriage group compared to the control. *Atopbium* spp. was enriched in the miscarriage cohort (OR, 2.9;95%CI, 1.4-5.8; *P*<0.01).	**Sequencing technique and experiments performed:** **-**16S rRNA metataxonomics (V3-4) at 5-8 weeks gestation **Cohort demographics:** -48 first trimester miscarriage (no cytogenetics) -116 normal pregnancies
Seo et al. 2017 [302]	-Multivariate logistic regression analysis identified *A. vaginae , L. amnionii* and *S. sanguinegens* strongly associated with miscarriage compared to control.	**Sequencing technique and experiments performed:** **-**16S rRNA metataxonomics (V1-3) cervical swabs **Cohort demographics:** **-**36 pregnant controls (no history of miscarriage) -23 miscarriage (unknown cytogenetics) -88 termination of pregnancy
Fan et al 2020[272]	-Alpha diversity higher in the RMC compared to the termination group. -*Pseudomonas, Roseburia, Collinsella, Aerofaciens and Arthrobacter* increased expression in the RMC compared to the termination group. -Increase CCL2, 3,4,5and8 in the villus tissue of women with RMC.	**Sequencing technique and experiments performed:** **-**16S rRNA metataxonomics (V3-4) -Chemokine testing on the villi of 3 miscarriages and 3 termination patients. **Cohort demographics:** **-**31 miscarriage (euploid) RMC with normal parental karyotype. -27 termination of pregnancy
Jiao et al. 2022 [303]	-*Atopbium* spp. was over represented in the RMC group -*Lactobacillus* spp. and *Gardnerella* spp. were more abundant in the control group.	**Sequencing technique and experiments performed:** -16S rRNA metataxonomics (V3-4) **Cohort demographics:** 16 non pregnant with history of RMC (≥ 2 miscarriages) 20 non pregnant healthy volunteers
Shahid et al. 2022 [275]	-*Lactobacillus iners* dominant in 50% women with history of miscarriage/current miscarriage compared to those with no history/ control (*P*=0.011). -*Finegoldia, Peptoniphillus Prevotella, Dialister* and *Staphylococcus* expressed in the miscarriage group.	**Sequencing technique and experiments performed:** **-**16S rRNA metataxonomics (V4) **Cohort demographics:** x2 vaginal swabs sampled in the first trimester of pregnancy -8 Miscarriage (cytogenetics unknown) -14 control term delivery

Abbreviations; RMC- recurrent miscarriage

Placental microbiome and first trimester miscarriage		
Oliveira et al. 2020 [137]	-Women with *Mollicutes* detected in placenta had a seven fold higher chance of miscarriage. -A positive association between *U. parvum* in placental tissue and miscarriage.	**Sequencing technique and experiments performed:** **-**qPCR to detect *M. genitalium, M. hominis, U. parvum, U. urealyticum* and *N. gonorrhoeae* -Endocervical swabs and placenta tissue collected **Cohort demographics:** -89 miscarriage -20 pregnant control delivered at term (no history of miscarriage).
Gut microbiota and first trimester miscarriage		
Gao et al. 2021 [276]	-Alpha and beta diversity of gut microbiota lower in miscarriage compared to control ($P<0.05$). -Actinobacteria high abundance in miscarriage group -*Prevotella, Lactobacillus, and Paracoccus* were significantly more abundant in the control group than in the case group at the genus level ($P < 0.05$).	**Sequencing technique and experiments performed:** 16S rRNA metataxonomics (V1-9) **Cohort demographics:** Stool samples collected in the first trimester -50 miscarriage -50 pregnant control
Liu et al. 2021 [243]	-Diversity and richness significantly higher in control compared to miscarriage group ($P<0.001$). – Î Firmicutes/Bacteroidetes ratio in the miscarriages compared to controls ($P=0.039$) -IL-2, IL-17, TNF-α, and IFN-γ are significantly increased in serum of miscarriage patients.	**Sequencing technique and experiments performed:** **-**16S rRNA metataxonomics V3-4 of stool samples -IL-2, -4, -5, -6, -9, -10, -13, -17A, -17F, -21, -22, IFN-γ, and TNF-α multiplex panel -10 miscarriage and 10 controls randomly chosen for metabolomics **Cohort demographics:** 41 miscarriage (euploid) 19 control (termination)

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Review Article

The reproductive tract microbiota in pregnancy

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The reproductive tract microbiota plays a crucial role in maintenance of normal pregnancy and influences reproductive outcomes. Microbe–host interactions in pregnancy remain poorly understood and their role in shaping immune modulation is still being uncovered. In this review, we describe the composition of vaginal microbial communities in the reproductive tract and their association with reproductive outcomes. We also consider strategies for manipulating microbiota composition by using live biotherapeutics, selective eradication of pathogenic bacteria with antibiotics and vaginal microbiota transplantation. Finally, future developments in this field and the need for mechanistic studies to explore the functional significance of reproductive tract microbial communities are highlighted.

Introduction

Infection has long been recognised as an important risk factor of poor reproductive success. In early pregnancy, infection is implicated in 15% of early and 66% of late miscarriage [1]. Towards the end of pregnancy, it is associated with approximately 40% of all preterm birth cases [2]. Like other body niches, the lower reproductive tract has co-evolved alongside a rich microbial community that has permitted the formation of important symbiotic relationships that play a crucial role in health and disease [3,4]. While clinical microbiology has enabled the identification of specific reproductive tract pathogens associated with increased risk of adverse pregnancy outcomes (e.g. *Chlamydia trachomatis* [5]), recent application of high-throughput bacterial DNA sequencing methods has deepened our understanding of how microbiota composition and host interactions effect pregnancy outcomes.

Techniques used to characterise the reproductive tract microbiota

The 21st century has seen a dramatic improvement in our ability to study the human microbiome because the limitations of culture and microscopy-based investigations have largely been superseded by molecular-based approaches, many of which are based upon high-throughput sequencing of bacterial DNA. Culture-based techniques, which have been used since the early 20th century, are labour-intensive and provide a limited view of the diversity of bacteria in any particular body site. The great majority of bacteria present in the human body require very specific culture conditions which makes comprehensive analysis of bacterial communities by culture almost impossible. Although more sophisticated culture approaches using enhanced culture techniques and microbial culture chips have been developed, the growth of some organisms depends on the metabolic activity of others, which leads to a number of limitations to these techniques [6]. High-throughput DNA sequencing approaches have become increasingly affordable enabling their widespread use for characterisation of complex microbial communities and estimation of the relative abundances of microorganisms in a given body site. Two main sequencing strategies have emerged. Firstly, shotgun metagenomics involves sequencing whole community DNA (bacterial, viral, fungal and host). This technique has the advantage that it potentially explores the genetic diversity and

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function of the microbiota, and is not limited just to bacteria. A disadvantage is that under certain circumstances, a significant proportion of the DNA being sequenced is of host origin. A more widely used technique, commonly termed metataxonomics, metabarcoding or amplicon sequencing, focuses on sequencing and amplifying specific regions of the bacterial 16S ribosomal RNA gene (16S rRNA) [3,6]. This gene is present in all bacterial species in a variety of copy numbers. It consists of nine variable regions flanked by regions of greater homology across bacterial species. PCR primers can be designed to hybridise to the conserved regions and amplify across one or more variable regions. The identity of the microorganism at genus, species and sometimes strain level can be determined from the DNA sequence of the variable region [6,7].

Metataxonomics-based characterisation of vaginal microbiota communities

It has been long established that the vagina is not a sterile environment. Gustav Doderlein first described Lactobacilli in 1892 and subsequent work has shown that Lactobacilli dominate most vaginal microbial communities [8]. There is a large body of evidence to suggest that microbial communities that colonise the vagina promote homoeostasis and have a substantial impact on reproductive health [9–11]. Taxonomic profiles of vaginal microbial communities can be sorted into a discrete number of categories based on hierarchical clustering of the pairwise distances between samples. This is advantageous because collapsing a hyperdimensional taxonomic profile into a single categorical variable facilitates data exploration, epidemiological studies and statistical modelling. One of the first studies to apply this approach to vaginal microbial communities was by Ravel and colleagues [12] who examined samples taken from 396 asymptomatic reproductively aged women. At species level, hierarchical clustering analysis characterised the vaginal microbiota into five community state types (CSTs), four of which were characterised by high relative abundance of specific *Lactobacillus* species [12]: CST I – *Lactobacillus crispatus* (*L. crispatus)*, CST II – *Lactobacillus gasseri (L. gasseri)*, CST III – *Lactobacillus iners* (*L. iners)*, CST IV – *'high diversity'*, CST V – *Lactobacillus jensenii (L. jensenii).* The CST IV (high diversity) group was characterised by a low abundance of *Lactobacillus* spp. and an overrepresentation of anaerobic bacteria such as *Atopbium, Prevotella, Sneathia, Gardnerella* and *Mobiliuncus* [13]. A number of these taxa have been associated with bacterial vaginosis (BV), a polymicrobial disorder that is associated with preterm birth [14], higher risk of acquiring sexually transmitted infections [15] and late miscarriage [16–19].

Other studies using different patient populations have used various forms of clustering analysis to define vaginal microbiome groups or vagina community states specific to those individual patient populations. Recently Ravel and colleagues [20] sought to standardise and advance the assignment of samples to CSTs by creating VALENCIA (VAginaL community state typE Nearest Centroid clAssifier), a nearest centroid-based tool which classifies samples based on their similarity to a set of racially, ethnically and geographically diverse reference datasets. This approach allows any individual microbiota community to be assigned to 1 of 13 CSTs. There are six *Lactobacillus* spp. dominant CSTs, I-A, I-B, II, II-A, II-B and V which correspond to the original CST defined by Ravel and colleagues [12], but with the designation expanded to allow for community states that contain a combination of organisms. The original *Lactobacillus* spp. depleted CST IV is expanded in VALENCIA into CST IV-A, IV-B and five CST IV-C (0–4), to account for the variety of different *Lactobacillus* spp. deplete bacterial communities. *The main advantage of this classification system is that it can characterise the vaginal microbiome in a standardised way to allow comparison of different study datasets. There is a move away from individual classification systems that are not comparable and a drive towards laying different datasets on to this framework.*

Vaginal microbiota and reproductive outcomes

The composition of the vaginal microbiota in pregnancy displays a higher abundance of *Lactobacillus* spp. and more stability throughout the entire pregnancy. In both pregnant and non-pregnant women, the vaginal microbiota can fluctuate and transition from one CST to another. There are a variety of factors, such as ethnicity, hygiene practises, hormonal fluctuation and contraceptives, that influence the structure and composition of the vaginal microbiota. One of the first longitudinal studies that characterised the vaginal microbiota using DNA sequencing techniques in pregnant and non-pregnant women found that high diversity communities were rarely seen in pregnant women who delivered at term [13]. Even though bacterial communities in pregnancy did appear to shift between CSTs dominant in *Lactobacillus* spp., they rarely transitioned to CST IV. *Lactobacillus* spp. stability in pregnancy may represent an evolutionary adaptation to enhance reproductive fitness and protect against ascending infection. The stability may also be driven by high oestrogen levels in pregnancy as the post-partum state is characterised by a dramatic shift to less *Lactobacillus* spp. dominant communities with increased α diversity [21].

Assisted conception

Several studies have focused on how the vaginal microbiota influences assisted reproductive technology outcomes. In a prospective study of 130 infertility patients undergoing *in-vitro* fertilisation (IVF), the clinical pregnancy rate was significantly lower in women with an 'abnormal' microbiota (high concentrations of *Gardnerella vaginalis* and/or *Atopobium vaginae*) [22]. In a recent study the vaginal microbiota and metabolome was characterised in recurrent implantation failure (RIF) patients (n=27) compared with patients who achieved a clinical pregnancy with their first frozen embryo cycle (n=40). Vaginal microbiota dominant in *Lactobacillus* spp. was again related to clinical pregnancy while RIF patients had increased microbial diversity [23]. Other fertility studies have also shown that a *Lactobacillus* spp. dominant vaginal microbiota is associated with positive pregnancy outcomes. However, many of these studies have small samples sizes and heterogeneity in their patient populations [24,25].

Miscarriage

There is currently a relative paucity of data exploring the pregnancy vaginal microbiota and adverse early pregnancy events. Recent work has shown reduced abundance in *Lactobacillus* spp. and increased α diversity with first trimester miscarriage [26,27]. *Lactobacillus*-depleted vaginal microbiota also appears to be a risk factor for ectopic pregnancy [28]. There is limited evidence investigating the early placental pregnancy microbiome and how that relates to reproductive outcome. One recent study used quantitative polymerase chain reaction (qPCR) to test for presence of *Mollicutes* in endocervical swabs and placental tissue collected early in pregnancy from women experiencing miscarriage (n=89) and controls (n=20). Detection of *Mollicutes* in the placenta was associated with miscarriage and there was also a significant increase in microbial load of *Mycoplasma hominis, Ureaplasma urealyticum* and *Ureaplasma parvum* in miscarriage patients compared with the control group. While the authors proposed that this represented ascending infection of placental tissue leading to the adverse event, the presence of these organisms in cervical swabs suggests possibility of contamination during sample collection [29].

Table 1 gives examples of studies which have explored the relationship between the vaginal microbiota and early pregnancy as well as assisted reproduction.

