**A retrospective pilot study to examine if the reproductive tract microbiota differs in women with a history of infertility compared to fertile women.**

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**Acknowledgements**

The researchers would like to acknowledge The Wesley Medical Research Institute Tissue Bank for help with facilitating specimen collection. We especially acknowledge Emma Raymond and Rebecca Rogers from the Tissue bank. The authors acknowledge the clinical team and staff at The Wesley Hospital who helped specimen collections on the day of surgery.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

This research was funded by The Wesley Medical Research Institute project grant awarded to JAA and WMH. The authors gratefully acknowledge the Institute for the funding. The Wesley Medical Research funding agency did not have any role in the interpretation or analysis of the data or manuscript drafting. Dr Huston and Associate Professor Allan held honorary appointments at the Institute.

**Author contributions**

BAW conducted all bioinformatics analysis and contributed to the drafting of the manuscript. MT conducted laboratory components, RT-qPCR and related statistical analysis and contributed to the drafting of the manuscript. ELS contributed to the design and interpretation of RT-qPCR and contributed to the drafting of the manuscript. FDF contributed to the design and interpretation of RT-qPCR and contributed to the drafting of the manuscript. MS contributed to participant recruitment, questionnaire design and data collection, and contributed to the drafting of the manuscript. JR contributed to bioinformatics design and analysis of the microbiota data and contributed to the drafting of the manuscript. PG contributed R scripts, bioinformatics design and analysis of the microbiota data and contributed to the drafting of the manuscript. GM contributed to bioinformatics design and analysis of the microbiota data and contributed to the drafting of the manuscript. PT contributed to design and interpretation of RT-qPCR and contributed to the drafting of the manuscript. JAA conceived the concept of the study, conducted clinical recruitment, epidemiological and questionnaire analysis and contributed to the drafting of the manuscript. WMH developed the case-control study design, contributed to statistical analysis and interpretation and contributed to the drafting of the manuscript.

## Abstract

**Background:** We know very little about the microbiota inhabiting the upper female reproductive tract and how it impacts on fertility.

**Aims:** This pilot study aimed to examine the vaginal, cervical, and endometrial microbiota for women with a history of infertility compared to women with a history of fertility.

**Materials and Methods:** Using a retrospective case-control study design, women were recruited for collection of vaginal, cervical and endometrial samples. The microbiota composition was analysed by 16S rRNA gene amplification and endometrial expression of selected human genes by qRT-PCR.

**Results:** Sixty-five specimens from the reproductive tract of 31 women were successfully analysed using 16S rRNA gene amplicon sequencing (16 controls and 15 cases). The dominant microbial community members were consistent in the vagina and cervix, and generally consistent with the endometrium although the relative proportions varied. We detected three major microbiota clusters that did not group by tissue location or case-control status. There was a trend that infertile women more often had *Ureaplasma* in the vagina and *Gardnerella* in the cervix. Testing for the expression of selected genes in the endometrium did not show evidence of correlation with case-control status, or with microbial community composition, although Tenascin-C expression correlated with a history of miscarriage.

**Conclusions:** There is a need for further exploration of the endometrial microbiota, and how the microbiota members or profile interplays with fertility or assisted reproductive technologies.

## Background

The vaginal microbiota has an established role in female reproductive tract physiology, pathogen defense, and function [1, 2]. In 2011, the vaginal microbiota of reproductive-age women was characterized by 16S rRNA gene amplicon sequencing, leading to the identification of five community state types (CST) [3]. These were defined by the presence or absence of a dominant *Lactobacillus* spp., including CST I: *Lactobacillus (L.) crispatus*, II: *L. gasseri,* III: *L iners*, IV: a diverse array of strict and facultative anaerobes lacking a dominant *Lactobacillus* spp., and V: *L. jensenii* [3]. Longitudinal analysis subsequently showed that the vaginal microbiota is highly dynamic, with changes observed after sexual contact, menses, douching, and lubricant use [4]. Although, a separate study using CPN60 profiling reported community stability throughout the menstrual cycle [5].

