Influence of Pregnancy History on the Vaginal Microbiome of Pregnant Women in their First Trimester

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Pregnancy permanently alters maternal anatomy, physiology and immunity. We evaluated if the vaginal microbiome differed between women with a first or subsequent conception. Relative abundance of bacteria in the vaginal microbiome in first trimester pregnant women, 52 with their first known conception, 26 with a prior spontaneous or induced abortion but no deliveries and 77 with at least one prior birth, was determined by classifying DNA sequences from the V1-V3 region of bacterial 16S rRNA genes. Lactobacillus crispatus was the numerically most abundant bacterium in 76.4% of women with a first conception, 50.0% with only a prior spontaneous or scheduled abortion and 22.2% with a prior birth (p ≤ 0.01). L. iners was the most abundant bacterium in 3.8% of women with a first conception as compared to 19.2% (p = 0.03) and 20.8% (p = 0.03) in those with a prior abortion or birth, respectively. Gardnerella was the most abundant bacterial genus increased from 3.8% in women with a first conception to 15.4% and 14.3% in those with a prior abortion or birth, respectively (p > 0.05). L. iners dominance was also associated with a history of spontaneous abortion (p ≤ 0.02). The composition of the vaginal microbiome and its influence on pregnancy outcome varies with pregnancy history.

Lactobacillus species tend to dominate the vaginal microbiome to a greater extent during pregnancy than when women are not pregnant1,2. Their increased abundance and stability may be due to a multitude of factors that favor their selection. These might include the absence of cyclic hormonal variations or menstruation, decreased sexual activity, dietary and hygiene changes and alterations in the composition of cervical-vaginal secretions. The increased availability of specific nutrients might also enrich for lactobacilli. For example, estrogen production following conception promotes glycogen production in vaginal epithelial cells3, and glycogen degradation by α-amylase is thought to provide high levels of maltose oligomers that are readily metabolized by lactobacilli4. Additional contributing factors could be the immunological and physiological changes that occur during the transition from the non-pregnant to the pregnant state.

The influence of the vaginal microbiome on pregnancy outcome remains unsettled. It has been reported that the vaginal microbiota differs5 or does not differ6 between women who deliver prematurely or at term. However, the influence of a prior conception on the composition of the vaginal microbiota during gestation has not previously been investigated. In the present study we evaluated whether the vaginal microbiota in first trimester pregnant women varied depending on whether or not this was their first conception or first delivery. This time period was studied because successful implantation of the embryo into the uterine wall and initiation of vascular changes leading to development of the placenta during the first trimester may be the stages most susceptible to pregnancy disruption7.

Results

Characteristics of the study population – women with their first known conception, women with no deliveries who had either a prior pregnancy termination or a spontaneous abortion, women with a prior delivery - are

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Women with their first conception were younger (22.3 years) than women in the other groups (p ≤ 0.01). There were no differences between groups in body mass index, the percentage who conceived by in vitro fertilization, the occurrence of a preterm delivery, gestational age at delivery or the neonatal birthweight. There were also no differences in the racial distribution of subjects in each group (Table 1).

Characterization of bacterial communities in relation to pregnancy history.

The association between a prior conception or birth and the relative abundance of various bacterial taxa in the vaginal microbiome is shown in Table 2. *L. crispatus* was the numerically most abundant bacterium in 76.4% of women with a first conception, in 50.0% of women with a prior spontaneous or induced abortion but no births and in 22.1% of women with a prior birth. These differences in *L. crispatus* abundance between women with their first conception and those with a prior birth (p = 0.0001) and between women with a prior abortion only and those with a prior birth (p = 0.0116) were significant. Conversely, *L. iners* was the numerically most abundant bacterium in only 3.8% of women with a first conception as opposed to 19.2% with a prior abortion (p = 0.0378) and 20.8% in women with a prior birth (p = 0.0032). Similarly, detection of *L. gasseri* as the most abundant vaginal bacterium occurred in 1.9% of women with a first conception as opposed to 7.7% with a history of spontaneous abortions and 18.2% of women with a previous birth (p = 0.0043 vs. first conception). The presence of *Gardnerella* as the numerically most abundant bacterial genus also increased from 3.8% of women in the first conception group to 15.4% in the abortion only group and 14.3% in the prior birth group. However, these differences did not reach statistical significance (p > 0.05), possibly due to the small number of women in these groups. The relative abundance of the different bacterial taxa were similar between White and non-White subjects (Table 2).