Preterm birth

A strong body of evidence exists linking the risk of preterm birth and vaginal microbial composition, which has been reviewed in detail elsewhere [30,31]. The broad themes which emerge from these studies is that in many patient populations *Lactobacillus* spp. depletion is linked to the risk of both spontaneous preterm birth and preterm pre-labour rupture of membranes (PPROM). Several studies have shown that *L. crispatus* appears to be protective. There are also some data, largely in white Anglo-Saxon populations that *L. iners* is also a risk factor for both cervical shortening and for preterm birth. Our group has characterised the vaginal microbial communities longitudinally from 6-week-gestation and shown that dominance of the vaginal niche by non-*Lactobacillus* spp. associated with PPROM at all gestational age time points [32], highlighting that the early pregnancy microbiome can influence outcomes that occur at a later timepoint. Women who deliver at term are more likely to have a vaginal microbiota dominant in *L. crispatus* and patients who deliver preterm consistently show increased richness and diversity within the vaginal communities [33–36]. A prospective study examining the vaginal microbiota from patients with a dilated cervix pre- and post-rescue cerclage, identified reduced *Lactobacillus* spp. abundance in patients with premature cervical dilation and that that *G. vaginalis* was associated with unsuccessful rescue cerclage [37]. A recent study analysing the vaginal samples of 90 pregnancies that delivered at term and 45 spontaneous preterm birth patients, confirmed that those who delivered at term had a predominance in *L. crispatus*. The present study also found specific taxa were more abundant in preterm birth including BV associated bacterium 1 (BVAB-1), *Prevotella* species and *Sneathia amnii*. Metagenomic and metatranscriptomics analysis showed that expression of genes linked to the taxa identified by 16S rRNA and encoding for bacterial secretion systems, key in pathogenicity, was higher in the preterm birth cohort [38]. Table 2 gives examples of studies which have explored the relationship between the vaginal microbiota and risk of preterm birth.

The relationship between preterm birth and neonatal microbiota

The neonatal gut microbiota plays a crucial role in early life, especially in the maturation of the immune system and the metabolism of nutrients. *Bifidobacterium* is a key player in the neonatal gut microbiota, particularly present in vaginally delivered and breastfed infants. *Bifidobacterium* selectively digest sugars in breast milk and amino acids into lactic acid which helps to improve infant gut integrity [39]. The mode of delivery has been shown to affect the neonatal intestinal colonisation. Infants born vaginally acquire microbial communities that are related to the maternal

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Table 1 Examples of studies which explore the relationship between the vaginal microbiota and early pregnancy and assisted reproduction

Author/year	Sample	Sample size	Population	Risk of adverse outcome		Findings
				High diversity	*L. crispatus/L. dominance*	
Early pregnancy and assisted conception						
Hyman et al./2012, *J. Assist. Reprod. Genet.* [24]	Vaginal swabs	30 women	IVF	Increased diversity in those who failed to achieve clinical pregnancy	No protective effect of *Lactobacillus* spp.	The vaginal microbiota on the day of embryo transfer affects pregnancy outcome.
Haahr et al./2016, *Hum. Reprod.* [22]	Vaginal swabs	130 women (qPCR-specific for *Lactobacillus* spp., *G. vaginalis*, *A. vaginae*)	Completed IVF treatment (*n*=84)			Significantly lower clinical pregnancy rate in those patients with abnormal vaginal microbiota
Haahr et al./2019, *J. Infect. Dis.* [99]	Vaginal swabs	120 women	IVF	Clinical pregnancy and live birth rate was less likely higher diversity		Overall there was no significant association between CST and reproductive outcome
Koedooder et al./2019, *Hum. Reprod.* [100]	Vaginal swabs	192 women	IVF (fresh embryo transfer)		Higher chance of pregnancy when dominated by *L. crispatus*	Women with a lower percentage of *Lactobacillus* spp. were less likely to have successful embryo implantation
Wee et al./2019, *Aust. N.Z. J. Obstet. Gynaecol.* [101]	Vaginal swabs Cervical swabs Endometrial sample	31 women Control (*n*=16) Cases (*n*=15)	History of infertility compared with those with history of fertility			A trend towards infertile women having more *Ureaplasma* in their vagina and *Gardnerella* in cervix
Al-Memar et al./2020, *BJOG* [26]	Vaginal swabs	Miscarriage (*n*=78) Term (*n*=83)	Early pregnancy	Increased risk first trimester miscarriage		First trimester miscarriage associated with reduced *Lactobacillus* spp. dominance and increased diversity and richness
Fu et al./2020, *mBio* [23]	Vaginal swabs	RIF (*n*=27) Control (*n*=40)	RIF and Control (clinical pregnancy in first frozen embryo transfer)	Higher diversity in the RIF group	Positive correlation with pregnancy rate	Significant differences are found between RIF patients and those who were pregnant in first frozen embryo cycle
Kong et al./2020, *Front. Med.* [102]	Vaginal	Total patients (*n*=475)	IVF Pregnancy (*n*=238) Non-pregnant (*n*=237)	Increased risk of IVF failure	Higher abundance of *Lactobacillus* in pregnancy group	Age, endometrial thickness, reduction in *Lactobacillus* and overgrowth of *Gardnerella*, *Atopbium* and *Prevotella* was strongly connected with IVF success

gut and vagina. However, those born via caesarean section are mainly colonised by environmental bacteria and have lower numbers of Bifidobacteria and Bacteroides leading to lower diversity and impaired immune responses [40–42]. However, during the first year of life diet influences the gut microbiota and increases diversity [43]. Many studies have also shown that intestinal microbiota of preterm infants show less bacterial diversity, especially in the context of necrotising enterocolitis and late-onset sepsis, and differ considerably from the healthy term infant [44,45]. A longitudinal study collecting faecal samples in breast-fed extreme and moderately/very preterm infants (median gestational age: 26 and 30 weeks respectively) found that gestational age was the main driver of microbiota development. The predominance of *Enterococcus* was seen in the extremely premature infants but a transition to *Bifidobacterium* dominance occurred with increasing gestational age in both groups despite the mode of delivery. Antibiotics caused temporary changes in the microbial composition but there was recovery within 2–3 weeks. [46].

Group B *Streptococcus* (GBS) still remains one of the most common causes of neonatal sepsis. The most predictive factor is the presence of GBS in the maternal genital tract during childbirth [47]. A study investigating the relationship between GBS and the vaginal microbial composition in 428 non-pregnant patients found no correlation between CSTs and GBS status. However, specific taxa such as *Streptococcus* spp., *Prevotella biva* and *Veillonella* spp.

Table 2 Examples of original research articles that explore the vaginal microbiota in relation to preterm birth

Author/year	Sample	Sample size	Population	Risk of adverse outcome		Findings
				High diversity	*L. crispatus*/*L. dominance*	
Preterm birth						
Hyman et al./2013, *Reprod. Sci.* [103]	Vaginal swabs	Term (*n*=66) Preterm birth (*n*=17)	Low and high risk for preterm birth	Increased risk in White patients		Higher *Lactobacillus* spp. content in low versus high risk for preterm birth
Romero et al./2014, Microbiome [11]	Vaginal swabs	Term control (*n*=72) Spontaneous preterm birth <34 weeks (*n*=18)	n/a	No	No	Increased relative abundance of *Lactobacillus* spp. as pregnancy progressed No difference in bacterial taxa between those who delivered at term or had preterm birth
Petricevic et al./2014, *Sci. Rep.* [104]	Vaginal swabs	Term (*n*=98) Preterm (*n*=13)	Low risk pregnant women sampled in early pregnancy		Decreased risk of preterm birth	*L.iners* as a single *Lactobacillus* spp. in early pregnancy may be involved in preterm birth
DiGuilio et al./2015, *Proc. Natl. Acad. Sci. U.S.A.* [105]	Vaginal swabs	Term (*n*=34) Preterm (*n*=15)	Low risk for preterm birth	Increased risk for preterm birth		Risk for preterm birth was higher in those with CST 4 followed by raised *Gardnerella* and *Ureaplasma* abundance
Nelson et al./2016, *Am. J. Perinatol.* [106]	Vaginal swabs	Term (*n*=27) Preterm (*n*=13)	Nulliparous Black women	No		The Shannon diversity index was not significantly different between the groups
Kindinger et al./2017, *Microbiome* [33]	Vaginal swabs	Term (*n*=127) Preterm birth <34 weeks (*n*=18) Preterm birth 34–37 weeks (*n*=16)	High risk	No	*L. crispatus* shown to be protective against preterm birth	*L. iners* dominance at 16 weeks is a risk factor for preterm birth (<34weeks). Cervical shortening and preterm birth were not associated with vaginal dysbiosis.
Stout et al./2017, *Am. J. Obstet. Gynaecol.* [35]	Vaginal swabs	Term (*n*=53) Preterm (*n*=24)	Mixed risk for preterm birth Predominately Black women. Preterm cohort included PTL, PROM and pre-eclampsia	Decrease in diversity in preterm birth group		Preterm birth is associated with increased vaginal microbiome instability compared with term. No distinct bacterial taxa correlated with preterm birth
Callahan et al./2017, *Proc. Natl. Acad. Sci. U.S.A.* [107]	Vaginal swabs	Term (*n*=85) Mainly White (*n*=30) Black (*n*=55) Preterm (*n*=50) Mainly White (*n*=9) Black (*n*=41)	White low risk and Black at high risk of preterm birth	Increased diversity in preterm birth within the predominately White cohort	*L. crispatus* low risk of preterm birth in both cohorts	Co-occurrence between *L. iners* and *Gardnerella*. No co-occurrence between *L. crispatus* and *Gardnerella*
Frietas et al./2018, *Microbiome* [10]	Vaginal swabs	Term (*n*=170) Spontaneous preterm birth (<37 weeks) (*n*=46)	Mixed risk cohort	Higher risk of preterm birth in those with increased diversity		No community structure predicted spontaneous preterm birth but there was an increase in diversity and *Mollicutes* prevalence
Brown et al./2018, *BMC Med.* [50]	Vaginal swabs	Term (*n*=20) PPROM (*n*=102) Before PPROM (*n*=15) After PPROM (*n*=87)	High risk (recruited from prematurity surveillance clinic)	Increased risk of PPROM and exacerbated by erythromycin treatment		*Lactobacillus* spp. depletion and *Sneathia* spp. were associated with early-onset neonatal sepsis
Tabatabaei et al/2019 BJOG [36]	Vaginal swabs	Term (*n*=356) Spontaneous preterm birth (*n*=94) <34 weeks (*n*=17) 34–36 weeks (*n*=77)	Low risk preterm birth	Increased risk of early (<34 weeks) but not late (34–36 weeks) preterm birth	Decreased risk of early (<34 weeks) but not late (34–36 weeks) preterm birth	Vaginal *Lactobacillus* and *Bifidobacterium* may lower the risk of preterm birth

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Table 2 Examples of original research articles that explore the vaginal microbiota in relation to preterm birth (Continued)

Author/year	Sample	Sample size	Population	Risk of adverse outcome		Findings
				High diversity	*L. crispatus/L. dominance*	
Brown et al./2019, *Transl. Res.* [32]	Vaginal swabs	Term (*n*=36) PPROM (*n*=60)	Pregnant women high (*n*=38) and low (*n*=22) risk of preterm birth	Increased risk of PPROM	Decreased risk of PPROM	Increased instability of bacterial communities in PPROM patients in second trimester (increased *Prevotella*, *Streptococcus*, *Peptoniphilus* and *Dialister*
Elovitz et al./2019, *Nat. Commun.* [108]	Vaginal swabs	Term (*n*=432) Spontaneous preterm birth (*n*=107)	Mixed risk cohort for preterm birth	Increased risk of preterm birth in White women	Protective role in all subjects	Certain bacterial taxa were significantly associated with increased spontaneous preterm birth; *L. iners* and *A. vaginae* in white women and *M. curtsii/mulieris* and *Mageebacillus indolicus* in Black women
Brown et al./2019, *BJOG* [37]	Vaginal swabs	Term (*n*=30) Exposed foetal membranes (*n*=20)	High-risk patients undergoing rescue cerclage	Increased risk of cervical dilation and exposed membranes	Reduced risk	*G. vaginalis* prior to rescue cerclage was associated with cerclage failure
Fettweis et al./2019, *Nat. Med.* [38]	Vaginal swabs	Term (*n*=90) Preterm (*n*=45)	Predominately African ancestry (term and preterm cohort)	Increased risk of preterm birth	Decreased risk of preterm birth	Women who delivered preterm had higher levels of BVAB-1, *Sneathia amnii* and a group of *Prevotella* spp.
Payne et al./2021, *Am. J. Obstet. Gynaecol.* [109]	Vaginal swabs	Term (*n*=818) Spontaneous preterm birth (*n*=58) Non-spontaneous preterm birth (*n*=60)	Mainly White women	Increased risk of spontaneous preterm birth	Decreased risk of preterm birth	*G. vaginalis*, *L. iners* and *U. parvum* were strongly predictive of spontaneous preterm birth

Abbreviation: PTL, preterm labour.

were associated with GBS colonisation [48]. A study exploring the neonatal gut microbiota in GBS-positive women found enrichment of Enterococcaceae, Clostridiaceae and Ruminococcoceae in the infant gut at 6 months. However, long-term follow-up will be required to see whether these differences lead to adult disease later in life [49]. A prospective study examining the vaginal composition before and after PPROM correlated the findings to early-onset neonatal sepsis (EONS). The vaginal microbiota prior to delivery in those cases of placental chorioamnionitis and funisitis found an enrichment of *Prevotella, Sneathia, Peptostreptococcus* and *Catonella* spp. and reduced *Lactobacillus* spp. compared with patients with normal histology. In the cases with EONS, the maternal vaginal microbiota prior to delivery was enriched with *Catonella* spp *and Sneathia spp*. whereas *L. crispatus* was overrepresented in those who did not develop EONS [50]. This highlights the key involvement of the vaginal microbiota in the development of preterm neonatal sepsis and the potential role for modifying the composition to positively influence neonatal outcome.