There are few studies of the upper reproductive tract microbiota. Analysis of vaginal, cervical, and endometrial specimens after insertion of an intrauterine levonorgestrel device (LNG IUS) found a microbiota shift towards *Lactobacillus crispatus* [6]. Species-specific PCR analysis of hysterectomy samples demonstratedthat the upper reproductive tract microbiota did not correlate with selected host inflammatory markers [7]. Upon examining the tip of the transfer catheter used for embryo transfer during IVF [8], a lower relative abundance of *Pseudomonas* spp. and *Flavobacterium* spp. was present in women who proceeded to successful pregnancy [8]. The diversity of the vaginal microbiota in women undergoing *in vitro* fertilization embryo transfer was higher for women who had a live birth from the cycle [9]. The presence of a non-*Lactobacillus* dominant microbiota in the endometrium correlated with IVF embryo transfer failure [10]. While these reports support that the reproductive tract microbiota impacts fertility and pregnancy outcomes, much remains to be tested, such as examining infertility or pregnancy outcomes in the context of specific taxa, species level

analysis, or the community state types/profiles. In this pilot study, we sought to examine the possible association of the reproductive tract microbiota with women who have a current or recent past history of infertility.

## Method

### Study design

We designed a pilot case-control study of the reproductive tract microbiota of women with a recent previous or current history of infertility (Cases), compared to women with no history of infertility (Controls) (Table 1). Women already undergoing hysteroscopy for a variety of reasons, excluding suspected cancer, were invited to participate. Participants categorized as Cases were currently attending gynecological or fertility care, or attending for other gynecological reasons but had a recent previous history of infertility that required assisted reproductive technologies. Inclusion criteria for Cases were women who had reported greater than a year of trying to achieve pregnancy, and who currently or previously required assisted reproductive technologies to achieve pregnancy. Five women in the infertile group were undergoing treatment for infertility at the time of recruitment; the remaining ten were having treatment for other gynecological reasons, having previously required assisted reproductive technologies to achieve pregnancy. Given the retrospective and pilot nature of this study, type of infertility was not an inclusion or exclusion criteria. Participants recruited to the Control group were women who had a history of pregnancies with no need for assisted reproductive technologies, and who were attending gynecological care for a hysteroscopy procedure for gynecological care (hysteroscopy for an IUD (IUS) change, or hysterectomy, excluding suspected reproductive cancers). Gynecological and obstetric histories were collected using a self-completed questionnaire. Several participants who were recruited and consented to the

study, but were 50 years or over, were used for extraction method validation and were not included in the analysis. Participants whose vaginal and/or other specimens did not pass extraction quality assessment were not included for sequencing.

At the time of hysteroscopy in the operating theatre, a sterile speculum was inserted into the vagina, and swabs collected from the posterior fornix and the endocervical canal. The vagina was then prepared for surgery with betadine antiseptic surgical solution. An endometrial biopsy was collected using an endometrial curette and stored in 0.5 ml of RNALater. Study samples were immediately taken to the on-site Tissue Bank and frozen at -80°C, with the exception of the endometrial sample that was incubated overnight at 4°C to allow the preservative buffer to penetrate the tissue, and then frozen at -80°C the following day.

In addition to comparing participant microbiota profiles based on case and control categories, we also analyzed other aspects of their gynecological history. These included the type of contraceptive used, any history of miscarriages, any history of endometriosis, and age . For contraceptives, we grouped the participants into the categories of i) *pill* (oral contraceptives), ii) *LNG IUS* (intrauterine levonorgestrel device) or iii) *other*, based on self-reporting. The *other* contraceptive category included methods such as rhythm, condoms, tubal ligation or no use of active contraception at all. These were classified together (Other) because we assumed similar implications for endometrial gene expression as compared to hormonal contraceptives. We used the following age groups for categorical analysis: 28-33, 34-39, 40-45, and 46-49 years old.

**Ethics approval and consent to participate**

Ethical approval to conduct this study was granted by the Uniting Care Health Human Research Ethics committee (Approval number 1227), Queensland University of Technology Human Research Ethics Committee (Approval number 1300000004), and University of Technology

Sydney Human Research Ethics Committee (Approval number 2015000700). Participants provided informed written consent prior to participation in the study.

### Availability of data and material

The dataset is available in SRA database under accession number: PRJEB18626.