Table 1. Characteristics of the study population. Spont, spontaneous; SD, standard deviation.

| Characteristic | First conception | Prior conception but no births | Prior birth | White | Non-white |
|---------------|------------------|---------------------------------|------------|-------|-----------|
| Number women evaluated | 52 | 10 | 16 | 77 |
| Mean age (SD in years) | 32.3 (3.8) | 33.3 (4.7) | 36.0 (5.5) | 34.6 (3.7) |
| Mean body mass index (SD) | 22.5 (2.6) | 22.9 (2.2) | 23.0 (3.1) | 22.4 (3.1) |
| In vitro fertilization | 6 (11.3%) | 3 (30.0%) | 5 (29.4%) | 2 (2.6%) |
| Preterm delivery | 2 (3.8%) | 0 | 0 | 1 (1.3%) |
| Mean gestational age at birth (SD) | 39.2 (1.5) | 39.7 (0.6) | 39.3 (1.1) | 39.5 (1.0) |
| Mean neonatal birthweight (SD) g | 3294 (513) | 3306 (404) | 3355 (534) | 3287 (500) |
| Race | | | | |
| White | 52.9% | 37.5% | 46.7% | 63.5% |
| Asian | 13.7% | 25.0% | 13.3% | 10.8% |
| Hispanic | 5.9% | 0 | 6.7% | 2.7% |
| Black | 2.0% | 0 | 0 | 0 |
| Mixed | 25.5% | 37.5% | 33.3% | 23.0% |

Table 2. The numerically most abundant bacterial taxa in the vaginal microbiota of pregnant women in the first trimester in relation to pregnancy history and race. The data are presented as the percentage of cases in which a specific taxa was numerically most abundant in each of the three categories of subjects. a p = 0.0001 vs. prior delivery, 0.0217 vs. prior conception; b p = 0.0116 vs. prior delivery; c p = 0.0032 vs. prior delivery, 0.0378 vs. prior conception; d p = 0.0043 vs. first conception; e p = 0.0914 vs. prior conception, 0.0734 vs. prior delivery.

| Bacterial taxa | First conception | Prior conception but no births | Prior birth | White | Non-white |
|----------------|------------------|---------------------------------|------------|-------|-----------|
| N = 52 | N = 26 | N = 77 | N = 84 | N = 67 |
| *L. crispatus* | 76.4%a | 50.0%b | 22.1% | 46.4% | 38.8% |
| *L. iners* | 3.8%c | 19.2% | 20.8% | 7.1% | 10.4% |
| *L. gasseri* | 1.9% | 7.7% | 18.2%d | 10.7% | 11.9% |
| *L. jensenii* | 9.6% | 7.7% | 5.2% | 4.8% | 11.9% |
| Other lactobacilli | 0% | 0% | 6.5% | 3.6% | 0 |
| *Gardnerella* | 3.8%e | 15.4% | 14.3% | 8.3% | 10.4% |
| *Streptococcus* | 3.8% | 0% | 5.2% | 1.2% | 6.0% |
| Other genera | 0.7% | 0% | 7.7% | 3.6% | 7.5% |
most abundant. There were no statistically significant associations between the relative abundance of any bacterial taxa and maternal age, gestational age at birth, or neonatal gender.

A listing of all taxa in the vaginal microbiome of each subject in each of the three groups is presented in supplemental Tables 1–3.

### Vaginal communities and history of spontaneous abortions.

Among the women in both the prior conception only and birth groups, 39 (25.2%) had a history of spontaneous abortion. Twenty six women (16.8%) had one abortion, 11 (7.1%) had two abortions and two (1.3%) had three abortions. The association between spontaneous abortion history and bacterial abundance is detailed in Table 3. The only significant difference noted was with *L. iners*. This bacterium was numerically most abundant in 9.5% of the women who had never had a spontaneous abortion as compared to 26.9% with one abortion (p = 0.0240) and 45.4% with two abortions (p = 0.0049). In the two women with three spontaneous abortions *L. crispatus* or *L. jensenii* were most abundant in their vaginas. There was no association between a history of spontaneous abortion and maternal age or gestational age at the time of birth.

