DR. DAVID MACINTYRE (Orcid ID : 0000-0002-4186-5567)

Article type : Main research article

Title: The association between vaginal bacterial composition and miscarriage: a nested case-control study.

Authors: Maya Al-Memar[1,2], Shabnam Bobdiwala[1,2], Hanine Fourie[1,2,3], Ramona Manino[1,2], Yun S Lee[1,2,3,4], Ann Smith[5], Julian R. Marchesi[4,6,7], Dirk Timmerman[8], Tom Bourne[1,2,3,8], Phillip R. Bennett[1,2,3,4] and David A. MacIntyre[1,2,3,4*].

Affiliations:

1. Tommy's National Centre for Miscarriage Research, Imperial College London, W12 0NN, UK
2. Imperial College Parturition Research Group, Division of the Institute of Reproductive and Developmental Biology, Imperial College London, W12 0NN, UK
3. Queen Charlotte's Hospital, Imperial College Healthcare NHS Trust, London, W12 0HS, UK
4. March of Dimes European Preterm Birth Research Centre, Imperial College London, W12 0NN, UK
5. School of Medicine, Cardiff University, CF103AX, UK
6. Division of Integrative Systems Medicine and Digestive Disease, Imperial College London, W2 1NY, UK
7. School of Biosciences, Cardiff University, CF103AX, UK
8. Department of Obstetrics and Gynaecology, University Hospitals Leuven, KU Leuven, Belgium

***Corresponding author:**

Dr David MacIntyre, Imperial College Parturition Research Group, Division of the Institute of Reproduction and Developmental Biology, Imperial College London, Hammersmith Campus, London, W12 0NN, UK. Email: d.macintyre@imperial.ac.uk

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1471-0528.15972

Short Running title: Vaginal bacterial composition and miscarriage

Abstract

Objective

To characterise vaginal bacterial composition in early pregnancy and investigate its relationship with first and second trimester miscarriages.

Design

Nested case-control study.

Setting

Queen Charlotte's and Chelsea Hospital, Imperial College Healthcare NHS Trust, London.

Population

161 pregnancies; 64 resulting in first trimester miscarriage, 14 in second trimester miscarriage and 83 term pregnancies.

Methods

Prospective profiling and comparison of vaginal bacteria composition using 16S rRNA gene-based metataxonomics from 5 weeks gestation in pregnancies ending in miscarriage or uncomplicated term deliveries matched for age, gestation and body-mass index.

Main outcome measures

Relative vaginal bacteria abundance, diversity and richness.

Pregnancy outcomes defined as first or second trimester miscarriage, or uncomplicated term delivery.

Results

First trimester miscarriage associated with reduced prevalence of *Lactobacillus* spp.-dominated vaginal microbiota classified using hierarchical clustering analysis (65.6% vs. 87·7%; P=0·005), higher alpha diversity (mean Inverse Simpson Index 2.5 (95% confidence interval 1.8-3.0) vs. 1.5 (1.3-1.7), P=0·003) and higher richness 25.1 (18.5-31.7) vs. 16.7 (13.4-20), P=0·017), compared to viable pregnancies. This was independent of vaginal bleeding and observable before first trimester miscarriage diagnosis (P=0·015). Incomplete/complete miscarriage associated with higher proportions of *Lactobacillus* spp.-deplete communities compared to missed miscarriage. Early pregnancy vaginal bacterial stability was similar between miscarriage and term pregnancies.

Conclusions

These findings associate the bacterial component of vaginal microbiota with first trimester miscarriage and indicate suboptimal community composition is established in early pregnancy.

While further studies are required to elucidate the mechanism, vaginal bacterial composition may represent a modifiable risk factor for first trimester miscarriage.

Funding

This work was supported by Tommy's Charity (grant P62774), the UK National Institute for Health Research Biomedical Research Centre (grant P45272) and the Medical Research Council (grant MR/L009226/1). Funders did not contribute to conducting the research or writing the manuscript.

Key words

Vaginal bacteria, first trimester miscarriage, second trimester miscarriage, vaginal microbiome

Tweetable abstract

Vaginal bacterial composition in first trimester miscarriage is associated with reduced *Lactobacillus* spp. abundance and is independent of vaginal bleeding.

Introduction

Miscarriage is the most common adverse pregnancy outcome, complicating up to 25% of pregnancies [1]. Around 50% of all miscarriages are thought to be due to aneuploidy or other chromosomal aberrations with causal drivers of the remaining cases largely unknown [1]. However, evidence supports an infectious aetiology in both first and second trimester miscarriage [2]. While earlier studies using non-objective, symptomatic measures suggested infection as a rare cause of pregnancy loss [3, 4], a recent study reported histological chorioamnionitis in 78/101 (77%) of miscarriage samples compared to 0/103 (0%) of controls, with 47% of chorioamnionitis cases culture positive [5]. Moreover, a relationship between bacterial vaginosis (BV) and increased risk of miscarriage [6-9] has been described and chlamydial infection is thought to cause miscarriage through impairment of endometrial decidualisation [10, 11].

Recent application of culture-independent methods for the assessment of vaginal microbial composition have shown that healthy pregnancy is characterised by stable vaginal bacterial composition with *Lactobacillus* spp. dominance and low bacterial richness and diversity [12-14]. Increased vaginal microbiota stability in pregnancy has been partly attributed to increased oestrogen levels, which are thought to stimulate glycogen deposition in vaginal epithelial cells favouring colonisation of *Lactobacillus* spp., as well as decreased sexual activity, hygiene changes and the lack of cyclic hormonal variations [15, 16]. In contrast, adverse pregnancy outcomes such as preterm prelabour rupture of membranes and preterm birth are associated with reduced *Lactobacillus* spp. dominance and a shift towards high bacterial diversity community compositions [17-22]. However, vaginal microbial composition is poorly described prior to 8 weeks of gestation, which represents a crucial time period of pregnancy given that most miscarriages occur early in the first trimester [23, 24].

We hypothesised that vaginal bacterial instability and *Lactobacillus* spp. depletion may be associated with miscarriage. We tested this hypothesis by comparing early vaginal bacterial composition in 161 pregnancies of which 64 resulted in first trimester miscarriage, 14 in second trimester miscarriage and 83 healthy term delivery.

Methods

Study Design and Ethical Approval

This study was a prospective observational cohort study based at Queen Charlotte's & Chelsea Hospital, London, between March 2014 and March 2016. The study was approved by NHS National Research Ethics Service (NRES) Riverside Committee London (REC 14/LO/0199), and all participants provided written informed consent. Patients were not involved in the development of the research.