### Microbiota processing, amplification and analysis

The protocols for sample processing, 16S rRNA gene amplification, sequencing, statistical analysis of microbiota data, PCR for *Ureaplasma*, and qPCR for endometrial gene expression are provided as Supplementary Material 1.

### Statistical analysis

To identify differences between cases and controls, we performed a non-parametric t-test using species level classifications [11], fully described in the Supplementary material 1. Plots were generated using the R Package Phyloseq v1.12.2 [12]. We compared the proportions or distributions of the demographic (age), obstetric, and gynecological factors between the case and control groups using SPSS (specific test indicated with the results).

## Results

### Case-control groups were comparable for gynecological factors, although age was significantly different

This was a retrospective pilot case-control examination of the reproductive tract microbiota for women with a current or past history of infertility needing assisted reproductive technologies to conceive (Cases), compared to women with no history of infertility (Controls) (Table 1).

The groups had similar proportions of the types of contraceptives in use, endometriosis, and miscarriage (Table 1). However, the number of live births, seeking pregnancy, and age were significantly different between the Cases and Controls.

**The endometrial microbiota appeared to have different relative abundances of microbiota to that in the vagina and cervix in some participants**

16S rRNA gene amplicon sequencing was performed on DNA isolated from the vagina, cervix, and endometrium, for samples where this could be successfully extracted (summarized in Table 2 and Supplementary Table S2). The sequence depth per sample was variable, with the highest number of filtered sequences obtained from vaginal (mean=88,302) and cervical (mean=95,401) samples (Supplementary Table S2). Endometrial samples produced fewer reads ( average of 7,815 per sample).

The genus *Lactobacillus* was most frequently observed (Figure 1). The most abundant taxonomic units were the same in both cervical and vaginal specimens for all 24 of the 33 women where both were sequenced (Figure 1). In women with sequences from both the endometrium and vagina, half had some differences in the proportions of the most abundant taxa relative to the vagina. A principal coordinates analysis of the Jensen-Shannon Divergence showed no grouping of specimens by reproductive site, age, case-control status (Supplementary data Figure S1). Similarly, Alpha Diversity analysis showed a variety of richness across the sites and case-control status with no clear associations (Supplementary data Figure S1).

Community clusters were analyzed using a cluster dendrogram [3] that is based on dominant or absent *Lactobacillus* species (Figure 2). Microbiota profiles were distributed across these

community state types, with three dominant clusters apparent. Community state type I (CSTI; *L. crispatus* dominant), CSTIII (*L. iners* dominant), and CSTIV were most common. Generally, each individual's specimens (vaginal, cervical, and endometrial) clustered together with the exception of three endometrial samples and one vaginal sample that featured a mix of *Corynebacterium*, and *Propionibacterium*, that formed a cluster.

There were possible sub-clusters within the CSTIV (with CST II or dominant *L. gasseri* within this group). These were dominated by *Gardnerella vaginalis* (with *Prevotella* in some cases) (consistent with CSTIV [3], CST IV-B [4]), or *Streptococcus agalactiae* /*Streptococococcus anginosus* were dominant (consistent with CST IV-A [4]); or *Bifidobacterium* (also as described in another study [5]). However, while consistent with previously reported sub-clusters, these clusters would need a larger sample size to resolve.

**Women with a past history of infertility showed a trend towards having *Ureaplasma* spp. in the vagina and *Gardnerella* in the cervix**

We observed a trend for two organisms to be more abundant or prevalent in the Cases (*Ureaplasma* spp., p=0.042 (vagina) and *G. vaginalis*, p=0.044 (cervix); unadjusted). A *Ureaplasma*-specific PCR was used to further confirm that all five positive samples contained *Ureaplasma parvum* (Supplementary Figure S2 and S3). Four of the five women with a history of infertility (Cases) colonised with *Ureaplasma* also had a vaginal CST III dominated by *L. iners* (p= 0.015; unadjusted for multiple testing).