Placental microbiota

Much recent research attention has been directed toward establishing whether there is a physiologically normal and functional placental microbiome, abnormalities or imbalances of which may contribute to adverse pregnancy outcome. The belief that reproduction occurs in a sterile environment was supported by studies using culture-based techniques, which failed to detect bacteria in the placenta of healthy pregnancies [51]. However, using highly sensitive bacterial DNA sequencing approaches, Aagaard et al. published the first study that proposed a unique non-pathogenic placental microbiota niche [52] and that the placental microbiota that differed between term and preterm deliveries [53–55]. While it was originally suggested that that these organisms contribute to metabolic function, their low biomass in the placenta questioned the physiological significance. Organisms reported to contribute to the placental

microbiota included those commonly found in soda lakes and marine environments [30] suggesting that this apparent placental microbiome is actually reagent and laboratory contamination and that apparent differences, for example between term and preterm cases, were due to batch effects [56]. Recent studies have addressed this issue by extensively matching the cases being investigated with laboratory controls and could not identify a microbiota within the placenta that was distinguishable from background technical controls [57,58]. Even in studies where distinct bacteria have been detected by molecular techniques, it is unclear whether these are viable organisms or dead material. The placenta has a role in removing organisms and there is a risk such sensitive techniques are amplifying these cleared microbes. The anatomical, physiological and immunological barriers that exist at the maternal–foetal interface to prevent microbial invasion also argue against the likelihood of a normal placental microbiome. If a unique microbiome existed in the placenta an immunologically naïve foetus could be overwhelmed [51]. Therefore, there may be bacteria present at a low level in the placenta but given the function of the placenta, normal bacterial colonisation and development of a placental niche seems unlikely [59].

A recent study by Goffau et al. [60] found no evidence of bacterial signals from placental samples of pregnancies complicated by preterm birth, pre-eclampsia and small for gestational age infants (n=318) or uncomplicated pregnancies (n=219). This study found that the main source of bacterial DNA was from the laboratory reagents. However, *Streptococcus agalactiae* (GBS) was detected using 16S rRNA amplification and verified by metagenomics and qPCR, in the placenta of 5% of women prior to the onset of labour, although there was no association with complicated pregnancies. Therefore, while the study concluded that a resident placental microbiome did not exist, bacterial placental colonisation can be present although is unlikely to contribute to the majority of complicated pregnancies. Studies that have examined pathogens such as *Salmonella* on human placental villous explants from different gestational ages demonstrated that the bacterial burden was highest in the first trimester explants. Therefore, the first trimester may be a more vulnerable time and placental colonisation infection needs to be carefully considered in relation to poor outcomes at this gestation [61]. In the case of spontaneous preterm birth associated with chorioamnionitis-specific bacteria such as *Mycoplasma* spp. and *Ureaplasma* spp. have been identified in the placenta [62,63]. Therefore, while the evidence to support a functional placental microbiota is weak, in some cases there may be placental pathogenic colonisation seeded from ascending vaginal infection or haematogenous spread. It is also notable that the majority of studies investigating evidence of placental microbial colonisation have focused on term and preterm placentas collected vaginally or by caesarean section. Table 3 summarises the current evidence regarding the placental microbiome in pregnancy at any gestational age.

Endometrial microbiome in reproduction

An increasing body of work has focused on the existence of the endometrial microbiome. While many studies are confounded by vaginal contamination and low biomass there is emerging evidence that the lower uterine microbiome is distinct and could be contributed to by the vaginal microbiota [64]. A systematic review assessing the effect of the endometrial microbiome on artificial reproductive technologies (ARTs) showed that an abnormal endometrial microbiome was associated with poor ART outcomes [65]. More recent work has described the use of molecular approaches to characterise the endometrial microbiota at the time of single euploid embryo transfer which amplified bacteria within the embryo catheter tip [66]. A study evaluating paired endometrial fluid and vaginal aspirates samples in 13 women found different bacterial genera in the uterine cavity compared with paired vaginal samples. The presence of non-*Lactobacillus* dominated microbiota in the endometrium was also associated with decreased implantation and live birth rates [67]. These findings were corroborated in slightly larger studies where a non-*Lactobacillus* dominated endometrial microbiota was higher in infertile patients [68]. Nonetheless small sample sizes and limited laboratory contamination controls have not been able to address the impact of cross-contamination from the high biomass in the vagina. The relatively low biomass in the uterine cavity can also lead to molecular techniques being susceptible to background contamination. Studies have tried to account for these limitations by collecting samples from abdominal hysterectomies and incorporating extraction kit controls. Winters et al., reported a resident microbiota in the middle endometrium of 60% of women undergoing a hysterectomy that principally consisted of *Acinetobacter*, *Pseudomonas*, Comamonadaceae and *Cloacibacterium* that were not present in background technical controls, or other body sites except the cervix [69]. Studies that collect samples abdominally also corroborate these microbial profiles and rarely detect high levels of *Lactobacillus* spp. within the endometrium [64]. Nonetheless these results need to be verified by complementary techniques such as microscopy and culture and further studies are required to understand the signalling pathways activated by these microbes and the metabolites synthesised to appreciate the impact on reproduction and fertility [70]. Table 4 demonstrates the current studies to date that have evaluated the endometrial microbiota.

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Table 3 Examples of current evidence to date investigating the human placental microbiota at any gestation

Author/year	Sample	Sample size	Mode of delivery	Techniques	Contamination Control	Findings
Aagaard et al./2014/Science translational medicine [52]	Villous tree	Term (*n*=231) Preterm (*n*=89)	Term Caesarean (*n*=53) Term Vaginal (*n*=178) Preterm Caesarean (*n*=20) Preterm Vaginal (*n*=69)	16S rRNA gene sequencing V1-3 Metagenomics (subset *n*=48)	1 blank extraction kit per 11 placental samples (no bands routinely amplified) Reagents were sequenced when non-human sources were identified but details not provided	There is a unique low abundance placental microbiome. There are observed similarities in non-pregnant oral and placental microbiomes. The placental microbiome differs between preterm and term women and in those with and without antenatal infection
Doyle et al./2014/Placenta [54]	Placental membranes (chorion and amnion)	Spontaneous preterm birth (*n*=14) Term (*n*=10)	Preterm Vaginal (*n*=14) Term Caesarean (*n*=4) Term Vaginal (*n*=6)	16S rRNA gene sequencing V1-2 and V5-7	No	Bacterial DNA present in preterm and term placental membranes irrespective of mode of delivery A consistently identifiable bacterial species in preterm labour
Antony et al./2015/Am J Obstet Gynecol. [55]	1 × 1 × 1 cm cuboidal section excised from different areas of placenta	Term (*n*=175) Preterm (*n*=62)	Caesarean (*n*=54) Vaginal (*n*=183)	16S rRNA gene sequencing V1-3	No	Excess gestational weight gain associated with altered placental microbiome and metabolic profile in preterm birth patients
Zheng et al./2015/Nutrients. [110]	Placenta 4 × 1 cm^3 cuboidal sections (decidua and foetal chorion discarded)	Low birth weight <3 kg (*n*=12) Normal birth weight ≥3 kg to <4 kg (*n*=12)	Vaginal (*n*=24) Caesarean (*n*=0)	16S rRNA gene sequencing V3-4	No	There is a placental microbiome. The placentas of low birthweight neonates had lower bacterial richness and evenness compared with normal birthweight neonates
Bassols et al./2016/Pediatric research [111]	Villous tree	Gestational Diabetes (*n*=11) Without Gestational Diabetes (*n*=11)	Vaginal (*n*=22) Caesarean (*n*=0)	16S rRNA gene sequencing V3-4	No	A distinct microbiota profile is present in the placental samples of patients with gestational diabetes
Collado et al./2016/Scientific reports [53]	Placenta Amniotic fluid Colostrum Meconium	Infant mother pairs (*n*=15)	Term Caesarean (*n*=15)	16S rRNA gene sequencing V1-3 Anaerobic culture of placenta and amniotic fluid samples	No	Placenta and amniotic fluid harbour unique microbial communities. Meconium shares features with the microbiota in placenta, amniotic fluid and colostrum. Foetal intestinal colonisation could be initiated *in utero*. *Staphylococcus* and *Propionibacterium* were cultured from placenta
Lauder et al./2016/Microbiome. [57]	Placenta (basal plate biopsy and foetal side biopsy)	Term (*n*=6)	Caesarean (*n*=1) Vaginal delivery (*n*=5)	16S rRNA sequencing V1-2 qPCR	Laboratory air swabs (*n*=11) Sterile swabs (*n*=8) Blank extraction kits (*n*=8)	Microbial signatures in placental tissue could not be distinguished from technical controls
Prince et al./2016/Am J Obstet Gynecol [63]	Swabs from chorion or villous membrane adjacent to foetal side	Term (*n*=27) Preterm (*n*=44) Term Chorioamnionitis (*n*=12) Preterm Chorioamnionitis Mild (*n*=11) Severe (*n*=20)	Term Cesarean (*n*=7) Term Vaginal (*n*=20) Preterm Caesarean (*n*=7) Preterm vaginal (*n*=37)	Metagenomics Culture for *Ureaplasma* or *Mycoplasma spp.*	No Only yields with high reads were included in analysis without concern for contamination	Spontaneous preterm birth patients have a placental microbiota that differed by severity of chorioamnionitis

Continued over

Table 3 Examples of current evidence to date investigating the human placental microbiota at any gestation (Continued)

Author/year	Sample	Sample size	Mode of delivery	Techniques	Contamination Control	Findings
Doyle et al./2017/Placenta. [112]	Amnion and Chorion	1097 subjects *Rural Malawi setting	Unreported vaginal, caesarean, preterm and chorioamnionitis cases	16S rRNA gene sequencing V5-7 qPCR	Reagents from blank extraction kit sequenced for every ten extractions. Only placental samples that were positive for bacterial DNA (defined as 40 CFU/μl) were sequenced. Sample processing delays increased the chance of positive qPCR	A distinct placental microbiome exists. 68.1% of amnion–chorion and 46.8% placental samples had positive qPCR. A varied placental microbial structure is associated with severe chorioamnionitis. The source of bacteria in the placenta overlapped with the vagina and not the oral cavity
Gomez-Arango et al./2017/Scientific reports [113]	Placental biopsy from foetal side. Matched oral and faecal samples	37 patients Overweight (*n*=13) Obese (*n*=24)	Term Caesarean (*n*=17) Term Vaginal (*n*=20)	16S rRNA gene sequencing V6-8	Reagent, DNA extraction and PCR control pooled and sequenced for each kit type. Any OTUs detected were removed from analysis	A placental microbiome was identified irrespective of mode of delivery. Placental communities shared more similarity to oral microbiome than gut but this declined with each taxonomic level
Parnell et al./2017/Scientific reports. [114]	Placenta: Basal plate Villous tree Foetal membrane	57 Term Women	Term Cesarean (*n*=34) Term Vaginal (*n*=23)	16S rRNA gene sequencing V1-9 (V7/8 did not amplify.V1,5,9 amplified less than half and V2 showed environmental contaminants) qPCR conducted on V4 region	Water Control *n*=5 and Regent test blanks *n*=8 Negative controls occasionally had 34 copies/μl. Only positive qPCR in placental samples were included (if >34 copies/μl)	Tissue-specific profiles identified in placental microbiome. Variation is seen in the placental microbiota between amnion–chorion and basal plate.
Zheng et al./2017/Oncotarget [115]	Placenta 4 × 1 cm^3 cuboidal sections (decidua and foetal chorion discarded)	Term without macrosomia (*n*=10) Macrosomia birth weight > 4 kg (*n*=10)	Caesarean (*n*=20)	16S rRNA gene sequencing V3-4	No	Distinct placental microbiota profile in foetal macrosomia
Leon et al./2018/Appl Environ Microbiol [62]	Placental Villous tree	256 patients Term (*n*=165) Preterm (*n*=91)	Caesarean Term (*n*=81) Vaginal Term (*n*=84) Caesarean Preterm (*n*=55) Vaginal Preterm (*n*=36)	16S rRNA gene sequencing V5-7	Negative extractions and PCR blanks were examined. Samples ≥ 500 reads were analysed (*n*=19)	Low level relatively diverse placental microbial signature is present in normal and complicated pregnancies. There was overlap between technical controls and placental samples A unique preterm placenta did not exist but *Ureaplasma* and *Mycoplasma* enriched the spontaneous preterm birth cohort.
Seferovic et al./2019/Am J Obstet Gynecol [116]	Placental villous tree	53 patients Term (*n*=26) Preterm (*n*=26) One positive control with histological chorioamnionitis	Term Caesarean (*n*=22) Term Vaginal (*n*=4) Preterm Caesarean (*n*=8) Preterm Vaginal (*n*=18)	*In situ* hybridisation against conserved region of 16 S ribosome. 16S rRNA sequencing V4 Metagenomics	Environmental swab cultures (inside and outside placental containers). Kit-negative extractions, *n*=6	Very low biomass bacteria were observed by histological and 16S rRNA gene sequencing distinct from environmental controls. Unclear if commensal microbial abundance varies in preterm and term pregnancies. Viability of organisms unknown

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Table 3 Examples of current evidence to date investigating the human placental microbiota at any gestation (Continued)