Women in the first trimester of pregnancy with an intrauterine pregnancy were invited to participate. The first trimester was defined as less than 14 weeks gestation by last menstrual period (LMP) or where LMP was not known, ultrasound scan dating based on crown rump length measurements (CRL) [25]. 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. Participants were recruited via open advertisements (using posters) in local GP surgeries, in local hospitals, and at the university where the study is 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 16 years of age, multiple pregnancies, sexual intercourse within 72 hours of sampling and human immunodeficiency virus (HIV) or Hepatitis C positive status.

A detailed questionnaire including details on demographic information, past medical, gynaecological and obstetric history was completed. Validated symptoms scores were used at each study visit to assess vaginal bleeding based upon a pictorial blood assessment chart score at the time of sampling [26]. Ethnicity was self-reported as White, Black or Asian. Depending on the gestational age at the time of recruitment and clinical need, participants were seen a minimum of twice and up to five times in the first trimester during pregnancy. Serial ultrasound scans were performed until the end of the first trimester with routine ultrasonographic measurements collected at each visit. Participants were encouraged to contact the research team if they had any complications, such as bleeding, and when necessary were invited to attend for an additional ultrasound scan with the team. Participants were subsequently seen at the time of their routine dating scan (11 to 14 weeks gestation).

Vaginal Sampling

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) within the gestational time windows of 5-8, 8-10, and 10-14 weeks and >14 weeks gestation. Swabs were placed immediately on ice before being frozen and stored at -80°C within 5 minutes of collection. Negative control swabs were also collected by exposing swabs to clinic and laboratory environments prior to freeze storage.

Pregnancy Outcomes

Pregnancy outcomes were collected and documented using hospital medical records Cerner Millennium® and Powerchart®. The core outcome sets were miscarriage and live birth at term. First trimester miscarriage was defined as pregnancy loss confirmed sonographically before 14+0 weeks gestational age using previously described criteria for cut-off values [23, 27]. Missed miscarriage was confirmed when there was an empty gestational sac with a mean sac diameter of greater than 25mm or more, if an embryo with a crown-rump length (CRL) measurement of 7mm or more was visualised without an embryonic heart beat or if there was absence of an embryonic heart beat irrespective of the size of the CRL where one had previously been observed [23, 27]. A diagnosis of complete miscarriage was made when transvaginal ultrasound scan showed an empty uterus after a previous ultrasound scan had demonstrated an intrauterine pregnancy [24]. 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]. Second trimester miscarriage was defined as pregnancy loss under 24+0 weeks gestational age. Women with ongoing pregnancies were followed through to delivery. Any women experiencing antenatal delivery or neonatal complications (e.g. preterm birth, preeclampsia, gestational diabetes) were excluded from the study. Women who experienced uncomplicated term deliveries were included as controls and were matched to cases for age, ethnicity and body mass index (BMI) at a ratio of approximately 1:1 miscarriages cases:control.

DNA extraction and 16S rRNA gene amplicon sequencing

DNA extraction from vaginal swabs and sequencing were performed as previously described [13]. Briefly, the V1-V2 hyper variable regions of 16S rRNA genes were amplified for sequencing using a forward primer consisting of an Illumina i5 adapter (5′-

AATGATACGGCGACCACCGAGATCTACAC-3′), an 8–base pair (bp) bar code, a primer pad (forward, 5′-TATGGTAATT-3′), and the 28F primer (5′-GAGTTTGATCNTGGCTCAG-3′) [28]. The reverse fusion primer was constructed with an Illumina i7 adapter (5′-CAAGCAGAAGACGGCATACGAGAT-3′), an 8-bp bar code, a primer pad (reverse, 5′-AGTCAGTCAG-3′), and the 388R primer (5′-TGCTGCCTCCCGTAGGAGT-3′). Sequencing was performed at RTL Genomics (Lubbock, TX, USA) using an Illumina MiSeq platform (Illumina Inc.). The MiSeq SOP Pipeline of the Mothur package was used to process the sequence data [29]. Sequence alignment was performed using the Silva bacterial database (www.arb-silva.de/), and classification was performed using the RDP (Ribosomal Database Project) database reference sequence files and the Wang method [30]. Determination of operational taxonomic unit taxonomies (phylum to genus) was performed using the RDP MultiClassifier script and USEARCH was used for species-level taxonomies [31]. To account for potential sequencing bias, data were resampled and normalised to the lowest read count.

Statistical Analysis

Statistical comparisons of continuous variables describing clinical and demographic parameters were calculated using Analysis of variance (ANOVA) where data was normally distributed or Kruskall-Wallis where data was not normally distributed. For categorical variables, Chi-squared test was applied.

Potential contaminants were identified as Operational Taxonomic Units (OTUs) that were detected in at least half of negative control swabs at a proportional ratio of greater than one to one with the assumption that sequences found in negative controls are likely to have arisen from contamination from the sampling environment or extraction kit itself. These were removed and not considered for further downstream analyses.

Statistical comparisons between vaginal microbiota at genera and species level were performed within the Statistical Analysis of Metagenomic Profiles software package (STAMP) [32]. Hierarchical clustering analysis (HCA) using centroid linkage was performed using both bacterial genera and species level data in Clustvis (https://biit.cs.ut.ee/clustvis) to classify samples into community groups on the basis of bacterial compositional similarity.

Chi-squared analyses were performed to test for significant differences in proportions of the vaginal community state type groups in the following groups; 1) All miscarriage samples versus

viable control samples, 2) First trimester miscarriages versus viable pregnancies matched for gestational age and excluding longitudinal samples, 3) Samples taken preceding diagnosis of first trimester miscarriage versus controls matched for gestational age and excluding longitudinal samples, 4) Samples collected prior to diagnosis of second trimester miscarriage and controls, 5) Samples taken from first trimester miscarriages versus viable pregnancies, excluding those with a reported bleeding score of greater than one, matched for gestational age and excluding longitudinal samples, and 6) Cases of missed miscarriages versus incomplete/complete miscarriage.

Differences in bacterial richness (species observed) and diversity (Inverse Simpson Index) between groups were analysed using one-way ANOVA or the factorial Kruskal-Wallis test where appropriate. For pairwise comparisons in the same parameters in miscarriage and viable pregnancies, non-parametric testing using Mann-Whitney U was applied.

To assess changes in the vaginal bacterial composition in the first trimester, longitudinal samples collected in a subset of women experiencing term delivery, first trimester and second trimester miscarriages were analysed. Vaginal bacterial community stability, or "transition index" was calculated by dividing the number of different community group types observed by the number of samples collected from that individual throughout pregnancy.