**Endometrial gene expression significantly correlated with a history of miscarriage and contraceptive use, but not with a history of infertility or microbiota composition**

We next assessed endometrial expression of selected genes with known endometrial function (IL-1α, IL-6, IL-8, TN-C, TNFα, and SDC1). There were no significant differences in the expression of these genes in relation to case-control status (Figure 3A, B). High expression of TN-C grouped in two clusters on a linkage dendrogram and was higher in women with a history of miscarriage ($p<0.001$, Figure 3C). IL-6 expression levels were higher in LNG IUS users ($p=0.05$), whereas SDC1 was significantly lower in LNG IUS users compared to OCP users ($p<0.01$, Figure 3D).

## Discussion

The objective of this pilot study was to examine the reproductive tract microbiota (vagina, cervix, and endometrium) in women with a history of needing fertility treatment for pregnancy compared to fertile controls. We observed some differences in the upper reproductive tract (endometrium) compared to the vagina and cervix within individual participants. Furthermore, we showed a trend of *Gardnerella* in the cervix and *Ureaplasma* in the vagina being associated with women with a history of infertility, adding further support to the case that the microbiota should be further evaluated for relevance to fertility and assisted reproductive treatment.

Our study has a number of limitations that should be considered when interpreting these results. The time of fertility status is in the past, meaning the samples were not collected when the women were experiencing infertility/fertility. Also,the Control group were significantly older. Although three reproductive sites were sampled from each participant, inconsistencies in DNA extraction quality and/or sequence results limited the number of individuals with sequenced microbiota from all three sites. This may reflect the expected relatively lower

numbers of bacteria in the endometrium, or could indicate difficulties in DNA extraction from this site. The sampling protocol specified the need to avoid the surface of the vaginal canal when removing the cervical/endometrial sample, however we do note the potential for cross contamination by this technique. Additionally, it is known that the vaginal microbiome (and presumably also the endometrial microbiome) is affected by ethnicity, age, pregnancy, sexual activity, smoking and exogenous hormones [13-17]. While we were not able to control for several of these factors in this pilot study, we recommend that any future large-scale studies should attempt to do so.

In spite of these limitations, our findings are consistent with other published studies. Many individuals within this study had a dominant *Lactobacillus* species [3, 4]. The possible CSTIV sub-clusters may be significant for future research. A potential *Bifidobacterium* dominant sub-cluster was present in some endometrial samples, consistent with previous work [10]. *Ureaplasma* spp. (confirmed to be *U. parvum)* trended towards an association with infertility cases in the vagina and *Gardnerella* in the cervix. *Ureaplasma* spp. have been identified within the reproductive tracts of both fertile and infertile women [18, 19], and have been associated with preterm delivery (< 37 weeks of gestation) [20, 21] and other adverse pregnancy outcomes [21]. Here we provide a further association of *Ureaplasma* with infertility. The different abundance and presence/absence of taxa between the upper and lower genital tract specimens warrants further investigation, and our pilot study provides further baseline data to support the need for continued investigation into the endometrial microbiota. We speculate that the microbiome of the endometrium could be an important factor in unexplained infertility and/or repeat IVF cycle failures.

There were no differences in endometrial gene expression of our selection of target genes based on case-control (fertility) status. TN-C had significantly high expression in

participants with history of miscarriage, consistent with prior reports of an association with recurrent miscarriage [22-25]. We also detected the expected differences in IL-6 endometrial gene expression in women using LNG IUS compared to other contraceptives, as this has been previously reported [26, 27]. SDC1 has been reported as a marker of endometritis [28, 29], and here we found that SDC1 was significantly downregulated in LNG IUS users.

Our pilot study supports that the endometrium microbiota is likely to have subtle yet important differences from the lower reproductive tract, and even the lower reproductive tract microbiota may have an impact on infertility. Future studies should include much larger sample sizes and prospective recruitment during fertility treatment. This will permit the monitoring of the microbiota and endometrial tissue responses during assisted reproductive treatment cycles. There is already a common practice of endometrial scratching (reviewed, [30]) during the menstrual cycle prior to embryo transfer. We propose scratching material could be harvested and tested for the endometrial microbiota and expression of select endometrial genes to optimize embryo transfer timing. Such an innovation will require larger, longitudinal studies of the reproductive tract microbiota on women undergoing fertility treatment during their cycles.