Author/year	Sample	Sample size	Mode of delivery	Techniques	Contamination Control	Findings
De Goffau et al./2019/Nature [60]	Placental terminal villi	537 women Adverse pregnancy outcome (*n*=318) Controls (*n*=219)	Caesarean SGA (*n*=20) PET (*n*=20) Control (*n*=40) Vaginal and Caesarean SGA (*n*=100) PET (*n*=100) Control (*n*=198) Preterm (*n*=100)	16S rRNA V1-2 Metagenomics qPCR for *S. agalactiae*	Positive control using *S. bongori* to compare 16S rRNA with metagenomics For each DNA isolation kit extraction blanks were carried out	No evidence to support a placental microbiome. No relationship between placental infection and SGA, PET or preterm birth. The major source of bacterial DNA in the samples was contamination from laboratory reagents. The only organism consistently present in the placenta of 5% of women prior to labour (detected by three methods) was *S. agalactiae*
Theis et al./2019/Am J Obstet Gynecol [58]	Amnion–chorion plate Villous tree	Healthy Term women (*n*=29)	Term Caesarean (*n*=29)	16S rRNA V4 qPCR Metagenomic surveys Bacterial culture	DNA extraction kits (*n*=6) Laboratory environmental controls (*n*=16) Operating rooms (*n*=21)	No consistent evidence the placenta harbours a unique microbiota. 28/29 placental samples did not yield bacterial cultivars 18 prominent OTUs accounted for 90% of placental tissue and 86.4% of background technical controls There were no consistent differences in the composition of placental samples and technical controls
Gschwind et al./2020/PLoS One [117]	Chorionic villi Umbilical cords Foetal membranes	Healthy Term pregnancy (*n*=38)	Caesarean (*n*=29) Vaginal (*n*=9)	16S rRNA V8-9 qPCR V4 Bacterial culture Metagenomics	16 Extraction blanks (*n*=16) Reagent extraction kit controls (*n*=3) Culture media and incubation condition controls (*n*=38)	Placenta does not harbour specific consistent functional microbiota No significant viable bacteria or bacterial DNA in the *in utero* samples collected from caesarean section
Sterpu et al./2020/Am J Obstet Gynecol [59]	Placenta (maternal, middle and foetal side) Saliva Vaginal Rectal Amniotic fluid Vernix	76 Term pregnancies	Term Caesarean (*n*=50) Term Vaginal (*n*=26)	Metagenomics qPCR 16S rRNA V6-8 Bacterial culture	PCR reagents DNA extraction controls	16S rRNA gene sequencing and qPCR found bacterial signals that were not distinguishable from background controls No meaningful comparisons could be made to oral, faecal or vaginal samples Very few genera detected by16S rRNA sequencing could be confirmed by culture
Olomu et al./2020/BMC Microbiol. [118]	Parenchymal placental tissue Vaginal Rectal Maternal blood Cord blood	Term patients (*n*=47) GDM (*n*=16) Obese (*n*=16) Normal weight (*n*=15)	Term Caesarean (*n*=47)	16S rRNA V3-4 qPCR	Multiple negative or blank controls. Sterile swabs exposed to operating rooms or air in sampling room. Reagent, Kit and sequencing reaction controls	No distinct microbiome existed in placental samples that differed from blank controls An additional source of cross contamination was identified from high biomass samples being analysed adjacent to low biomass samples

Continued over

Table 3 Examples of current evidence to date investigating the human placental microbiota at any gestation (Continued)

Author/year	Sample	Sample size	Mode of delivery	Techniques	Contamination Control	Findings
Oliveira et al./2020/Epidemiol Infect [29]	Endocervical swabs Placental tissue	Miscarriage patients (*n*=89) Control with no history of miscarriage (*n*=20)	Miscarriage patients undergoing curettage 8–20 weeks gestation (*n*=89) Term pregnancies vaginal deliveries (*n*=20)	qPCR to detect *M. genitalium, M. hominis, U. parvum, U. urealyticum* and *N. gonorrhoeae*	No	Women with *Mollicutes* detected in placenta had a seven-fold higher chance of miscarriage. A positive association between *U. parvum* in placental tissue and miscarriage

Abbreviations: CFU, colony forming unit; GDM, gestational diabetes mellitis; OTU, operational taxonomic unit; PET: pre-eclampsia; SGA, small for gestational age.

Mechanisms that link the vaginal microbiota to pregnancy outcomes

Many of the studies exploring the link between the vaginal microbiota and pregnancy outcomes have been associated with little insight into the mechanisms that trigger adverse events. Nonetheless, the protective effects of *Lactobacillus* species against pathogen colonisation are quite well described. Lactobacilli utilise breakdown products of glycogen within the vagina to produce lactic acid which creates an acidic pH that deters the growth of many other bacteria, as well as up-regulating autophagy which clears intracellular pathogens from vaginal epithelial cells [71]. Lactobacilli also produce bacteriocins to eliminate other bacteria and strengthen their dominance [72,73]. *L. crispatus*, *L. gasseri* and *L. jensenii* produce both the L and D-isomers of lactic acid whereas *L. iners* has a smaller genome that lacks the gene encoding enzyme required to produce D-lactic acid. Relevant to reproductive tract health, the D-isomer of lactic acid has been shown to down-regulate matrix metalloproteinase-8 (MMP-8) production which breaks down the cervical plug that inhibits entry of bacteria to the upper genital tract [71]. Moreover, vaginal microbiota dominant in *L. crispatus* demonstrate increased autophagy and lower cellular stress compared with women dominated by *L. iners* [74]. Therefore even within the *Lactobacillus* genera, certain species such as *L. iners* are not as effective at out competing other species and thus associated with transitions to 'high diversity' microbial states [75]. A recent study investigating the interaction between the different strains of Lactobacilli and decidualised endometrial cells *in vitro* found that *L. crispatus* was significantly more successful at attaching to the host cells compared with other *Lactobacillus* strains. In addition the interaction between *Lactobacillus* and endometrial cells did not cause inflammation or host cell death [76].

Many studies have focused on the correlation between high-diversity vaginal microbial composition and inflammatory mediators as an explanation for poor outcomes. Kindinger et al. [77] reported in a case–control study of nearly 700 patients that pregnancy outcome in women at high risk of preterm birth who had undergone cervical cerclage was highly dependent upon the suture material used for the procedure. Use of the commonly used braided suture material, compared with monofilament material, was associated with increased risk of both intrauterine foetal death and preterm birth. The braided material was shown to induce, in some women, a persistent shift towards a reduced *Lactobacillus* spp. composition and enrichment of pathobionts. This was associated with increased inflammatory cytokines and interstitial collagenase excretion into the cervicovaginal fluid and early remodelling of the cervix. This uncovered how the interaction with the host could induce an adverse microbial composition and therefore alter reproductive outcomes. Other longitudinal cohort studies have also demonstrated that preterm birth associated taxa correlate with pro inflammatory cytokines in the cervicovaginal fluid [38], although this is influenced by host ethnicity and probably genetics and the ultimate adverse outcome involves the interplay between the microbiota and immune system.

Modifying the cervicovaginal microbiota

Antibiotics

The current international guidance for the treatment of vaginal conditions such as BV recommends metronidazole, clindamycin or tinidazole administered orally or vaginally. However, high recurrence rates are still reported following treatment and many studies report antimicrobial resistance [78]. Antibiotics themselves may be directly harmful in early pregnancy and can increase the risk of spontaneous miscarriage. Macrolides, quinolones and tetracyclines all increased the risk of miscarriage and should be given with caution [79]. However, the large body of evidence that

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Table 4 Examples of current evidence exploring the endometrial microbiota in reproduction

Author/year	Sampling	Sample size	Population	Techniques	Contamination controls	Findings
Kyono et al./2019, *Reprod. Med. Biol.* [119]	Endometrial fluid samples collected using IUI catheter	92 women	IVF	Endometrial flora test (Varinos Inc.)	No	Pregnancy rates were not significantly different between *Lactobacillus* dominant and non-dominant groups
Grau et al./2019 *Pathogens* [120]	Endometrial fluid (six samples from one patient)	Case report	Infertile patient with history of ectopic pregnancy and two miscarriages	16S rRNA sequencing and whole metagenome sequencing	No	This patient had persistent endometrial *G. vaginalis* colonisation and virulence genes involved in biofilm and antibiotic resistance
Liu et al./2019 *Fertil. Steril.* [121]	Endometrial biopsy and fluid (7 days after LH surge)	130 infertile women	Infertile women with chronic endometritis (*n*=12) and without (*n*=118)	16S rRNA sequencing V4	16 negative controls (8 air swabs and 8 collection controls) Extremely low sequence reads	Bacteria such as *Dialister*, *Bifidobacterium*, *Prevotella Gardnerella* and *Anaerococcus* are more abundant in the endometrial microbiota of women with CE than those without *Lactobacillus* spp. was more abundant in non-CE microbiota
Kyono et al./2018, *Reprod. Med. Biol.* [68]	Endometrial fluid and vaginal samples	102 women	IVF (*n*=79) Non-IVF (*n*=23) Healthy controls (*n*=7)	16S rRNA sequencing V4	No	Increased *Lactobacillus* spp. in endometrium of healthy volunteers compared with infertility patients
Hashimoto et al./2019, *J. Assist. Reprod. Genet.* [122]	Endometrial fluid	99 women	IVF	16S rRNA sequencing V4	Yes-blank extraction controls and known regent contaminants such as *Sphingomonas* and *Stenotrophomonas* were excluded	No difference in pregnancy or miscarriage rate between eubiotic or dysbiotic endometrium
Winters et al./2019, *Sci. Rep.* [69]	Mid endometrial Rectal Vaginal samples	25 women	Patients having a hysterectomy primarily for fibroids	16S rRNA sequencing V1-2 and qPCR	Background DNA controls	60% of the mid endometrial samples had a bacterial load that exceeded background controls and was distinct from other body sites
Chen et al./2017, *Nat. Commun.* [64]	Endometrial Vagina Cervical mucus Peritoneal fluid Fallopian tubes	95 women having surgery for non-infectious conditions	Samples from peritoneal and uterine sites were taken during abdominal surgery	16S rRNA sequencing V4-5 qPCR	Negative controls (sterile swabs from surgeons gloves, and patients skin) Negative laboratory controls	Distinct communities were identified in uterus, fallopian tubes, peritoneal fluid that differed from the vagina
Kitaya et al./2019, *Mediators Inflamm.* [123]	Endometrial fluid and vaginal samples	46 women	History of RIF (*n*=28) Infertile women undergoing first IVF (*n*=18)	16S rRNA sequencing V4	Blank water controls Known contaminants were excluded from endometrial samples	Endometrial microbiota showed significant variation between RIF and control group
Carosso et al./2020, *J Assist. Reprod. Genet.* [124]	Vaginal Swab Embryo catheter tip	15 women	Does ovarian stimulation and progesterone supplementation modify the microbiota in women having IVF	16S rRNA sequencing V3-4-6	Blank extraction kit controls *Sphingomonas* excluded from analysis as known contaminant from previous work	Endometrial microbiota was heterogeneous Endometrial *Lactobacillus* spp. was reduced following controlled ovarian stimulation and progesterone supplements

Abbreviation: CE, chronic endometritis.

vaginal microbial composition can influence reproductive outcomes, suggests the possible therapeutic role of agents that can change that composition. Several have examined the role of antibiotics in pregnant patients with BV in relation to the risk of preterm birth. The largest randomised control trial, which screened 84530 women in early pregnancy and randomised 3105 women with BV to an oral clindamycin treatment arm and placebo arm, found no

risk reduction for late miscarriage (16–22 weeks) or spontaneous very preterm birth (22–32 weeks) [80]. A subsequent metanalysis confirmed these findings [81] although highlighted heterogeneity of the studies included, with different patient cohorts and antibiotic regimes being compared. Antimicrobial resistance genes are present in the vaginal microbiome of patients with BV symptoms may also influence the use of antibiotics in this field [82]. The formation of biofilms are now implicated in BV and the inability of antimicrobials to penetrate this matrix is also likely to result in treatment failure and resistance [83].

There is currently no evidence that antibiotic prophylaxis reduces the risk of preterm birth [84]. The ORACLE-II Study showed that, in women in spontaneous preterm labour, neither erythromycin, co-amoxiclav or a combination of the two had any effect upon a composite outcome of neonatal death, chronic lung disease, or major cerebral abnormality on ultrasonography before discharge from hospital [85]. Its follow-up study showed that the prescription of antibiotics was associated with an increase in functional impairment among their children at 7 years of age although the overall risk was low. The ORACLE-I study, in women with PPROM showed that the composite outcome of short-term respiratory function, chronic lung disease and major neonatal cerebral abnormality was reduced with the use of erythromycin. But the use of co-amoxiclav was associated with a significant increase in the occurrence of neonatal-necrotising enterocolitis [86]. At the 7-year follow-up neither antibiotic had a significant effect on the overall level of behavioural difficulties experienced, on specific medical conditions or on the proportions of children achieving each level in reading, writing or mathematics at key stage one [87]. A recent study has shown that prescription of erythromycin in after PPROM has a tendency to convert a healthy *Lactobacillus*-dominant vagina microbiota into *Lactobacillus* depletion which is then a risk factor for early adverse neonatal outcomes [50]. It is likely that for antibiotics to have any benefit in these contexts; we will need to develop tools to allow them to be properly targeted at well-phenotyped individuals.

Live biotherapeutics: probiotics

There is a growing interest in the potential to modulate the vaginal microbiota using probiotics or live biotherapeutic products. A systematic review of oral probiotic use in pregnant women at low risk for preterm birth did not find a reduction in the incidence of preterm birth (<37 weeks) [88]. Recent studies have shown that oral probiotics administered in early pregnancy do not modify the vaginal microbiota [89,90]. Subsequently, a systematic review evaluated the use of vaginal probiotics in BV and vulvovaginal candidiasis. The use of vaginal probiotics was promising in BV cure and prevention, but of the 13 studies included, 5 had medium and 8 had high overall risk of bias. There was also minimal detection of probiotic strains after the dosing period, implying a lack of colonisation. There was considerable heterogeneity in these trials in terms of probiotic strain, length of use and duration between last probiotic use and vaginal sample collection [91]. It is probably the case that it will not be possible to colonise the vagina with live biotherapeutics administered orally. The apparent protective effect of *L. crispatus* in preterm birth, and perhaps also in miscarriage and other adverse pregnancy outcomes suggests that a live biotherapeutic containing that organism and administered vaginally, might be therapeutically valuable. A recent randomised double-blind placebo controlled trial in 228 women found vaginally administered *L. crispatus* prevented BV recurrence after metronidazole treatment [92]. This work encourages future trials to focus on vaginal administration of *L. crispatus* in pregnancy to influence the vaginal composition and improve pregnancy outcome.