This work was supported by the Tommy's National Centre for Miscarriage Research (grant P62774), the UK National Institute for Health Research Biomedical Research Centre (grant P45272), the UK National Institute for Health Research Biomedical Research Centre (grant P45272), the March of Dimes European Preterm Birth Research Centre at Imperial College London, the NIHR Collaboration for Leadership in Applied Health Research & Care, North-West London and the Medical Research Council (grant MR/ L009226/1).

Results

A total of 1003 women were recruited to the study. Those who underwent termination of pregnancy (*n*=20), withdrew from the study (*n*=5), were lost to follow up (*n*=32) or who subsequently experienced pregnancy complications other than miscarriage (e.g. preterm birth, preeclampsia; n=593) were excluded (Figure 1A). There were 99 first trimester miscarriages and 14 second trimester miscarriages. Of the 99 first trimester miscarriages, 64 had vaginal swabs taken at least once (sample *n*=74). All women who had a second trimester miscarriage were sampled in the first trimester at least once (sample *n*=24). Of the remaining 240 uncomplicated pregnancies, 83 were selected as control cases of term delivery matched for maternal age, ethnicity and BMI at a ratio of approximately 1:1 miscarriage cases:controls (sample *n*=139, Table 1).

A total of 237 swab samples were sequenced providing an average of 3267 reads per sample. After removing singletons and rare OTUs (<10 average reads per sample), a total of 244 bacterial species were identified within the total sample cohort. Using hierarchical clustering of relative abundance data from the top 50 bacterial species (accounting for >95% of all sequence reads), samples could be classified into five major groups similar to previously described vaginal community states types (CSTs) (Figure 1B)[12]. Note that for visualisation purposes, the top 30 taxa are presented in Figure 1B. Four groups were characterised by dominance of either *Lactobacillus crispatus* (CST I), *L. gasseri* (CST II), *L. iners* (CST III) or *L. jensenii* (CST V) whereas CST IV was characterised by low relative abundance of *Lactobacillus* spp. and enrichment of facultative or strict anaerobic species often associated with BV. Consistent with this finding, the highest alpha diversity and richness was observed in CST IV type communities (Figure 1C and 1D). Additionally, 2 samples from 2 viable control patients, and 2 samples from a patient experiencing second trimester miscarriage were dominated by *Lactobacillus acidophilus*, which were grouped as CST-Other. Due to a lack of power, samples from these patients were excluded from additional comparative analyses resulting in n=81 for viable controls and n=13 for second trimester miscarriage.

Comparison of vaginal bacterial composition at genera level at the time of clinical confirmation of first or second trimester (*n*=77) miscarriage with gestational-aged matched samples from healthy controls (*n*=81) showed that miscarriage is associated with *Lactobacillus* spp. depletion (*P* =

0.0053, chi-squared) and at species level, significantly higher proportion of samples dominated by CST IV (P = 0.031, chi-squared) (Figure 2A). Consistent with this, bacterial alpha diversity, as estimated using the Inverse Simpson Index (P= 0.0031, Mann Whitney), and richness (number of species observed, P= 0.0287, Mann Whitney) were significantly higher in miscarriage samples compared to matched control samples (Figure 2B and 2C). Vaginal bacterial composition from first trimester miscarriages (n=64) was compared with viable control pregnancies (n=81) matched for gestational age. A significant difference in the distributions of vaginal bacterial communities was observed between first trimester miscarriage samples and controls at both genera (P = 0.0030) and species level (P =0.0170) (Figure 2D) with the former associated with *Lactobacillus* spp. depletion and an increased proportion of CST IV. This association was maintained when samples collected from participants reporting a vaginal bleeding score greater than 1 were excluded (Figure S1). A similar relationship was also observed in samples collected prior to miscarriage when the pregnancy appeared to be viable on ultrasound (n=20) (Figure 3E), but not in samples taken prior to second trimester (n=13) (Figure 3F).

We next examined whether observed differences in vaginal bacterial composition were consistent across clinical miscarriage phenotypes, specifically missed miscarriages (n=61) compared to incomplete and complete miscarriages (n=13) (Figure 3). A significantly higher proportion of *Lactobacillus* spp. depleted (P=0.0006) and CST IV (P=0.0098) vaginal communities was observed in incomplete and complete miscarriages compared with missed miscarriages.

Comparison of vaginal bacterial community stability in longitudinal samples collected from control (n=34) and miscarriage participants (first trimester miscarriage, n=10: second trimester miscarriages, n=9) showed no significant difference in the proportion of patients experiencing a transition between one genera (P = 0.8167) or CST (P = 0.5296) level composition group to another (Figure S2). This comparable stability was reflected by similar mean transition index scores between control and miscarriage participants (Figure S2).

Discussion

Main findings

First trimester is associated with reduced prevalence of *Lactobacillus* spp. dominated vaginal bacterial communities in the first trimester, irrespective of vaginal bleeding.

Lactobacillus spp. deplete, high diversity vaginal bacterial community composition precedes diagnosis of miscarriage.

Incomplete/complete miscarriage is associated with increased prevalence of *Lactobacillus* spp. depletion.

Early pregnancy vaginal bacterial stability in women who experience miscarriage is similar to those who have healthy viable pregnancies that deliver at term.

Strengths and limitations

Strengths include the analysis of a large, well-characterised cohort that includes longitudinal sampling prior to the miscarriage event and information regarding vaginal bleeding. In addition, the use of bacterial DNA sequencing approaches permits detection, identification and assessment of relative abundance of a broad range of commensal and pathogenic vaginal bacteria, including high resolution of key *Lactobacillus* spp. However, this does not capture information on other important microbiota constituents including viruses and fungi. Other limitations include the relatively low number of samples collected from women having second trimester miscarriage and those sampled prior to first trimester miscarriage diagnosis. Further, a lack of karyotyping information limits our ability to assess if the observed association between first trimester miscarriage and *Lactobacillus* spp. depletion is similar between chromosomally normal and abnormal pregnancies. It is also important to note that our data is associative, and further studies are required to establish causal relationship between vaginal microbiota and miscarriage.