**Acknowledgements**

The authors acknowledge the Wesley Medical Research Biobank team members for working with us to facilitate this study (especially Emma Raymond). The authors acknowledge and are thankful for valuable funding from Wesley Medical Research awarded to JA and WMH to support this research project.

## Figures

**Figure 1. The relative proportion of the 25 most abundant taxonomic assignments.** The key is to the right. The results are grouped according to the participants' Case-Control status (indicated at the top). The participant codes are below the graphs with each of the three specimen sites shown by row(indicated to the right: Vagina, Cervix, Endometrium). Only samples with more than 1,000 assembled and assigned sequences are displayed.

**Figure 2. Community state types dominated by *Lactobacillus* were present in the majority of specimens.** Heat map showing the proportions of the top 20 most abundant taxonomic groups in the reproductive tract microbiota. Contraceptive, age, history of endometriosis, history of miscarriage, and case/control status are displayed above each sample (color keys are indicated to the right). Linkage clustering of the samples to the previously defined community state types (CST I, II, III and IV) [3] is shown at the top of the figure.

**Figure 3. Endometrial expression of six selected genes with known implications for endometrial pathology or function.** (**A)** The dendrogram shows hierarchical linkage clustering of IL-1α, IL-6, IL-8, SDC1, TN-C and TNF-α gene expression. The relevant participant data are shown on the right; including case-control status, presence of *Ureaplasma* spp., age, miscarriage, and contraceptive (color keys indicated to the right). Colors represent the transformed mean ΔCt values for each specimen, calculated as $2^{-\Delta Ct}$. **(B)** miscarriage

history, (**C**) contraceptives, (**D**) case-control status. Graphical representation of the expression levels of six genes of interest relative to the geometric mean of GAPDH and PPIA levels, grouped according to participant data. The data displayed on the Y-axis of each graph represents the transformed mean ΔCt values of each group, calculated as $2^{-\Delta Ct}$ and multiplied by an arbitrary factor of 100. Error bars denote the standard error of the mean, while the X-axis displays gene names. Bar color indicates participant grouping based on (**A**) Statistical testing of data was performed using either the Kruskall-Wallis or Mann-Whitney tests (* $p<0.05$; *** $p=0.0006$).

## Supplementary Data

Supplementary Data are available for the manuscript.

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**Table 1**

| | Categories/ groups | CONTROL (n=16)* (fertile) | CASE (n=15) (infertile) | |
|---|---|---|---|---|
| **Age** | Range (mean) | 35-48 (42.75) | 28-45 (37.6) | p= 0.05! |
| | 28-33 | 0 | 3 | p=0.035$ |
| | 34-39 | 4 | 7 | |
| | 40-45 | 8 | 5 | |
| | 45-49 | 4 | 0 | |
| **Ever Pregnant** | | 16 (100%) | 12 (80%) | p=0.203$ |
| **Ever had a Live Birth** | | 16 (100%) | 11 (73.3%) | p=0.086$ |
| **# live births median (interquartile range)** | | 2.5 (2-3) | 2.0 (0-2) | p=0.049^ |
| **No longer seeking pregnancy** | | 16 (100%) | 10 (66%) | p= 0.018$ |
| **Contraceptive # (%)** | Other | 6 (37.5%) | 9 (60%) | p=0.149$ |
| | Pill | 4 (25%) | 5 (33.3%) | |
| | Mirena | 6 (37.5%) | 1 (6.6%) | |
| **History of endometriosis** | Y | 6 (37.5%) | 6 (40.0%) | p=0.589$ |
| | N | 10 (62.5%) | 9 (60.0%) | |
| **History of miscarriage** | Y | 8 (50.0%) | 5 (33.3%) | p=0.283$ |
| | N | 8 (50.0%) | 10 (66.7%) | |