Vaginal microbiome transplant

Although the vaginal microbiota is much less rich and diverse than the microbiota of other body compartments, especially the gut, it nevertheless remains possible that most effective colonisation strategy would be achieved by biotherapeutic treatment using a community of organisms, rather than a single pure organism. The use of faecal microbiota transplantation has been successful in treating recurrent *Clostridium difficile* infection and this has led to subsequent interest in the use of transplanted human material to alter the vaginal microbial composition [93]. The first exploratory study that used vaginal microbiome transplantation (VMT) targeted five patients with recurrent and antibiotic non-responsive BV. In this pilot study, four of the five patients had long-term remission, which was defined as symptom improvement and microscopic vaginal fluid appearance of a *Lactobacillus*-dominated vaginal microbiome at 5–21 months after VMT. Recurrent VMT was needed in three patients but overall long lasting improvements were seen with a post-VMT compositional change dominated in *Lactobacillus* genus. These preliminary results call for randomised control trials to help understand the therapeutic efficacy in women with intractable BV. Given the small sample size the potential risks of this procedure cannot be discounted even though no adverse effects were reported [94].

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As mentioned above, the mode of delivery is also thought to have an impact on the microbial composition in newborns and associations have been reported between caesarean section deliveries and an increased risk of obesity and asthma [95,96]. Although a causal link and mechanism has not been identified, reports suggest the altered microbial composition may impact development of the host immune system and metabolism [97]. A recent pilot study explored exposing newborns to maternal vaginal contents following a caesarean section and found the neonatal gut, oral and skin microbiome was enriched with vaginal bacteria similar to those infants born vaginally. Such organisms were often underrepresented in unexposed caesarean section infants. Nonetheless the sample size was limited, and sampling was only within the first month after birth [98]. Therefore, it is unclear whether such vaginal communities continue to persist in the infant or have any influence on future disease outcomes. Given the complex nature of labour and the lack of understanding between the host–microbe interactions and neonatal immune system, further research is required to evaluate the full potential of this process.

Conclusion

There is a great deal of evidence that demonstrates the reproductive tract microbiota can influence pregnancy outcome. Nonetheless, a great deal needs to be uncovered with regard to the mechanisms that trigger adverse events and the relationship between microbial composition and the immune system. A recurring theme that populates the current literature is that *L. crispatus* is beneficial to the host and possess key properties that create a stable environment. This paves the way for therapeutic intervention that modifies the microbiome and provides exciting new developments for translational research.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

ART, artificial reproductive technology; BV, bacterial vaginosis; CST, community state type; EONS, early-onset neonatal sepsis; GBS, Group B *Streptococcus*; IVF, *in-vitro* fertilisation; PPROM, preterm pre-labour rupture of membrane; qPCR, quantitative polymerase chain reaction; RIF, recurrent implantation failure; VALENCIA, VAginaL community state typE Nearest Centroid clAssifier; VMT, vaginal microbiome transplantation.

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Chromosomally normal miscarriage is associated with vaginal dysbiosis and local inflammation

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Abstract

Background: Emerging evidence supports an association between vaginal microbiota composition and risk of miscarriage; however, the underlying mechanisms are poorly understood. We aim to investigate the vaginal microbial composition and the local immune response in chromosomally normal and abnormal miscarriages and compare this to uncomplicated pregnancies delivering at term.

Methods: We used 16S rRNA gene based metataxonomics to interrogate the vaginal microbiota in a cohort of 167 women, 93 miscarriages (54 euploid and 39 aneuploid using molecular cytogenetics) and 74 women who delivered at term and correlate this with the aneuploidy status of the miscarriages. We also measured the concentrations of IL-2, IL-4, IL-6, IL-8, TNF-α, IFN-γ, IL-1β, IL-18 and IL-10 in cervical vaginal fluid.

Results: We show that euploid miscarriage is associated with a significantly higher prevalence of *Lactobacillus* spp. deplete vaginal microbial communities compared to aneuploid miscarriage ($P = 0.01$). Integration of matched cervicovaginal fluid immune-profiles showed that *Lactobacillus* spp. depleted vaginal microbiota associated with pro-inflammatory cytokine levels most strongly in euploid miscarriage compared to viable term pregnancy (IL-1β; $P < 0.001$, IL-8; $P = 0.01$, IL-6; $P < 0.001$).

Conclusions: Our data suggest the vaginal microbiota plays an important aetiological role in euploid miscarriage and may represent a target to modify risk of pregnancy loss.

Keywords: Miscarriage, Microbiota, Infectious disease, Early pregnancy, Translational research

Background

Miscarriage, defined as pregnancy loss before the fetus reaches viability, is a distressing disorder associated with pain and bleeding as well as significant psychological morbidity. Early miscarriage (pregnancy loss before 12 weeks) occurs in one in five pregnancies of which half are due to chromosomal abnormalities [1]. Infection is implicated in 66% of late miscarriages (12–24 weeks) but is less prevalent in early miscarriage [2]. However, the mechanisms driving miscarriage in these groups are poorly defined. Despite its prevalence, there are no interventions that prevent sporadic miscarriage and only few treatments, such as progesterone supplements, have been shown to modestly reduce the recurrence risk of miscarriage in subsequent pregnancies [3].

Pregnancy has a unique and dynamic immunological milieu that is required to support a healthy pregnancy [4]. Initially, a pro-inflammatory state is required for implantation which involves a release of inflammatory

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mediators inducing tissue injury and repair [5]. Infection may disrupt the immunological synergy at implantation and trigger adverse outcome [6]. For example, chlamydial infection has been shown to cause dysregulation of decidualisation [7], which can contribute to miscarriage by impairing implantation and trophoblast invasion. As pregnancy progresses, there is broadly resolution of inflammation until close to term, a pro-inflammatory state then returns and contributes to the mechanisms of the onset of labour [8]. However, a continuous anti-inflammatory state in pregnancy is considered over simplified and has since been disputed. Further work has described localized changes in response to infection or 'dangerous' signals [9]. Therefore, failed tolerance in certain women at the maternal fetal interface and inappropriate, premature activation of inflammation in the reproductive tract may lead to miscarriage or preterm birth [10].

Emerging evidence implicates the reproductive tract microbiota as a key modulator of local inflammatory and immune pathways throughout pregnancy. During pregnancy, increased oestrogen levels promote glycogen deposition in the vaginal epithelia, consequently supporting *Lactobacillus* spp. dominance and stability during pregnancy [11–13]. This relationship provides protection against pathogenic bacteria by producing lactic acid and antimicrobial compounds such as bacteriocins [14]. Depletion in vaginal *Lactobacillus* spp. is linked to adverse pregnancy outcomes, including preterm birth and preterm prelabour rupture of fetal membranes (PPROM) [15–17]. There is a correlation between vaginal communities deplete in *Lactobacillus* spp. and levels of pro-inflammatory cytokines within the cervicovaginal fluid (CVF), suggesting a mechanistic link between an aberrant vaginal microbial environment and adverse pregnancy outcome [18, 19].

In a recent longitudinal study of the pregnancy vaginal microbiome starting at 6 weeks of gestation, we showed that women with vaginal communities dominated by species other than *Lactobacillus* spp. at any point during gestation were at increased risk of PPROM [20]. We have also recently shown that a *Lactobacillus* spp. depleted vaginal microbiome in early pregnancy is a risk factor for first trimester miscarriage [21]. However, this study did not distinguish between euploid and aneuploid pregnancy losses, nor did it explore the relationships between the vaginal microbiota and inflammatory mediators. We hypothesise that whilst aneuploid pregnancies are lost mainly because of intrinsic developmental errors, euploid miscarriages may be caused by inflammatory signals triggered by an adverse vaginal microbiota composition. To test this hypothesis, we characterized the vaginal microbiota and host immune response in women with chromosomally normal and abnormal miscarriages as well as in uncomplicated pregnancies that successfully progressed to term.

Methods

Study population and study design

This study was a prospective observational cohort study based at Queen Charlotte's & Chelsea Hospital, Early Pregnancy Unit, London between March 2014 and February 2019. The study was approved by NHS National Research Ethics Service (NRES) (REC 16/WA/0357 and REC 14/LO/0199). All participants provided written informed consent. Patients were not involved in the development of the research. Patients were recruited either when they presented initially with a confirmed miscarriage diagnosis, or when they presented with pain and/or bleeding without an initially confirmed miscarriage diagnosis in the first trimester of pregnancy. The first trimester was defined as < 14 weeks' gestation by last menstrual period (LMP) or, where LMP was not known, ultrasound scan dating based on crown-rump length measurements (CRL). An intrauterine pregnancy was defined on the basis of an ultrasound scan showing an intrauterine gestation sac with or without a visible embryo and heartbeat. Missed miscarriage was confirmed when an empty gestation sac was present with a mean sac diameter of 25 mm or more, if an embryo with CRL measurement of 7 mm or more was identified without an embryonic heartbeat or if the embryonic heartbeat was absent irrespective of the size of the CRL, if one had previously been observed [22, 23]. A diagnosis of incomplete miscarriage was made when a transvaginal ultrasound demonstrated irregular heterogeneous tissue in the endometrial cavity in keeping with retained products of conception after a previous ultrasound scan had shown an intrauterine pregnancy [24].

Participants were recruited via open advertisements (using posters) within the hospital and at the university where the study was being conducted (Imperial College). The majority of women were recruited after attending the hospital Ultrasound Department or Early Pregnancy Assessment Unit. Exclusion criteria for this study included women under 18 years of age, sexual intercourse within 72 h of sampling and human immunodeficiency virus (HIV) or hepatitis C-positive status. All patients using antibiotics, probiotic supplements or progesterone supplements within 2 weeks of sample collection were excluded. A detailed questionnaire including demographic information, past medical, gynaecological and obstetric history was completed. Validated symptom scores were used to assess vaginal bleeding based upon a pictorial blood assessment chart score at the time of sampling [25]. In this methods, bleeding score 0 represents no bleeding, 1 minimal bleeding, 2 moderate bleeding, 3 soaking sanitary towels and 4 passing clots.

Sample collection

Cervicovaginal fluid samples were collected from each participant from the posterior vaginal fornix using a BBL CultureSwab MaxV Liquid Amies swab (Becton, Dickinson and Company, Oxford, UK) prior to surgical management of miscarriage (the minimum time between transvaginal ultrasound scan and sample collection was 48 h). Swabs were stored in 500 µl of liquid amies and immediately placed on ice before being frozen and stored at − 80 °C within 5 min of collection. A subset of swabs (n = 96) were directly stored at − 80 °C without liquid amies. All swabs were weighed before and after collection to determine the wet weight. Negative control swabs were also collected by exposing swabs to clinic and laboratory environments prior to freeze storage. The degree of vaginal bleeding was assessed at the time of surgical evacuation.

Cytogenetic analysis

Chorionic villous material was collected at the time of surgical evacuation of the uterus and analysed for molecular cytogenetics using QF-PCR (quantitative fluorescent polymerase chain reaction) or BACs (bacterial artificial chromosomes) on Beads [26]. For molecular cytogenetics using QF-PCR, DNA was amplified using two multiplexes that include a total of 31 markers; assay 1 contains primers for chromosomes 13, 18, 21 and 22, and assay 2, primers for chromosomes 14, 15 and 16 and the X and Y chromosomes. Supplementary markers were used as required. PCR products were separated on an ABI 3100 capillary genetic analyser, and results were analysed using ABI Genotyper software [27]. The Karyo-Lite bacterial artificial chromosomes-on-Beads (KL-BoBs™) assay was performed using a prenatal chromosome aneuploidy and microdeletion detection test kit (Perkin Elmer, Waltham, MA, USA), according to the manufacturer's instructions. Briefly, genomic DNA from specimens as well as reference DNA were biotinylated and purified. The genomic DNA and BoBs™ was then subjected to single-cell hybridization overnight before washing and incubation with streptavidin-phycoerythrin, which was used as the reporter. Fluorescence of DNA bound to the microbeads was measured using a Luminex 200 (Austin, TX, USA), and BoBsoft™ analytical software (Perkin Elmer) was used for data analysis whereby a ratio of specimen fluorescence to reference fluorescence greater than 1.0 indicated the chromosome fragments were repeated and a ratio less than 1.0 indicated a deletion [26].

DNA extraction and bacterial 16S rRNA gene amplicon sequencing

The methods for DNA extraction from the vaginal swabs (BBL™ CultureSwab™) were followed from those outlined in the Manual of Procedures for the Human Microbiome Project with minor modifications [28]. The details of the DNA extraction from vaginal swabs was performed as previously outlined [12]. In brief, mixed universal primers 28F-YM GAGTTTGATYMTGGCTCAG, 28F-Borrellia GAGTTTGATCCTGGCTTAG, 28F-Chloroflex GAATTTGATCTTGGTTCAG and 28F-Bifdo GGGTTCGATTCTGGCTCAG at a ratio of 4:1:1:1 with 388R TGCTGCCTCCCGTAGGAGT reverse primers were used to amplify the V1-V2 region of 16S rRNA. Sequencing was performed at RTL genomics (Lubbock, TX, USA) using the Illumina MiSeq platform (Illumina Inc.). The data was processed and analysed using the MiSeq SOP Pipeline of the Mothur package [29]. Sequence alignment was performed using blastn (16SMicrobial.tar.gz) and classification used RDP (Ribosomal Database Project) [30]. To account for sequencing depth bias, data were resampled and normalised to the lowest read count. Rarefaction curves were constructed and analysed to guide selection of depth for rarefaction and Good's coverage index calculated to facilitate assessment of adequacy of sub-sampling of the data.