Interpretation

Over half of all miscarriages are thought to be caused by structural or chromosomal abnormalities affecting the developing embryo [1]. While the remaining causal factors are yet to be fully elucidated, an association between BV, as diagnosed using culture and microscopy based methods, and increased risk of miscarriage have previously been reported [8, 9]. Consistent with these

findings, our study reports a positive relationship between first trimester miscarriage and reduced vaginal *Lactobacillus* spp. abundance and increased prevalence of high diversity community compositions as assessed using 16S rRNA gene metataxonomics. Other recent studies using culture-independent profiling methods have also identified an association between vaginal *Lactobacillus* spp. depletion and adverse reproductive health outcomes including infertility, preterm birth and sexually transmitted diseases [17-20, 33, 34]. The protective role of *Lactobacillus* spp. in the lower reproductive tract is well described and can be partly attributed to lactic acid driven acidification of the vagina, which inhibits growth and colonisation of other pathogenic species [15]. Furthermore, lactic acid can suppress production of inflammatory mediators in the vagina [35-37]. Data from our cohort allows us to conclude that the protective role of *Lactobacillus* spp. extends into the early stages of pregnancy where samples deplete of *Lactobacillus* spp. display increased richness and diversity and colonisation by potential pathogens including *Prevotella*, *Streptococcus*, *Peptoniphilus*, *Ureaplasma* and *Dialister* species. These bacteria have been shown to up-regulate the expression of metalloproteinases [38, 39] and pro-inflammatory cytokines [40], whilst reducing the inhibitory effect of tissue inhibitors of metalloproteinases (TIMPs) [41].

Precise regulation of inflammatory and tissue remodelling in the upper reproductive tract is a critical aspect of early pregnancy events including implantation and placental trophoblast invasion [42-44]. Vaginal microbiota may impact these pathways in a number of ways. Pathogen recognition and response by the maternal innate immune system following ascending vaginal infection accompanied by seeding and colonisation of the endometrium by pathogenic bacteria could drive untimely activation of inflammatory pathways that negatively modify endometrial receptivity and implantation. Consistent with this, ascending vaginal group B streptococcus (GBS; *Streptococcus agalactiae*) infection, which has recently been shown to be mediated by β-catenin-induced loss of vaginal epithelial barrier function and cellular detachment leading to exfoliation and subsequent bacterial ascension [45], is associated with increased rates of miscarriage, still birth and preterm birth [46]. Further, it has recently been shown that, in mice vaginal colonisation of bioluminescent *E. coli* leads to ascending infection into the uterine cavity, activation of intrauterine inflammation and subsequent preterm birth and neonatal brain injury [47].

There is also emerging evidence that despite previously being considered sterile [48], the uterus may harbour a functionally relevant microbiome [49-54]. While the origin of the endometrial microbiome,

and when it might be established, is as yet to be determined, seeding by vaginal bacteria is a plausible mechanism. Female subfertility is associated with bacterial vaginosis, which is often characterised by presence of an adherent vaginal polymicrobial biofilm[8]. A structured polymicrobial *Gardnerella vaginalis* biofilm has been reported to be present in the endometrium of approximately half of patients presenting with bacterial vaginosis [55]. It is also feasible that vaginal microbiota may modulate upper reproductive tract inflammatory pathways without direct colonisation of the endometrium. In mice, vaginal administration of bacterial lipopolysaccharide is sufficient to induce cervical remodelling, macrophage infiltration and subsequent preterm birth [56]. Collectively, these data support a role for vaginal bacteria in an inflammatory aetiology that may underpin a proportion of miscarriage cases. This notion is supported by our findings showing that high diversity, CST IV communities, which we and others have previously shown to evoke local inflammatory activation [18, 57], are overrepresented in incomplete/complete miscarriages where the pregnancy is more likely to be expelled.

Due to the current study design, it is difficult to determine when *Lactobacillus* spp. depletion occurs in patients experiencing miscarriage, or what is mechanistically driving it. Vaginal bleeding is a symptom of miscarriage [24], but can also cause vaginal microbial instability and shifts towards *Lactobacillus* spp. deplete communities through alkalisation of the vaginal environment and provision of energy substrates that can be readily utilised by opportunistic colonisers [13]. Concordant with this, we observed the highest proportions of *Lactobacillus* spp. deplete, high diversity community compositions, in incomplete/complete miscarriages where bleeding is a common symptom. However, the relationship between *Lactobacillus* spp. depletion and first trimester miscarriage identified in our study appears to be independent of bleeding, as it remains following exclusion of samples with reported bleeding, and is seen where samples are taken prior to the miscarriage.

An alternative explanation for increased rates of vaginal *Lactobacillus* spp. depletion in miscarriage involves interaction between dysregulated hormonal signalling in miscarriage, which is associated with reduced oestradiol and progesterone levels [58, 59] and microbial energy substrate availability. Oestrogen production in pregnancy is thought to promote glycogen accumulation in vaginal epithelial cells, which can ultimately be used preferentially by *Lactobacillus* spp. as a primary carbon source [12]. Therefore, low oestrogen levels may fail to sufficiently promote *Lactobacillus* spp. dominance in some pregnancies that subsequently miscarry. Alternatively,

vaginal microbiota communities associated with miscarriage may already be established prior to conception. Support for this second point is provided by our longitudinal analysis that indicates high levels of vaginal microbial stability in our patient cohort during early pregnancy. Further insight would be obtained through the analysis of pre-conception samples.

It has been previously reported that bacterial vaginosis at 11-13 weeks gestation is associated with late miscarriage [6]. In our study, a trend increase in the proportion of high diversity vaginal bacterial communities in the first trimester and subsequent second trimester miscarriage was observed, although this trend did not reach statistical significance. Second trimester miscarriages are associated with multiple aetiologies and therefore the small cohort included in this study may not have had sufficient power to detect an association with vaginal bacterial composition. Alternatively, this may be due to sampling predominately in the early first trimester so that any later shifts towards *Lactobacillus* spp. deplete communities were not observed in this study.

Conclusions

This study provides an insight into the first trimester vaginal microbiota composition and its relationship with miscarriage. Through the application of 16S rRNA based metataxanomics, we show that *Lactobacillus* spp. deplete, high diversity vaginal bacterial communities are a risk-factor for miscarriage. Unlike other causes of miscarriage, such as chromosomal abnormalities, the vaginal microbiome is a risk factor that can be potentially modifiable through the use of targeted antibiotic, prebiotic or probiotic treatments and other novel therapies e.g. bacteriophage. Further studies designed to determine when and how high-risk vaginal microbiota communities are established are needed to better understand this relationship in order to further elucidate the role of infection in miscarriage and how treatment strategies might help us to prevent some miscarriages.

Disclosure of interests

All authors declare that they have no competing interests. Completed disclosure of interest forms are available to view online as supporting information.

Contribution to authorship: TB, PB and DAM conceived and designed the study. Patient recruitment and sample collection were undertaken by MA-M, SB and HF. Experiments and data collection were performed by MA-M, YSL and RM. Data analyses and interpretation were performed by MA-M, AS, JRM, DT, TB, PRB and DAM. All figures and tables were generated by MA-M and DAM. The manuscript was written by MA-M and DAM and critically reviewed by all authors. All authors read and approved the final manuscript.

Details of ethics approval

This study was approved by the National Health Service (NHS) National Research Ethics Service (NRES) Riverside Committee London (REC 14/LO/0199, approval date 15/01/2014).