! t test; $ Fishers exact chi-square; ^Wilcoxon rank sum test

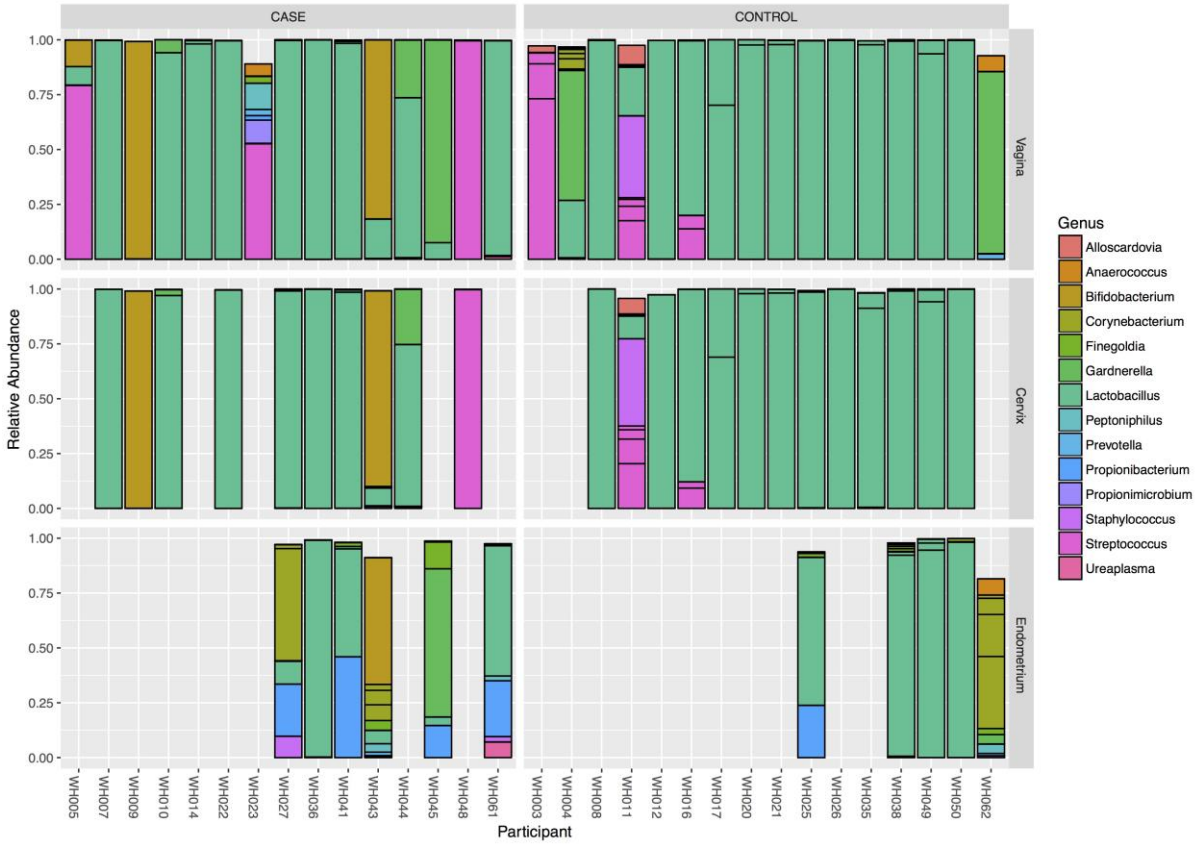
CASE
CONTROL
Vagina
Cervix
Endometrium
Relative Abundance
1.00
0.75
0.50
0.25
0.00
WH005
WH007
WH009
WH010
WH014
WH022
WH023
WH027
WH036
WH041
WH043
WH044
WH045
WH048
WH061
WH003
WH004
WH008
WH011
WH012
WH016
WH017
WH020
WH021
WH025
WH026
WH035
WH038
WH049
WH050
WH062
Participant
Genus
Alloscardovia
Anaerococcus
Bifidobacterium
Corynebacterium
Finegoldia
Gardnerella
Lactobacillus
Peptoniphilus
Prevotella
Propionibacterium
Propionimicrobium
Staphylococcus
Streptococcus
Ureaplasma