Cytokine analysis

The vaginal swabs used for microbiome analysis were thawed slowly on ice and vortexed. For the subset of swabs not stored in amies solution, a total of 500 µl of PBS supplemented with 2.5 µl protease inhibitor (PI) was added to the swab to provide a comparable dilution volume as those stored in Amies transport solution. A constant volume was used as the mean wet weight for all swabs were highly comparable (mean 0.07 g ± 0.02). The samples were then centrifuged (8000 rpm for 10 min) and the supernatant removed and stored at − 20 °C for cytokine studies. In cases where supernatant was not collected and only dry swabs were available, we suspended the swab in protease inhibitor (PI) and PBS (5 µl PI/1 ml PBS) to make up the same volume of as those stored in Amies transport solution. The supernatants (50 µl) were analysed by Human Magnetic Luminex Screening Assay (8-plex) to measure the concentration of the following analytes: IL-2, IL-4, IL-6, IL-8, TNF-α, IFN-γ, IL-1β, IL-18 and IL-10.

Statistical analysis

Analysis of statistical differences between the vaginal microbiota of samples according to pregnancy outcome was performed using hierarchical clustering analysis (HCA) using Ward linkage in CLUSTVIS (https://biit.cs.ut.ee/clustvis). Vaginal microbiota composition was classified into two groups at genus level according to the relative abundance of *Lactobacillus* spp. using Ward hierarachial clustering analysis; samples were also classified into CSTs using the recently developed *VA*gina*L*

community state typ*E* *N*earest *C*entro*I*d classifier (VALENCIA) [31]. The Fisher's exact test was used when comparing *Lactobacillus* spp. depleted and dominated in three different pregnancy outcome categories.

Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to identify taxa significantly overrepresented according to clinical outcome, through all taxonomic levels [32]. This analysis was performed using taxonomic relative abundance, with per-sample normalisation and default settings for alpha values (0.05) for the factorial Kruskal–Wallis test among classes and pairwise Wilcoxon test between subclasses. A logarithmic LDA score > 2 was used to determine discriminative features.

Other statistical analyses were performed using the statistical package GraphPad Prism v.8.4.3 (GraphPad Software Inc., CA, USA).

To infer the pattern of microbial relationships in the vaginal microbiome, we used BAnOCC [33] to construct correlation matrices at the genus and species level (we limited the latter to the top 50 species with the greatest overall abundance across the entire cohort). The MCMCs were run for 10,000 iterations and four chains (runs were checked for convergence). Co-occurrence networks were drawn using these correlation matrices in the Qgraph package in R [34].

Results

Patient cohort, characteristics and outcomes

Two hundred women were recruited, of whom 119 miscarried and 81 had a viable term pregnancy. Of the women who miscarried, 92 were recruited at first consultation, the time of miscarriage diagnosis. A live pregnancy was initially diagnosed in 108 cases of whom 27 went on to miscarry and 81 had a successful pregnancy and delivered at term. Cytogenetic analysis was unavailable in 26 cases (in 24 cases due to inadequate numbers of villi and in two cases because of technical failure). Of the 81 viable pregnancies, eight swabs were unavailable for cytokine analysis and seven unavailable for microbiota analysis. The final study cohort consisted of two patient groups, one miscarriage group for which vaginal microbiota, cytogenetic and vaginal cytokine concentrations were available for all cases and one term pregnancy group for which 74 microbiota data and 73 cytokine data were available. The final cohort therefore comprised a total 93 pregnancies that miscarried and 74 pregnancies that went to term (Fig. 1). Table 1 shows the clinical and demographic characteristics of the three patient groups. Median maternal age was 32 (range 17–46) in the euploid miscarriage group, 36 (range 27–45) in the aneuploid miscarriage group and 32 (range 20–44) in the term pregnancy group. Maternal age was significantly higher in the aneuploid miscarriage group (P = 0.0044, Kruskal-Wallis test). Women in the term pregnancy group were significantly less likely to have had one or more previous miscarriages (P = 0.0084, chi-squared test). Gestational age at sampling was categorised as 5–8, 8–10, or 10–14 weeks post last menstrual period (LMP). There were no significant differences in gestational age at the time of sampling between aneuploid and euploid miscarriage group, but the term pregnancy group were sampled significantly later (P = 0.0083, chi-squared test). Bleeding scores were similar between euploid and aneuploid miscarriage groups and higher bleeding scores were more common in the miscarriage groups compared to the term pregnancy group (P = 0.0002, chi-squared test). There were no significant differences in BMI, smoking status or ethnicity between the groups.

Baseline vaginal microbiota composition and pregnancy outcomes

In total 4,523,582 sequence reads were obtained from 167 samples with an average of 27,087 reads per sample and a median read length of 370 bp after bar code removal. Following removal of singletons and rare operational taxonomic units (OTUs), a total of 128 taxa were identified in the vaginal microbiota of the study cohort. All negative control samples failed to amplify or generate any read data following sequencing apart from 1,

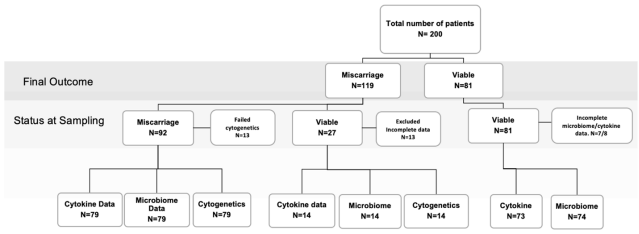

Fig. 1 Flowchart detailing entire study cohort including status at sampling and experiments performed

Table 1 Clinical and demographic characteristics of 167 patients included in study cohort

	Euploid miscarriage	Aneuploid miscarriage	Control (term pregnancy)	*p* value
Number of women	54	39	74	
Maternal age, years Median (range)	32 (17–46)	36 (27–45)	32 (20–44)	†0.0044
BMI (kg/m^2) Median (range)	25.1 (17.7–35)	24.4 (18.7–39.4)	24.5 (18–38.4)	†0.205
Smokers (%)	7 (13)	2 (5.1)	3 (3.7)	††0.189
Ethnicity (%)				
White	30 (56)	24 (62)	54 (73)	†††0.1271
Asian	15 (28)	11 (28)	10 (13.5)	
Black	7 (13)	4 (10)	10 (13.5)	
Mixed	2 (3)	0 (0)	0 (0)	
Previous miscarriage (%)				
0	30(55.6)	22 (56.4)	31 (42)	††††0.0084
1	9(16.7)	6 (15.3)	29 (39)	
2	9 (16.7)	7 (18)	10 (14)	
≥ 3	6 (11)	4 (10.3)	4 (5)	
Gestational age group at sampling (%)				
5–8 weeks	14 (26)	14 (36)	7 (10)	†††0.0083
8–10 weeks	24 (44)	19 (49)	41(55)	
10–14 weeks	16 (30)	6 (15)	26 (35)	
Bleeding score				
0	28	24	65	†††0.0002
1	13	11	9	
2	8	2	0	
3	3	2	0	
4	2	0	0	
Status at sampling				
Missed	40	35		†††0.1
Incomplete	4	0		
Viable	10	4		

†Kruskal-Wallis test
††Fisher's exact (two-tailed)
†††Chi-squared
††††Chi-squared combining euploid and aneuploid miscarriages and comparing with viable term pregnancies

which had a read count of 15. By comparison, the average read depth for patient samples was 27,087 (minimum 1402) reads. The sequencing depth was similar across the three groups with an average read count of 25,926 reads for Euploid miscarriage, 25,257 for Aneuploid and 28,899 for Viable (p = 0.1196, Kruskall and Wallis test). To avoid sequencing bias, OTUs were randomly sub-sampled to the lowest read count of 1402 which maintained a minimum Good's coverage value of 95.9% (range 95.9–99.9%) for all samples. Further analysis was restricted to the top 50 taxa which accounted for 98% of the total sequence reads in the dataset.

Ward hierarchical clustering of genus level relative abundance data for the whole cohort identified two major vaginal microbiome groups (VMG), which were characterized by *Lactobacillus* spp. dominated or *Lactobacillus* spp. depleted (vaginal microbiome grouping 1; Fig. 2). These were observed in 75% (125/167) and 25% (42/167) of samples respectively. The *Lactobacillus* spp. dominated group had a mean *Lactobacillus* content of 94.2%. The *Lactobacillus* spp. depleted group had a mean *Lactobacillus* content of 18.5%. The *Lactobacillus* spp. deplete cluster was further divided into Gardnerella dominated and non-Gardnerella (vaginal microbiome sub-grouping 1), and these clusters could be further divided into Gardnerella dominant, mixed Lactobacillus/Gardnerella, Prevotella or Streptococcus dominant (vaginal microbiome sub-grouping 2). Similar analyses were

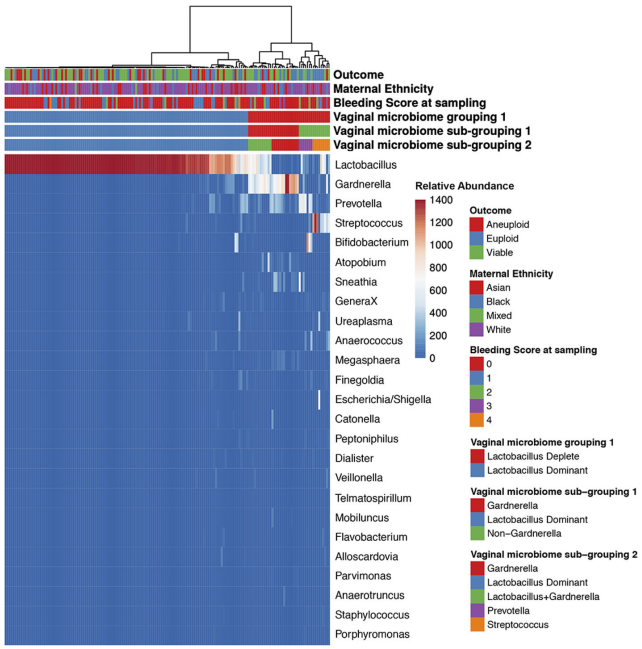

Fig. 2 Heat map of relative abundance data for the top 50 most prevalent vaginal bacterial genera and relationship with different clinical outcomes. Mixed universal primers 28F-YM GAGTTTGATYMTGGCTCAG, 28F-Borrellia GAGTTTGATCCTGGCTTAG, 28F-Chloroflex GAATTTGATCTTGG TTCAG and 28F-Bifdo GGGTTCGATTCTGGCTCAG at a ratio of 4:1:1:1 with 388R reverse primers were used to amplify the V1-V2 region of 16S rRNA followed by sequencing using the Illumina MiSeq platform. The data was processed using the MiSeq SOP Pipeline of the Mothur package and classification used RDP. Ward hierarchical clustering of genus level relative abundance data for the whole cohort of 167 individual samples identified two major vaginal microbiome groups (VMG), characterized by *Lactobacillus* spp. dominance 75% (125/167) and *Lactobacillus* spp. depletion 25% (42/167). The mean *Lactobacillus* content in the *Lactobacillus* spp. dominated group was 94.2% and in the *Lactobacillus* spp. deplete group was 18.5%. The *Lactobacillus* spp. deplete cluster was further divided into Gardnerella dominant and non-Gardnerella (VMG sub-grouping 1), and these clusters could be further divided into Gardnerella dominant, mixed Lactobacillus/Gardnerella, Prevotella or Streptococcus dominated (VMG sub-grouping 2)

performed at species level to identify the principal *Lactobacillus* spp. present in the *Lactobacillus* spp. dominant group of each individual patient. Ward hierarchical clustering separated patient samples into clusters that were dominated by either *Lactobacillus crispatus* (37%), *Lactobacillus iners* (19%), *Lactobacillus gasseri* (11%), *Lactobacillus jensenii* (10%), *Lactobacillus acidophilus* (2%), *Gardnerella vaginalis* (15%) and a highly diverse group (8%), Additional file 1: Figure S1. Using this classification, no particular *Lactobacillus* spp. was especially protective (chi-squared, $P = 0.3$). Classification of samples into equivalent CSTs using the recently developed VALENCIA classification tool [31] indicated broad agreement between the two clustering approaches (84%) (Additional file 1: Figure S1). In the remaining samples where classification differed, low similarity scores indicated poor fitting to the CSTs pre-defined by the VALENCIA algorithm. This indicated underlying composition differences in samples from our patient cohort and those used to train VALENCIA. The remaining analyses were therefore performed using our defined community clusters.

Analysis of co-occurrence between bacterial genera within our dataset identified a strong relationship between bacterial vaginosis (BV) associated genera including Gardnerella, Atopobium and Prevotella (Fig. 3a). In contrast, *Lactobacillus* was negatively correlated with most other genera, especially Gardnerella, Sneathia,

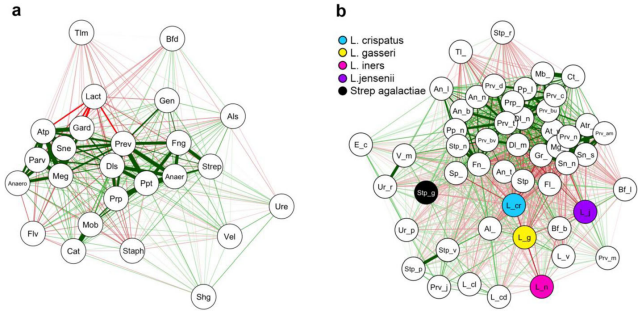

Fig. 3 Co-occurrence network analysis of vaginal bacterial taxa in the overall patient cohort. Bayesian Analysis of Compositional Covariance (BAnOCC) was used to generate co-occurrence networks between vaginal microbiota at genera (**a**) and species level taxonomy (**b**) using the 50 species with the highest overall abundance. This programme uses a Bayesian framework to analyse compositional covariance. We ran the MCMC for 10000 generations and four chains and restricted subsequent analyses to edges with [*r*] > 0.3. The red lines indicate negative correlations and the green lines indicate positive correlations and the lines are weighted according to the magnitude of the correlation. There were high levels of co-occurrence between BV associated genera, whereas *Lactobacillus* was negatively correlated with most other genera (**a**). At species level, *L.crispatus* had the most number of negative edges with all other bacterial taxa (**b**)

Atopobium and Megasphaera. When analysis was undertaken at the species level BV-associated taxa were again observed to positively correlate whereas *Lactobacillus* species, especially *L. crispatus*, tended to be negatively correlated with other bacterial taxa, including with other Lactobacilli. Strong co-occurrence between *Streptococcus vestibularis* and *Streptococcus pneumoniae* was also observed and *Streptococcus urinalis* showed a high density of negative edges with BV-associated bacteria but a positive edge with *Streptococcus agalactiae* (Fig. 3b).