Funding

This study was supported by the Tommy's National Centre for Miscarriage Research (grant P62774) and the UK National Institute for Health Research Biomedical Research Centre (grant P45272). TB, PRB and DAM are supported by the UK National Institute for Health Research Biomedical Research Centre (grant P45272), and the March of Dimes European Preterm Birth Research Centre at Imperial College London. S.B. was supported by NIHR CLAHRC NWL (Collaboration for Leadership in Applied Health Research & Care, North-West London) and DAM was supported by the Medical Research Council (grant MR/ L009226/1). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. The Division of Integrative Systems Medicine and Digestive Disease at Imperial College London receives financial support from the National Institute of Health Research (NIHR) Imperial Biomedical Research Centre (BRC) based at Imperial College Healthcare NHS Trust and Imperial College London. This article is independent research funded by the NIHR BRC, and the views expressed in this publication are those of the authors and not necessarily those of the NHS, NIHR, or the Department of Health.

Acknowledgements: We would like to thank all the participants that took part in the study.

Data availability

Public access to sequence data sets generated in this study along with accompanying metadata can be obtained from the Sequence Read Archive of the European Nucleotide Archive (PRJEB32479).

References

1. Larsen EC, Christiansen OB, Kolte AM, Macklon N. New insights into mechanisms behind miscarriage. BMC medicine. 2013 Jun 26;11:154.
2. Giakoumelou S, Wheelhouse N, Cuschieri K, Entrican G, Howie SE, Horne AW. The role of infection in miscarriage. Hum Reprod Update. 2016 Jan-Feb;22(1):116-33.
3. Simpson JL, Mills JL, Kim H, Holmes LB, Lee J, Metzger B, et al. Infectious processes: an infrequent cause of first trimester spontaneous abortions. Human reproduction (Oxford, England). 1996 Mar;11(3):668-72.
4. Simpson JL, Gray RH, Queenan JT, Barbato M, Perez A, Mena P, et al. Further evidence that infection is an infrequent cause of first trimester spontaneous abortion. Human reproduction (Oxford, England). 1996 Sep;11(9):2058-60.
5. Allanson B, Jennings B, Jacques A, Charles AK, Keil AD, Dickinson JE. Infection and fetal loss in the mid-second trimester of pregnancy. Aust N Z J Obstet Gynaecol. 2010 Jun;50(3):221-5.
6. Hay PE, Lamont RF, Taylor-Robinson D, Morgan DJ, Ison C, Pearson J. Abnormal bacterial colonisation of the genital tract and subsequent preterm delivery and late miscarriage. BMJ (Clinical research ed). 1994 Jan 29;308(6924):295-8.
7. Haahr T, Zacho J, Brauner M, Shathmigha K, Skov Jensen J, Humaidan P. Reproductive outcome of patients undergoing in vitro fertilisation treatment and diagnosed with bacterial vaginosis or abnormal vaginal microbiota: a systematic PRISMA review and meta-analysis. BJOG : an international journal of obstetrics and gynaecology. 2019 Jan;126(2):200-7.
8. van Oostrum N, De Sutter P, Meys J, Verstraelen H. Risks associated with bacterial vaginosis in infertility patients: a systematic review and meta-analysis. Human reproduction (Oxford, England). 2013 Jul;28(7):1809-15.
9. Ralph SG, Rutherford AJ, Wilson JD. Influence of bacterial vaginosis on conception and miscarriage in the first trimester: cohort study. BMJ (Clinical research ed). 1999 Jul 24;319(7204):220-3.
10. Baud D, Regan L, Greub G. Emerging role of Chlamydia and Chlamydia-like organisms in adverse pregnancy outcomes. Current opinion in infectious diseases. 2008 Feb;21(1):70-6.
11. Giakoumelou S, Wheelhouse N, Brown J, Wade J, Simitsidellis I, Gibson D, et al. Chlamydia trachomatis infection of human endometrial stromal cells induces defective decidualisation and chemokine release. Scientific reports. 2017 May 17;7(1):2001.
12. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, et al. Vaginal microbiome of reproductive-age women. Proceedings of the National Academy of Sciences of the United States of America. 2011 Mar 15;108 Suppl 1:4680-7.

13. MacIntyre DA, Chandiramani M, Lee YS, Kindinger L, Smith A, Angelopoulos N, et al. The vaginal microbiome during pregnancy and the postpartum period in a European population. Scientific reports. 2015 Mar 11;5:8988.

14. Freitas AC, Chaban B, Bocking A, Rocco M, Yang S, Hill JE, et al. The vaginal microbiome of pregnant women is less rich and diverse, with lower prevalence of Mollicutes, compared to non-pregnant women. Scientific reports. 2017 Aug 23;7(1):9212.

15. Spear GT, French AL, Gilbert D, Zariffard MR, Mirmonsef P, Sullivan TH, et al. Human alpha-amylase present in lower-genital-tract mucosal fluid processes glycogen to support vaginal colonization by Lactobacillus. The Journal of infectious diseases. 2014 Oct 1;210(7):1019-28.

16. Ayre WB. The glycogen-estrogen relationship in the vaginal tract. The Journal of clinical endocrinology and metabolism. 1951 Jan;11(1):103-10.

17. Brown RG, Marchesi JR, Lee YS, Smith A, Lehne B, Kindinger LM, et al. Vaginal dysbiosis increases risk of preterm fetal membrane rupture, neonatal sepsis and is exacerbated by erythromycin. BMC medicine. 2018 Jan 24;16(1):9.

18. Kindinger LM, MacIntyre DA, Lee YS, Marchesi JR, Smith A, McDonald JA, et al. Relationship between vaginal microbial dysbiosis, inflammation, and pregnancy outcomes in cervical cerclage. Science translational medicine. 2016 Aug 3;8(350):350ra102.

19. Kindinger LM, Bennett PR, Lee YS, Marchesi JR, Smith A, Cacciatore S, et al. The interaction between vaginal microbiota, cervical length, and vaginal progesterone treatment for preterm birth risk. Microbiome. 2017;5(1):6.

20. Freitas AC, Bocking A, Hill JE, Money DM. Increased richness and diversity of the vaginal microbiota and spontaneous preterm birth. Microbiome. 2018 Jun 28;6(1):117.

21. Brown RG, Al-Memar M, Marchesi JR, Lee YS, Smith A, Chan D, et al. Establishment of vaginal microbiota composition in early pregnancy and its association with subsequent preterm prelabor rupture of the fetal membranes. Translational research : the journal of laboratory and clinical medicine. 2018 Dec 27.