**Figure 1**

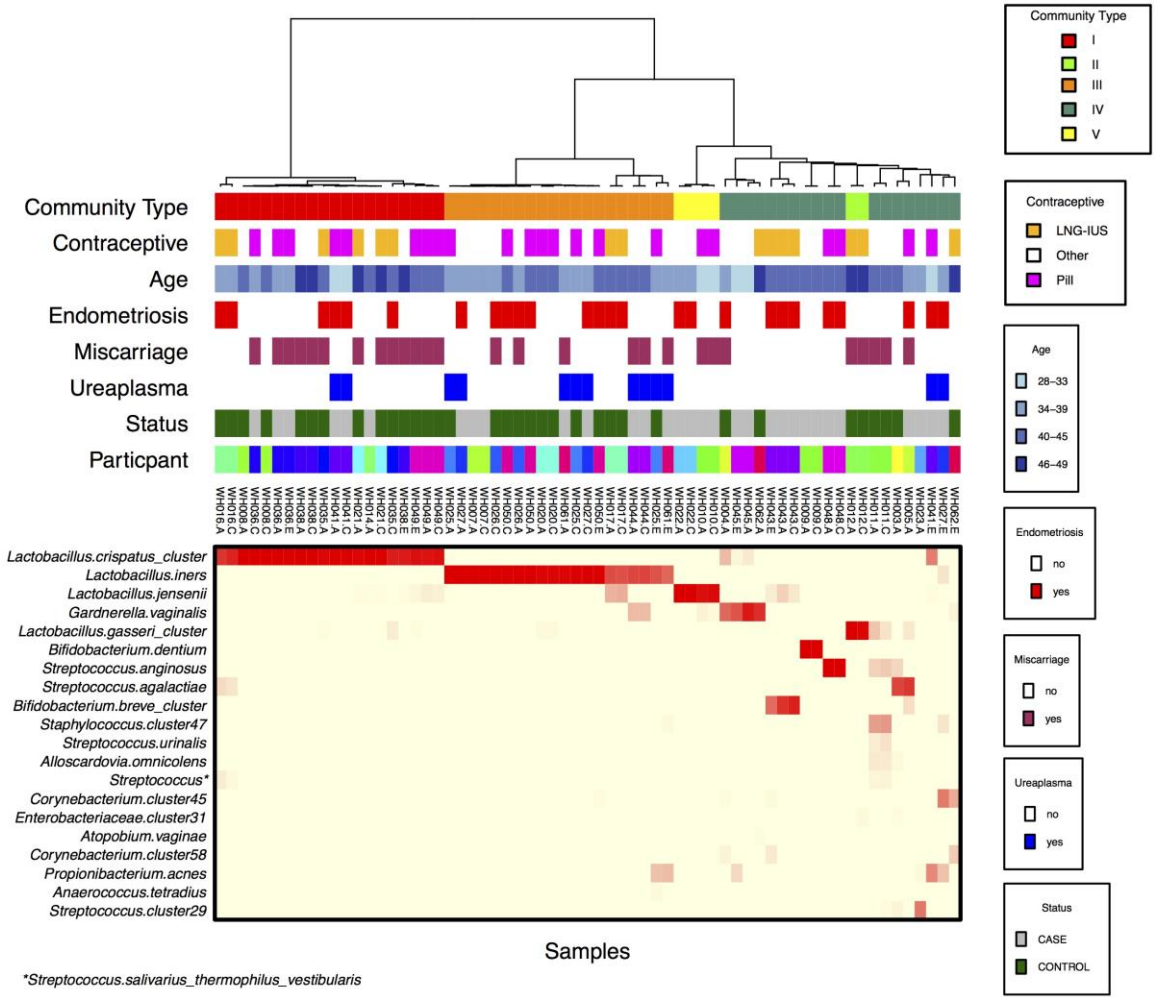
Community Type
Contraceptive
Age
Endometriosis
Miscarriage
Ureaplasma
Status
Particpant
Lactobacillus.crispatus_cluster
Lactobacillus.iners
Lactobacillus.jensenii
Gardnerella.vaginalis
Lactobacillus.gasseri_cluster
Bifidobacterium.dentium
Streptococcus.anginosus
Streptococcus.agalactiae
Bifidobacterium.breve_cluster
Staphylococcus.cluster47
Streptococcus.urinalis
Alloscardovia.omnicolens
Streptococcus*
Corynebacterium.cluster45
Enterobacteriaceae.cluster31
Atopobium.vaginae
Corynebacterium.cluster58
Propionibacterium.acnes
Anaerococcus.tetradius
Streptococcus.cluster29
Samples
*Streptococcus.salivarius_thermophilus_vestibularis
Community Type
I
II
III
IV
V
Contraceptive
LNG-IUS
Other
Pill
Age
28–33
34–39
40–45
46–49
Endometriosis
no
yes
Miscarriage
no
yes
Ureaplasma
no
yes
Status
CASE
CONTROL