We next compared vaginal microbiota composition between women who miscarried according to the pregnancy karyotype. Euploid miscarriage was associated with a significantly higher proportion of *Lactobacillus* spp. deplete VMG when compared to aneuploid miscarriage (P = 0.01, two-tailed Fisher's exact test; Fig. 4a). This difference remained significant when correcting for bleeding score by removing patients with a bleeding score > 1 (P = 0.02, two-tailed Fisher's exact test; data not shown). Euploid miscarriage was characterised by a significantly higher proportion of non-Gardnerella *Lactobacillus* spp. depleted VMG (P = 0.02, two-tailed Fisher's exact test, Figure 4b) which was enriched for *Streptococcus* spp. in 60% of cases and *Prevotella spp.* in 40% of cases (Fig. 4c). Consistent with these findings, both bacterial richness and alpha diversity were higher in the euploid miscarriage group (Fig. 4d, e). LEfSe analysis similarly identified decreased levels of *Lactobacillus* and increased levels of *Prevotella*, *Bacteriodia*, *Clostridia* and *Dialister* as characteristic features of euploid miscarriage compared to aneuploid miscarriage (Fig. 4f, g).

In our cohort, 26 women had at least two previous miscarriages, prior to a further miscarriage in the index pregnancy and were therefore defined as having 'recurrent miscarriage' [35]. Within these cases, there was no difference in the proportion of *Lactobacillus* spp. deplete VMG or *Lactobacillus* spp. dominant VMG between euploid and aneuploid miscarriage (Fig. 5b). Within the sporadic miscarriage and viable term pregnancy group the proportion of *Lactobacillus* spp. deplete VMG was greater in the euploid miscarriage group than either aneuploid miscarriage or viable pregnancy groups (P = 0.02 and P = 0.05 respectively, two-tailed Fisher's exact test, Fig. 5a). The prevalence of non-Gardnerella *Lactobacillus* spp. depleted VMG was also greater in the euploid miscarriage group than either aneuploid miscarriage or viable pregnancy groups (P = 0.03 and P = 0.04 respectively, two-tailed Fisher's exact test, Fig. 5c) and was particularly associated with *Streptococcus* spp. dominated compositions (Fig. 5d). Although the prevalence of *Lactobacillus* spp. depleted VMG was greater in the euploid miscarriage than the viable pregnancy groups, richness and diversity were not significantly different between the two groups. Similar results were obtained when re-analysis was performed on only those women where recurrent miscarriage was defined as being three consecutive miscarriages with no live births (n = 13, Additional file 1: Figure S2) [36]. LEfSe analysis identified increased relative abundance of *Prevotella* and *Streptococcus* spp. and reduced relative abundance of *Lactobacillus* spp. as being discriminatory for sporadic euploid miscarriage compared to viable term pregnancies (Fig. 5e).

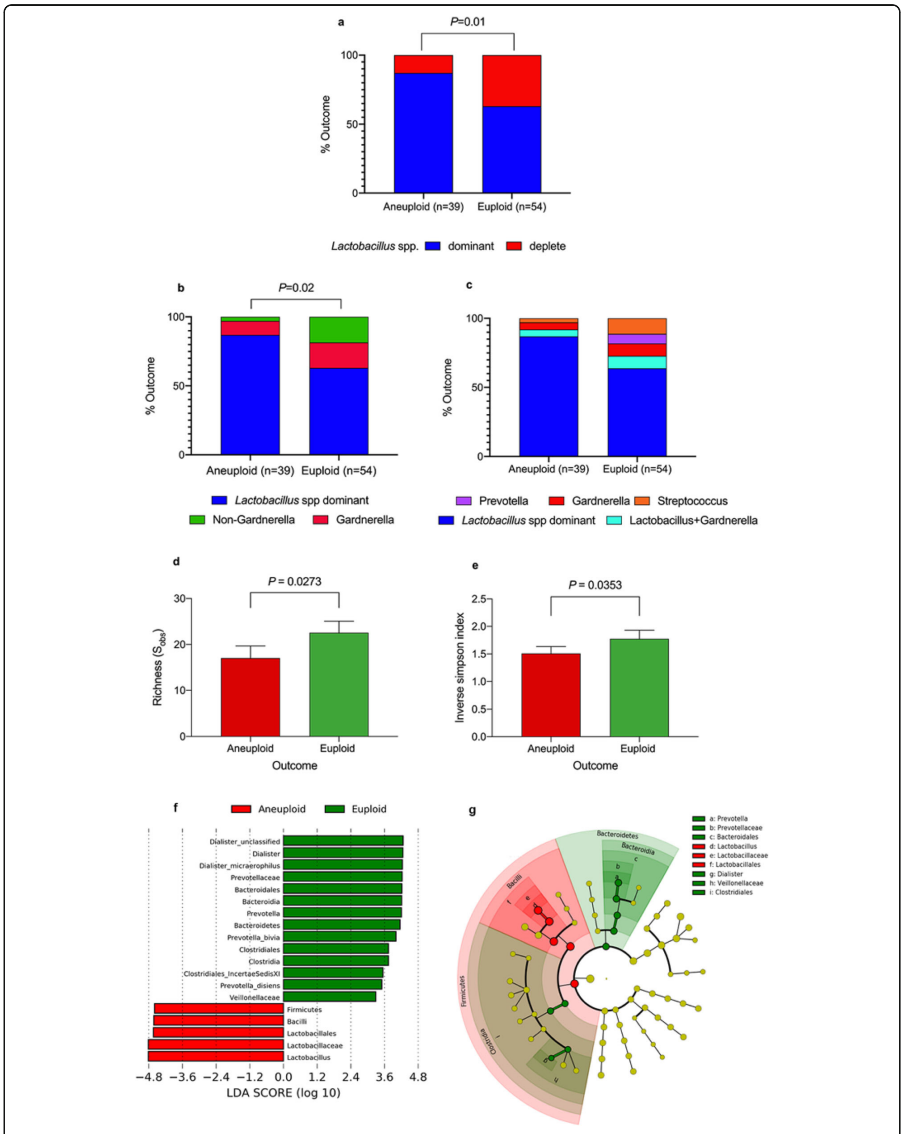

Fig. 4 (See legend on next page.)

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Fig. 4 Miscarriage outcome according to vaginal microbial classification. Stacked bar chart illustrating increased representation of *Lactobacillus spp.* depleted vaginal microbial communities in euploid miscarriages compared to aneuploid miscarriages ($P = 0.01$, two tailed Fisher's exact test **a**). Analysis of vaginal microbiome subgroups showed that this difference was largely driven by non-Gardnerella-dominance of the vaginal niche ($P = 0.02$, two-tailed Fisher's exact test **b**) and increased relative abundance of *Streptococcus* and *Prevotella* species in euploid miscarriages compared to aneuploid miscarriages (**c**). Consistent with this, significantly higher richness ($P = 0.0273$, two-tailed Mann-Whitney *U*, **d**) and diversity ($P = 0.0353$, two-tailed Mann-Whitney *U*, **e**) was observed in euploid miscarriage compared to aneuploid miscarriage. The linear discriminant analysis (LDA) effect size (LEFse) method (**f**) was then used to identify differentially abundant taxa in euploid compared to aneuploid miscarriage, which were presented at differing taxonomic levels using a cladogram (**g**). Data represented as percentages a, b and c and mean ± standard error of mean in **d** and **e**. LEfSe analysis (**f**, **g**) depicting particular vaginal microbial taxa associated with different clinical outcomes

Relationship between vaginal microbiota and cytokines concentrations across the entire cohort

To explore the relationship between the vaginal microbiota and cervicovaginal inflammatory markers, we compared the levels of nine cytokines between women with *Lactobacillus* spp. depleted or *Lactobacillus* spp. dominated VMG irrespective of pregnancy outcome. The *Lactobacillus* spp. depleted non-Gardnerella VMG subgroup had significantly higher concentrations of tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β and IL-18 compared to *Lactobacillus* spp. dominated VMG ($P = 0.001$,0.045,0.04,0.04 respectively, Fig. 6a). The individual data points for these four cytokines (TNF-α, IL-6, IL-1β and IL-18), their positions within the concentration range quartiles and their relationship to the proportion of *Lactobacillus* spp. relative abundance in the different pregnancy outcome groups is shown in Fig. 6b–e. High cytokine levels for TNF-α and IL-1β (defined as concentrations in the upper quartile) were more frequently observed in the *Lactobacillus* spp. depleted group ($P = 0.009$ and 0.002 respectively, two-tailed Fisher's exact test, Fig. 6f), particularly non-Gardnerella dominated samples, which also had elevated IL6 levels ($P = 0.009$, two-tailed Fisher's exact test, Fig. 6g). Further subdivision of the VMG groups showed that increased levels of TNF-α and IL-1β were largely associated with *Prevotella* spp. dominance ($P = 0.007$ and 0.018 respectively, chi-squared test) whereas IL6 was linked to *Streptococcus* dominance ($P = 0.02$ chi-squared test, Fig. 6 h).

Relationship between vaginal microbiota, cytokine concentrations and pregnancy outcome

The proportion of *Lactobacillus* spp. depleted vaginal microbiota in the aneuploid miscarriage, euploid miscarriage and viable pregnancy groups was, 13%, 37% and 23% respectively. Since nearly a quarter of the pregnancies with a viable outcome nevertheless had a *Lactobacillus* spp. depleted vaginal microbiota, we next compared cytokine concentrations between euploid miscarriage and viable term pregnancies in only women with *Lactobacillus* spp. depletion. IL-1β, IL-6 and IL-8 were significantly lower ($P < 0.001$, $P < 0.001$ and $P = 0.01$, respectively, two tailed Mann-Whitney *U* test) and IL-2 and IL-10 were significantly higher ($P = 0.004$ and $P < 0.001$ respectively, two tailed Mann-Whitney *U* test) in viable pregnancies (Fig. 7).

Discussion

We confirmed our primary hypothesis that euploid miscarriage is significantly more frequently associated with *Lactobacillus* spp. depleted vaginal microbial communities compared to aneuploid miscarriage. This association was reflected in increased richness and diversity in euploid miscarriage. A *Lactobacillus spp.* depleted vaginal microbiota characterized by Streptococci was the most significant vaginal microbiota risk factor for sporadic euploid miscarriage and the principal driver of proinflammatory mediators in these patients. We also found that aneuploid miscarriages and healthy pregnancies had similar vaginal microbial compositions. These data support the notion that aneuploid and euploid miscarriages generally have different causal mechanisms. In general, aneuploid miscarriage is assumed to be due to a genetic intrinsic failure whereas a proportion of sporadic euploid miscarriage are due to host-vaginal microbe interactions.

Of the 200 patients recruited to this study, 119 eventually miscarried and 81 went on to have a viable pregnancy. The relatively high rate of miscarriage in this cohort was because the majority of miscarriage patients ($n = 92$) were recruited at the time of miscarriage diagnosis. Molecular cytogenetics was available for 93 miscarriage patients, and results were reported after recruitment and sample collections, therefore eliminating selection bias. The proportion of aneuploid pregnancies in our miscarriage cohort is comparable with the aneuploidy rate reported in previous studies [37, 38]. In our cohort, there was a significantly higher maternal age in the aneuploidy miscarriage group, which is consistent with the known relationship between maternal age and the incidence of meiotic errors in embryos [39]. There was a significant difference in the gestational age at sampling between the viable pregnancies and miscarriages likely because women experiencing symptoms in a viable pregnancy tend to present at a later gestational age to the clinic. As expected, there was a significantly higher bleeding score in the miscarriage cohort compared to the viable term group [21]. Recurrent miscarriage is