22. DiGiulio DB, Callahan BJ, McMurdie PJ, Costello EK, Lyell DJ, Robaczewska A, et al. Temporal and spatial variation of the human microbiota during pregnancy. Proceedings of the National Academy of Sciences of the United States of America. 2015 Sep 1;112(35):11060-5.

23. Preisler J, Kopeika J, Ismail L, Vathanan V, Farren J, Abdallah Y, et al. Defining safe criteria to diagnose miscarriage: prospective observational multicentre study. BMJ (Clinical research ed). 2015 Sep 23;351:h4579.

24. Bottomley C, Bourne T. Diagnosing miscarriage. Best practice & research Clinical obstetrics & gynaecology. 2009 Aug;23(4):463-77.

25. Robinson HP, Fleming JE. A critical evaluation of sonar "crown-rump length" measurements. British journal of obstetrics and gynaecology. 1975 Sep;82(9):702-10.

26. Higham JM, O'Brien PM, Shaw RW. Assessment of menstrual blood loss using a pictorial chart. British journal of obstetrics and gynaecology. 1990 Aug;97(8):734-9.

27. Abdallah Y, Daemen A, Kirk E, Pexsters A, Naji O, Stalder C, et al. Limitations of current definitions of miscarriage using mean gestational sac diameter and crown-rump length measurements: a multicenter observational study. Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology. 2011 Nov;38(5):497-502.

28. Sundquist A, Bigdeli S, Jalili R, Druzin ML, Waller S, Pullen KM, et al. Bacterial flora-typing with targeted, chip-based Pyrosequencing. BMC Microbiology. 2007 Nov; 7:108.

29. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Applied and environmental microbiology. 2013 Sep;79(17):5112-20.

30. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and environmental microbiology. 2007 Aug;73(16):5261-7.

31. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010 Oct 1;26(19):2460-1.

32. Parks DH, Beiko RG. Identifying biologically relevant differences between metagenomic communities. Bioinformatics. 2010 Mar 15;26(6):715-21.

33. Eckert LO, Moore DE, Patton DL, Agnew KJ, Eschenbach DA. Relationship of vaginal bacteria and inflammation with conception and early pregnancy loss following in-vitro fertilization. Infectious diseases in obstetrics and gynecology. 2003;11(1):11-7.

34. van de Wijgert J. The vaginal microbiome and sexually transmitted infections are interlinked: Consequences for treatment and prevention. PLoS medicine. 2017 Dec;14(12):e1002478.

35. Witkin SS, Linhares IM. Why do lactobacilli dominate the human vaginal microbiota? BJOG : an international journal of obstetrics and gynaecology. 2017 Mar;124(4):606-11.

36. Smith SB, Ravel J. The vaginal microbiota, host defence and reproductive physiology. The Journal of physiology. 2017 Jan 15;595(2):451-63.

37. Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in female genital tract: cellular responses and interactions. Immunological reviews. 2005 Aug;206:306-35.

38. Guan SM, Shu L, Fu SM, Liu B, Xu XL, Wu JZ. Prevotella intermedia induces matrix metalloproteinase-9 expression in human periodontal ligament cells. FEMS microbiology letters. 2008 Jun;283(1):47-53.

39. Guan SM, Shu L, Fu SM, Liu B, Xu XL, Wu JZ. Prevotella intermedia upregulates MMP-1 and MMP-8 expression in human periodontal ligament cells. FEMS microbiology letters. 2009 Oct;299(2):214-22.

40. Borgdorff H, Gautam R, Armstrong SD, Xia D, Ndayisaba GF, van Teijlingen NH, et al. Cervicovaginal microbiome dysbiosis is associated with proteome changes related to alterations of the cervicovaginal mucosal barrier. Mucosal immunology. 2016 May;9(3):621-33.

41. Nakata K, Yamasaki M, Iwata T, Suzuki K, Nakane A, Nakamura H. Anaerobic bacterial extracts influence production of matrix metalloproteinases and their inhibitors by human dental pulp cells. Journal of endodontics. 2000 Jul;26(7):410-3.

42. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Seminars in reproductive medicine. 2007 Nov;25(6):445-53.

43. Gellersen B, Brosens JJ. Cyclic decidualization of the human endometrium in reproductive health and failure. Endocrine reviews. 2014 Dec;35(6):851-905.

44. Mor G, Kwon JY. Trophoblast-microbiome interaction: a new paradigm on immune regulation. Am J Obstet Gynecol. 2015 Oct;213(4 Suppl):S131-7.

45. Vornhagen J, Armistead B, Santana-Ufret V, Gendrin C, Merillat S, Coleman M, et al. Group B streptococcus exploits vaginal epithelial exfoliation for ascending infection. The Journal of clinical investigation. 2018 May 1;128(5):1985-99.

46. Seale AC, Bianchi-Jassir F, Russell NJ, Kohli-Lynch M, Tann CJ, Hall J, et al. Estimates of the Burden of Group B Streptococcal Disease Worldwide for Pregnant Women, Stillbirths, and Children. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2017 Nov 6;65(suppl_2):S200-S19.

47. Suff N, Karda R, Diaz JA, Ng J, Baruteau J, Perocheau D, et al. Ascending Vaginal Infection Using Bioluminescent Bacteria Evokes Intrauterine Inflammation, Preterm Birth, and Neonatal Brain Injury in Pregnant Mice. The American journal of pathology. 2018 Oct;188(10):2164-76.

48. Moller BR, Kristiansen FV, Thorsen P, Frost L, Mogensen SC. Sterility of the uterine cavity. Acta obstetricia et gynecologica Scandinavica. 1995 Mar;74(3):216-9.

49. Mitchell CM, Haick A, Nkwopara E, Garcia R, Rendi M, Agnew K, et al. Colonization of the upper genital tract by vaginal bacterial species in nonpregnant women. American journal of obstetrics and gynecology. 2015 May;212(5):611.e1-9.

50. Moreno I, Codoner FM, Vilella F, Valbuena D, Martinez-Blanch JF, Jimenez-Almazan J, et al. Evidence that the endometrial microbiota has an effect on implantation success or failure. American journal of obstetrics and gynecology. 2016 Dec;215(6):684-703.

51. Franasiak JM, Werner MD, Juneau CR, Tao X, Landis J, Zhan Y, et al. Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16S ribosomal subunit. Journal of assisted reproduction and genetics. 2016 Jan;33(1):129-36.

52. Franasiak JM, Scott RT. Endometrial microbiome. Current opinion in obstetrics & gynecology. 2017 Jun;29(3):146-52.

53. Romero R, Espinoza J, Mazor M. Can endometrial infection/inflammation explain implantation failure, spontaneous abortion, and preterm birth after in vitro fertilization? Fertility and sterility. 2004 Oct;82(4):799-804.