**Figure 2**

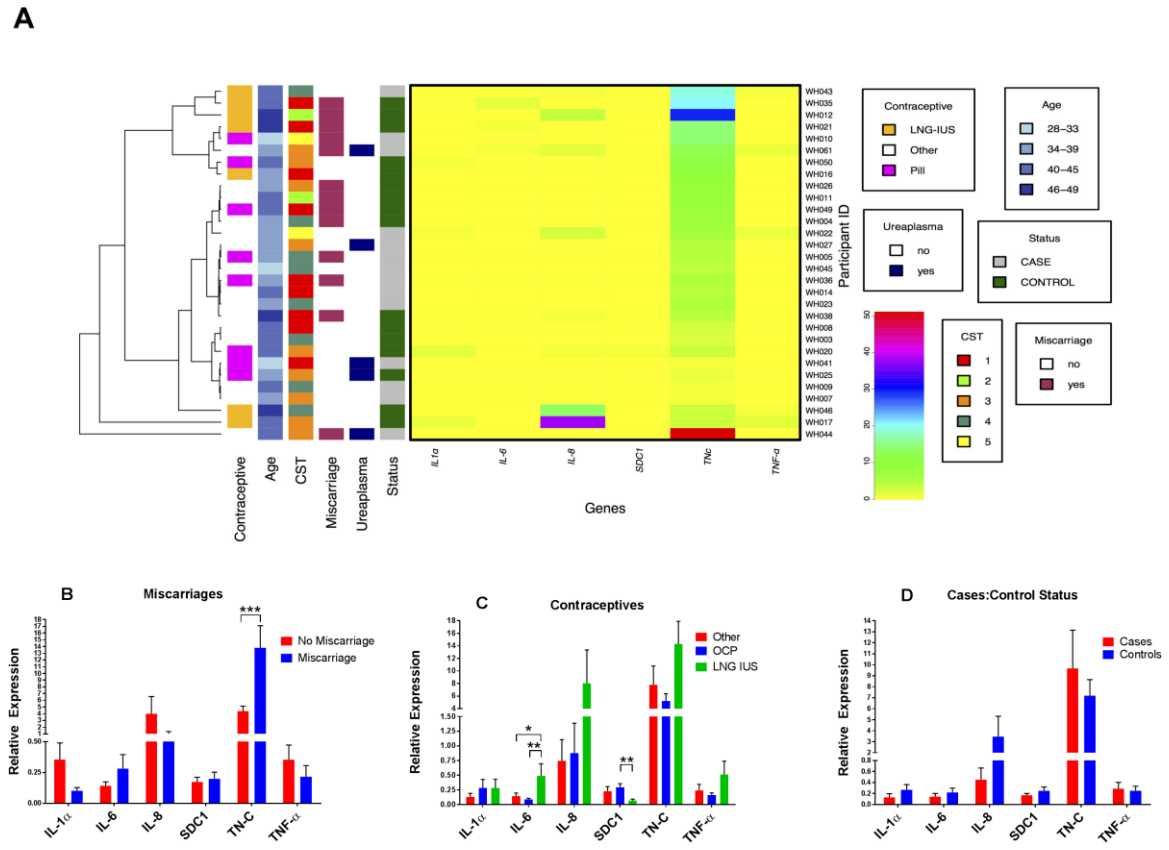
A
Contraceptive
LNG-IUS
Other
Pill
Age
28–33
34–39
40–45
46–49
Ureaplasma
no
yes
Status
CASE
CONTROL
CST
1
2
3
4
5
Miscarriage
no
yes
Participant ID
Genes
Contraceptive
Age
CST
Miscarriage
Ureaplasma
Status
B
Miscarriages
No Miscarriage
Miscarriage
Relative Expression
C
Contraceptives
Other
OCP
LNG IUS
D
Cases:Control Status
Cases
Controls
IL-1α
IL-6
IL-8
SDC1
TN-C
TNF-α

**Figure 3**