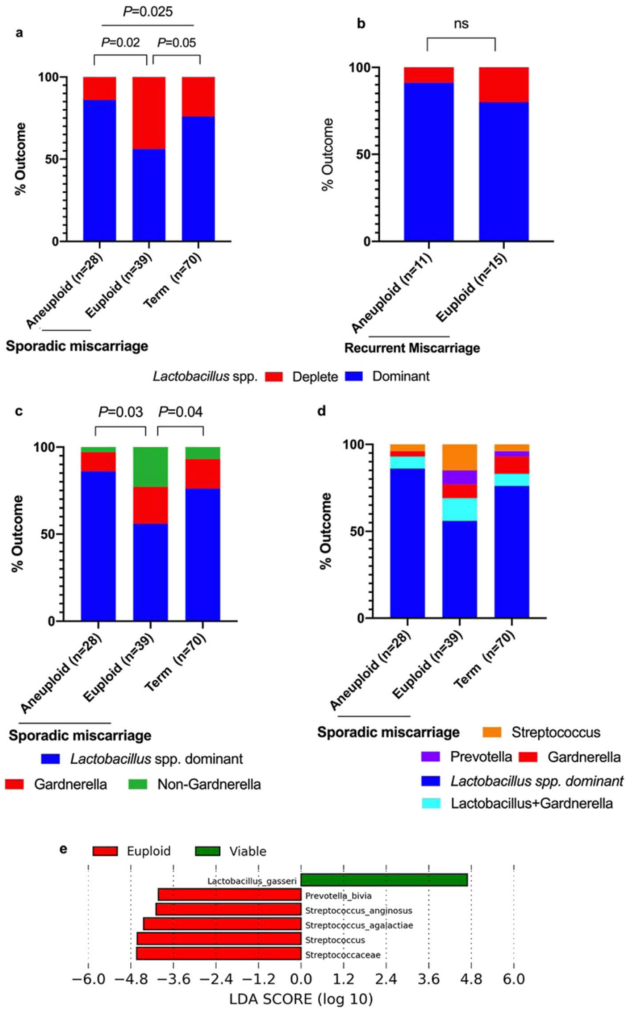

Fig. 5 Clinical outcomes according to vaginal microbial composition in sporadic miscarriage. Increased *Lactobacillus spp.* depleted vaginal microbial communities were observed in sporadic euploid miscarriages ($n = 39$) compared to sporadic aneuploid miscarriages ($n = 28$, $P = 0.02$ two-tailed Fisher's exact test) and viable term pregnancies ($n = 70$ $P = 0.05$ two-tailed Fisher's exact test, **a**). No significant difference was seen between euploid ($n = 15$) and aneuploid ($n = 11$) miscarriages in the recurrent miscarriage group (**b**). A significantly increased prevalence of non-Gardnerella vaginal bacterial communities was seen in euploid miscarriages compared to aneuploid miscarriage, $P = 0.03$ and viable pregnancy $P = 0.04$ (two-tailed Fisher's exact test, **c**). Differentially abundant taxa identified by LDA in euploid miscarriage compared to viable pregnancy are shown in **e**. Data represented as percentages in **a**, **b**, **c** and **d**. LEfSe analysis (**e**) depicting particular vaginal bacterial taxa associated with euploid and viable pregnancy, confined to sporadic miscarriage

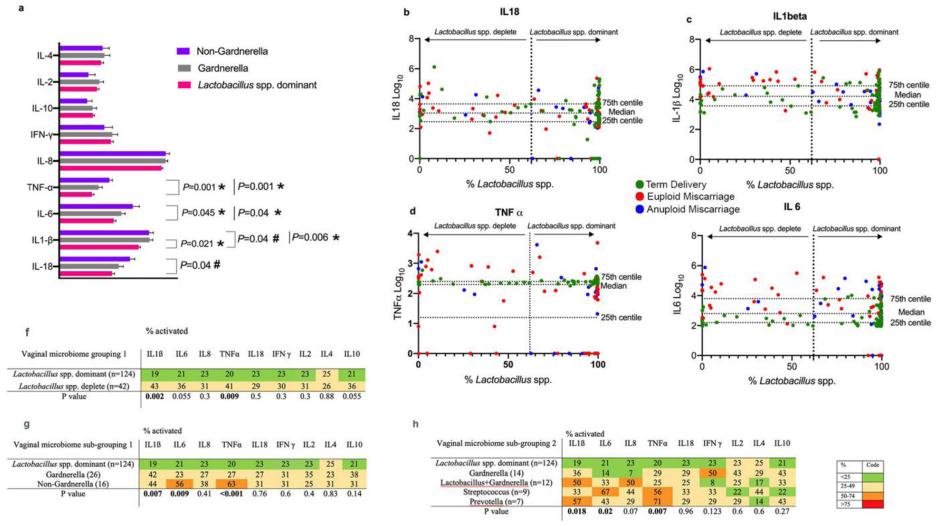

Fig. 6 Cytokine expression according to vaginal microbiome grouping 1 and subgroupings 1 and 2. Non-Gardnerella, *Lactobacillus spp.* depleted samples (16/166) had significantly increased vaginal levels of inflammatory cytokines TNF-α, IL-6, IL-1 β and IL-18 when compared to *Lactobacillus spp.* dominant samples (124/166). Gardnerella, *Lactobacillus spp.* depleted samples (26/166) had significantly increased IL-1 β when compared to *Lactobacillus spp.* dominant (**a**, **P* values, Kruskal-Wallis test with post hoc Bonferroni correction and $^{\#}P$ values, Mann-Whitney *U* test for non-Gardnerella versus *Lactobacillus spp.* dominant patients). Scatter plots representing the percentage of *Lactobacillus* spp. against the concentration of IL-18 (**b**), IL-1 β (**c**), TNF-α (**d**), IL-6 (**e**) and coloured according to different clinical outcomes with the 25th, median, and 75th centile of cytokine levels indicated. *Lactobacillus spp.* depleted samples had significantly increased levels of IL-1β and TNF-α compared to the *Lactobacillus spp.* dominated group (**f**). Increased inflammatory activation in the *Lactobacillus spp.* depleted samples could be largely attributed to non-Gardnerella dominated samples (**g**) and more specifically, high relative abundances of *Streptococcus* and *Prevotella* spp. (**h**). Data represented as a clustered bar chart with mean ± standard error of the mean for each cytokine by vaginal microbiome sub-grouping 1, *n* = 166 (**a**). The individual tables (**f**–**h**) illustrate the percentage of activated cytokines in each group. Activation was defined by cytokines expressed in the upper quartile. *P* values obtained by comparing activated versus non-activated cytokines using two-tailed Fisher's exact test (**f** and **g**) and chi-squared test (**h**)

thought to be aetiologically different to sporadic miscarriage and recent studies have highlighted the association with abnormal endometrial function, caused by lack of mesenchymal stem cells and heightened cellular senescence during the midluteal implantation window [40–42]. Our data supports the relationship between miscarriage and vaginal microbiota composition principally in sporadic miscarriage. Although the numbers of recurrent miscarriages in our cohort were small (n = 26) and the findings require confirmation in larger populations, this does reinforce the concept that different underlying causal mechanisms drive recurrent miscarriage.

The vaginal microbiota is an important regulator of innate immune response in the reproductive tract [18].

A significant body of evidence has linked inflammation within the cervicovaginal niche to adverse pregnancy outcomes, particularly in relation to second trimester pregnancy loss and preterm birth [43–47]. Consistent with previous findings in pregnant [18] and non-pregnant women [48, 49], we found that proinflammatory cytokines IL-1β, IL-6 and TNF-α were elevated in women with *Lactobacillus* spp. depleted VMGs. These findings support our primary hypothesis and reinforce the view that *Lactobacillus* spp. depleted vaginal microbiota correlate with local inflammatory activation which during pregnancy, can associate with adverse pregnancy outcome [18]. However, a *Lactobacillus* spp. depleted VMG was compatible with delivery at term in nearly a quarter of cases. Our exploration of local immune mediators revealed that women with a *Lactobacillus* spp. depleted VMG who delivered at term had comparably lower levels of proinflammatory cytokines in early pregnancy. Further, although no difference in the levels of anti-inflammatory cytokines IL-2 and IL-10 were observed between *Lactobacillus* spp. depleted and *Lactobacillus* spp. dominated VMGs across the whole cohort, there were significantly increased levels of these cytokines in *Lactobacillus* spp. depleted samples from viable term pregnancies compared to euploid miscarriage. Collectively, these observations implicate both adverse

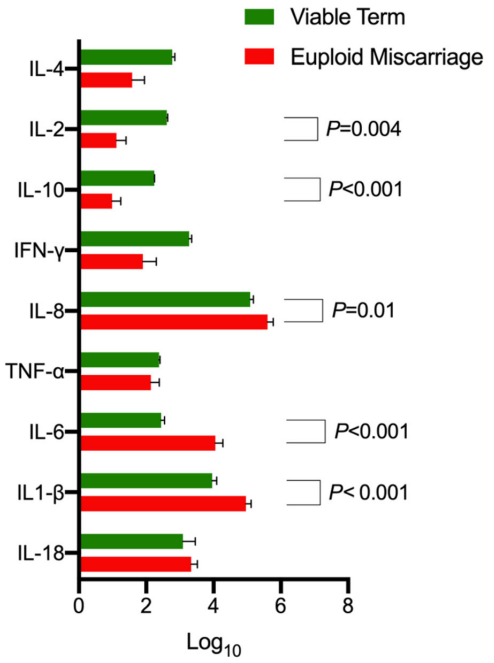

Fig. 7 Vaginal cytokine levels according to pregnancy outcome in patients with *Lactobacillus spp.* depleted vaginal microbial composition (n = 37, euploid n = 20 and viable term n = 17). In women with *Lactobacillus spp.* depleted vaginal microbial compositions, viable pregnancy was associated with significantly higher levels of anti-inflammatory cytokines (IL-2 P = 0.004, IL-10 P < 0.001, two tailed Mann-Whitney U test) and lower levels of pro-inflammatory cytokines (IL-6, P < 0.001, IL-1ß P < 0.001, and IL-8 P = 0.01, two tailed Mann Whitney U test) compared to euploid miscarriage. Data represented as clustered bar chart with mean ± standard error of the mean for each cytokine according to clinical outcome

vaginal microbiota composition and specific local host immune responses in early pregnancy with subsequent risk of miscarriage.

In this study, *Gardnerella vaginalis* was observed as being an important feature in high risk *Lactobacillus* spp. deplete compositions. Recent work has indicated the likely existence of difference *Gardnerella vaginalis* clades with potentially varying degrees of pathogenicity [50]. However, the metaxonomics approach used in our study was not capable of differentiating these. Future investigations may shed light on whether particular *Gardnerella vaginalis* clades are responsible for the observed relationship with adverse outcomes.

Furthermore, in our cohorts increased production of proinflammatory cytokines (IL-6, IL-1β, IL-18 and TNF-α) within the *Lactobacillus* spp. depleted VMGs was associated with dominance by *Prevotella* and *Streptococcus* species in women with euploid miscarriages. These species had low co-occurrence with *L. crispatus*, which has been shown to associate with vaginal bacterial stability and low levels of innate immune activation during pregnancy [18, 51]. Thus, whilst the underlying microbial structure may be the primary driver, it is the interplay between the vaginal microbiota and host immune response that determines the amplitude of the inflammatory response and the likelihood of miscarriage.

There are a number of potential mechanisms by which vaginal microbiota and host immune response could be causally linked to miscarriage. Suboptimal vaginal microbiota characterised by *Lactobacillus* spp. depletion and high bacterial diversity are associated with local inflammatory activation and damage to the cervical epithelial barrier [52] that can promote bacterial translocation [53]. Although studies of the endometrial microbiome are confounded by the difficulties of contamination and low biomass, the emerging evidence is that the lower uterine microbiome is distinct but maybe contributed to by the vaginal microbiota [54, 55]. In this study, microbiota and cytokine measurements were limited to CVF, thus it is not possible to determine if our observations are reflective of microbiota-host interactions in the endometrial mucosa. Logistical and ethical considerations make direct sampling of the early pregnancy uterine environment difficult. However, the embryological origin of Mullerian duct fusion means that the upper one third of the vagina shares similarities with the endometrial epithelium and therefore a similar proinflammatory response to pathobionts such as *Prevotella and Streptococcus* species would be expected [56, 57].

Lactobacilli confer protection in the vagina by promoting antimicrobial defense without initiating innate immune mediated inflammation [14]. Therefore it is possible in cases of *Lactobacillus* spp. depletion a proinflammatory environment can alter successful implantation, which is a process of tissue injury and repair that is regulated by immune mediators to allow the trophoblast to breach the decidual lining and invade the maternal decidua [5, 58]. Previous studies have also shown that altered levels of cytokines at the maternal fetal interface can trigger early pregnancy complications [59–61].

It is therefore plausible that an inflammatory response triggered by the vaginal microbiome could directly or indirectly contribute to dysregulation of the maternal decidua, promoting breakdown of the nascent maternal-fetal interface in early gestation [9].

Conclusions

In conclusion, we demonstrate that vaginal microbiota depleted of *Lactobacillus* spp. combined with a heightened local inflammatory response, predispose pregnant

women to euploid miscarriage. Although this may be a reflection of intrinsic maternal immune response, it appears that the cytokine response is largely driven by the bacterial taxa present in the vagina, which presents an opportunity for specific, directed intervention prior to conception or in early pregnancy. First trimester miscarriage occurs in 20% of pregnancies and is a major cause of physical and psychological pathology worldwide [62]. Whilst aneuploid miscarriage can be explained in terms of an intrinsic developmental defect, there is currently little evidence to explain the causative mechanisms underlying sporadic euploid miscarriage. There are currently no treatments to prevent this important clinical condition. The data presented here suggests that there is a group of women who would benefit from antibiotic or pre- or probiotic treatment to reduce the risk of miscarriage. Whilst further studies are needed to validate these findings and to understand what specifically triggers an inflammatory cascade in euploid miscarriage, an important next step will be to explore which interventional regimes might change the vaginal microbiota and positively influence pregnancy outcome.

Abbreviations

PPROM: Preterm prelabour rupture of fetal membranes; CVF: Cervicovaginal fluid; LMP: Last menstrual period; CRL: Crown-rump length; QF-PCR: Quantitative fluorescent polymerase chain reaction; BACs: Bacterial artificial chromosomes; OUT: Operational taxonomic units; VMG: Vaginal microbiome groups; BMI: Body mass index; DNA: Deoxyribonucleic acid; LDA: Latent discriminatory analysis; LEfSe: Linear discriminant analysis with effect size

A.Additional files

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Authors' contributions

PRB, DAM and TB contributed to the conception and design of the study. Ethical approval, recruitment and sample collection was undertaken by KG and MA-M. Experiments and data collection were performed by KG and YSL. Data analyses and interpretation of results was performed by KG, AS, PRB, SK, JJB and DAM. The Figures and tables were generated by KG, SK and PRB. The manuscript was written by KG, DAM and PRB. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was approved by NHS National Research Ethics Service (NRES) (REC 16/WA/0357 and REC 14/LO/0199). All participants provided written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors have no financial, commercial, personal, or professional competing interests to declare.

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