54. Verstraelen H, Vilchez-Vargas R, Desimpel F, Jauregui R, Vankeirsbilck N, Weyers S, et al. Characterisation of the human uterine microbiome in non-pregnant women through deep sequencing of the V1-2 region of the 16S rRNA gene. PeerJ. 2016;4:e1602.

55. Swidsinski A, Verstraelen H, Loening-Baucke V, Swidsinski S, Mendling W, Halwani Z. Presence of a polymicrobial endometrial biofilm in patients with bacterial vaginosis. PloS one. 2013;8(1):e53997.

56. Gonzalez JM, Franzke CW, Yang F, Romero R, Girardi G. Complement activation triggers metalloproteinases release inducing cervical remodeling and preterm birth in mice. The American journal of pathology. 2011 Aug;179(2):838-49.

57. Anahtar MN, Byrne EH, Doherty KE, Bowman BA, Yamamoto HS, Soumillon M, et al. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. Immunity. 2015 May 19;42(5):965-76.

58. Melnick AP, Pereira N, Murphy EM, Rosenwaks Z, Spandorfer SD. How low is too low? Cycle day 28 estradiol levels and pregnancy outcomes. Fertility and sterility. 2016 Apr;105(4):905-9.e1.

59. Arck PC, Rucke M, Rose M, Szekeres-Bartho J, Douglas AJ, Pritsch M, et al. Early risk factors for miscarriage: a prospective cohort study in pregnant women. Reproductive biomedicine online. 2008 Jul;17(1):101-13.

Figure Legends

Figure 1. Study design and characterisation of vaginal bacterial composition in early pregnancy. (A) Flow chart describing study design and selection of cases and controls. (B) Hierarchal clustering of vaginal bacterial species data (top 30 species shown) in early pregnancy collected from women subsequently experiencing first trimester or second trimester miscarriages, or viable control pregnancies. Samples clustered into 5 major groups, 4 of which were dominated by *Lactobacillus* spp. (CST I, II, III, V) and 1 which was characterised by *Lactobacillus* spp. depletion (CST IV). *indicates *Lactobacillus acidophilus* dominated. Both alpha diversity (C) and richness (D) was significantly higher in CST IV communities.

Figure 2. Vaginal *Lactobacillus* spp. depletion and high bacterial diversity is associated with first trimester miscarriage and precedes diagnosis. (A) Stacked bar chart showing that miscarriage (first and second trimester combined) is associated with a higher proportion of *Lactobacillus* spp. deplete, CST IV type vaginal microbiota community compositions as well as significantly greater vaginal (B) alpha diversity and (C) richness. (D) Sub-analysis showed a similar relationship in 1st trimester miscarriage which was also observed prior to diagnosis of first trimester (E) but this did not reach statistical significance for second trimester miscarriage (F). CST = community state type

Figure 3. *Lactobacillus* spp. deplete vaginal microbial communities are more frequently associated with incomplete/complete miscarriage. Analysis of vaginal microbiota composition on the basis of miscarriage type show that incomplete and/or complete miscarriages are associated with a significantly higher proportion of *Lactobacillus* spp. deplete, high diversity CST IV compared with missed miscarriages. CST = community state type

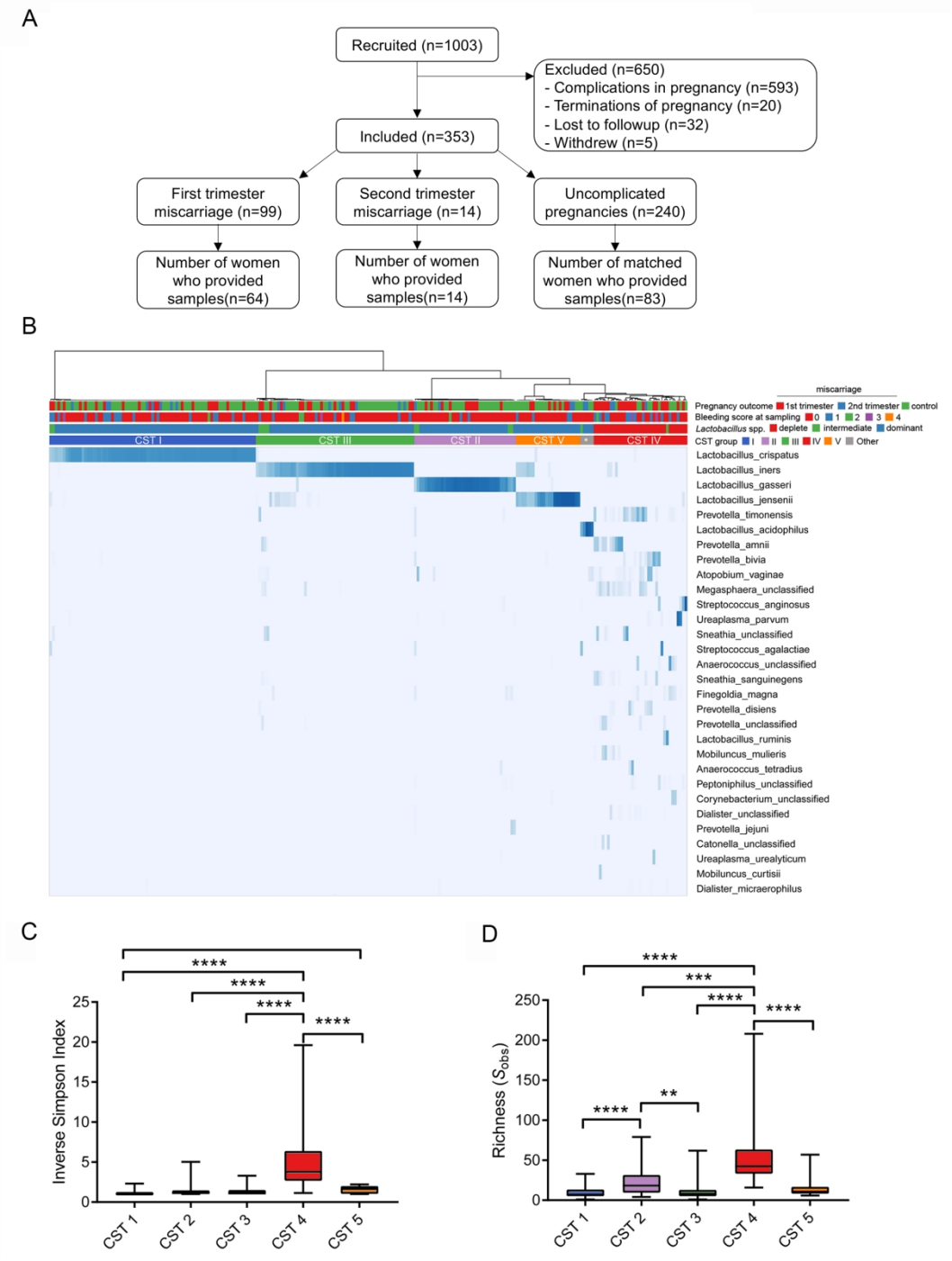
Table 1. Clinical and demographic characteristics of study cohort

**P* values for maternal age calculated using ANOVA. For BMI, *P*-values calculated using a Kruskall-Wallis test. For categorical variables, *P*-values calculated using a Chi-squared test.

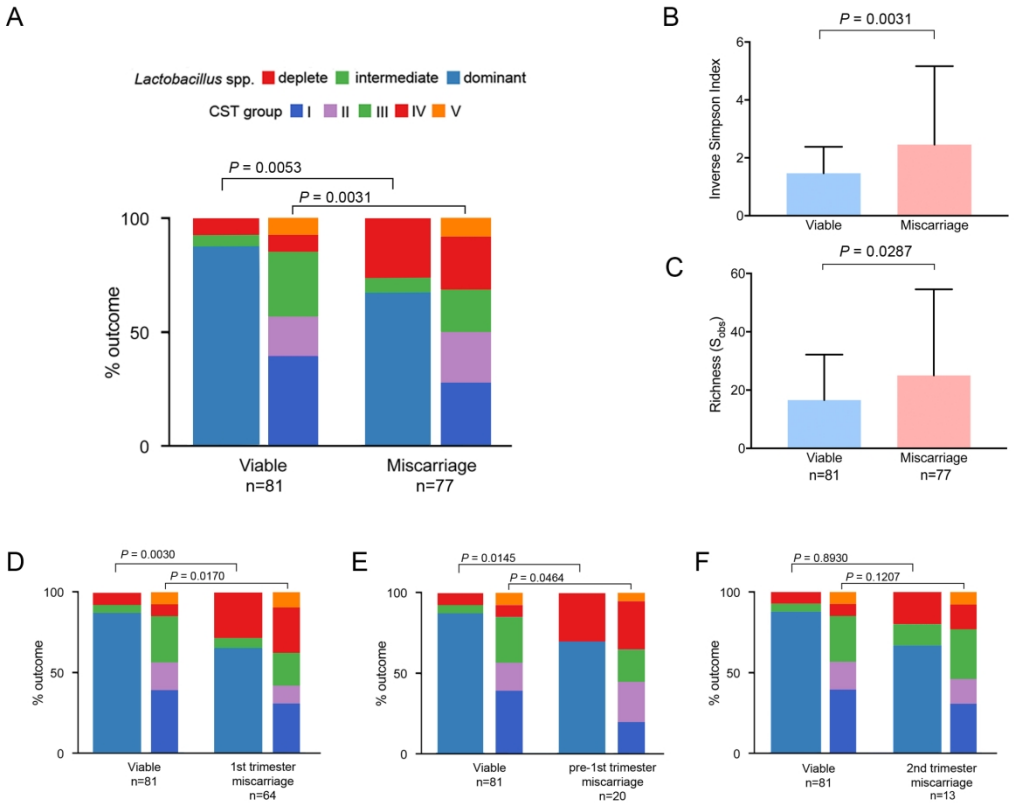
	1st Trimester Miscarriage	2nd Trimester Miscarriage	Controls	*P* value*

	n (%)	n (%)	n (%)	
Number of women (N)	64	14	83	
Number of samples (N)	74	24	139	
Maternal age median (IQR) (years)	34.5 (28.0-38.8)	31.5 (24.0-35.0)	34.0 (29.0-38.0)	0.176
BMI range, median (IQR) (kg/m2)	24.1 (22.6-27.0))	24.2 (21.1-27.4)	24.1 (21.7-26.6)	0.984
Number of smokers (%)	7 (10.9 %)	1 (7 %)	3 (3.6 %)	0.196
Gravidity				0.996
Primagravid	13 (20%)	3 (21%)	17 (20%)	
Multigravid	51 (80%)	11 (79%)	66 (80%)	
Parity				0.175
Nulliiparous	35 (55%)	11 (79%)	43 (52%)	
Multiparous	29 (45%)	3 (21%)	40 (48%0	
Previous miscarriage				0.141
0	23 (36%)	6 (43%)	36 (43%)	
1-2	31 (48%)	8 (57%)	43 (52%)	
≥3	10 (16%)	0 (0%)	4 (5%)	
Ethnicity				0.140
White	48 (75%)	6 (42%)	61 (73%)	
Asian	8 (12.5%)	5 (36%)	11 (13%)	
Black	8 (12.5%)	3 (21%)	11 (13%)	
Number of samples per gestational age group				
5-8 weeks	43 (58%)	3 (13%)	27 (19%)	
8-10 weeks	26 (35%)	8 (33%)	57 (41%)	
10-14 weeks	5 (7%)	12 (50%)	37 (27%)	
>14 weeks	0	1 (4%)	18 (13%)	
Number of samples with bleeding score >1 at time of sampling	10	0	0	
Type of Miscarriage				
Missed	52 (81%)			
Incomplete/complete	12 (19%)			

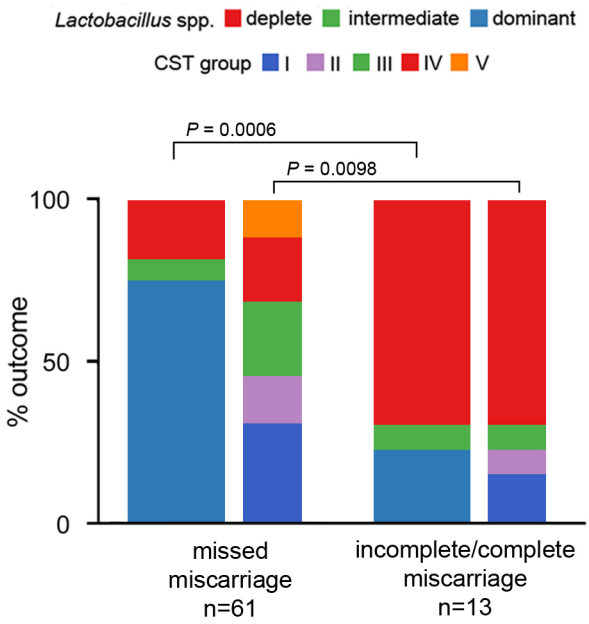
N = number, BMI = body mass index, IQR = interquartile range



bjo_15972_f1.jpg



bjo_15972_f2.jpg



bjo_15972_f3.jpg