### Discussion

Here we provide evidence that a prior conception influences the relative composition of the vaginal microbiota during the first trimester of pregnancy. Women with a prior birth or even only an induced or spontaneous abortion had a vaginal microbiota in the index pregnancy that was qualitatively and quantitatively different from that present in women experiencing their first conception. Specifically, there was a marked decrease in the relative abundance of *L. crispatus* and a concomitant increase in the relative abundance of other *Lactobacillus* species as well as *Gardnerella* in women with a prior conception, regardless of whether or not this proceeded to a birth. It appears that alterations in the vaginal microenvironment in women with a prior conception or delivery favor the proliferation of *Gardnerella*, *L. iners* and *L. gasseri* at the expense of *L. crispatus*.

What are the implications of our observation that the vaginal microbiota differs depending on prior pregnancy history? First of all it suggests that future studies designed to address the influence of the vaginal microbiome on pregnancy outcome must invariably take into account the pregnancy history of the women being evaluated. In addition, the generally accepted increase in *L. crispatus* abundance in the vagina during the first trimester of pregnancy appears to be limited to women with first conceptions. Thus, the contribution of specific vaginal bacteria or groups of bacteria to pregnancy outcome needs to be reevaluated and may differ depending on pregnancy history. Recurrent miscarriages and preeclampsia are most common in nulliparous women indicating that alterations in the vaginal microenvironment in women with a prior conception, regardless of whether or not this proceeded to a birth. It appears that alterations in the vaginal microenvironment in women with a prior conception or delivery favor the proliferation of *Gardnerella*, *L. iners* and *L. gasseri* at the expense of *L. crispatus*.

The expression of genes that inhibit pro-inflammatory immune system activation are up-regulated during pregnancy to minimize interference with proper fetal and maternal cell differentiation, fetal organ development and pregnancy progression. In addition, extrathyroidal regulatory FOXP3+ CD4+ T lymphocytes (Treg cells) that recognize paternal antigens expressed by the fetus begin to be produced shortly after implantation of the embryo in the uterus. These Treg cells are immunosuppressive and stimulate the release of anti-inflammatory mediators that limit cell-mediated immune responses not only to fetal antigens but also to inflammation induced by bacteria and other antigens that may be encountered by the mother. A population of these Treg cells persist in the circulation after a pregnancy is concluded. Their proliferation is induced by a subsequent conception which results in an elevated level of immunosuppression as compared to that which is present following a first pregnancy. Concomitant with variations in Treg-directed immune system modulation in nulliparous and multiparous women the present study identifies an alteration in the composition of the vaginal milieu with parity. Identification of the specific vaginal components that differ between a first and subsequent conception and

| Bacterial taxa | Number of prior abortions |
|---------------|--------------------------|
|               | None | One | Two | Three |
| *L. crispatus*| 48.3%| 38.5%| 27.3%| 50% |
| *L. iners*    | 9.5% | 26.9%| 45.4%| 0%  |
| *L. gasseri*  | 9.5% | 23.1%| 0%  | 0%  |
| *L. jensenii* | 8.6% | 0%  | 0%  | 50% |
| Other lactobacilli | 4.3% | 0% | 0% | 0% |
| *Gardnerella* | 12.1% | 0% | 27.3% | 0% |
| *Streptococcus* | 5.2% | 0% | 0% | 0% |
| Other genera  | 2.5% | 11.5%| 0% | 0% |

Table 3. The numerically most abundant bacterial taxa in vaginal communities of 155 pregnant women during the first trimester in relation to history of spontaneous abortion. The data are presented as the percentage of cases in which a specific taxa was numerically most abundant in each of the abortion categories. *p* = 0.0240 vs. none; *p* = 0.0049 vs. none.
their contribution to an altered vaginal microbiota and to pregnancy-related immunity remains to be more fully
determined.

The association between *L. iners* as the dominant member of the vaginal microbiota and a history of spontane-
ous abortions is consistent with a previous study showing that this bacterium does not promote maintenance of
a stable vaginal microbiota during pregnancy but, instead, is associated with the proliferation of other bac-
terial genera. The occurrence of a non-lactobacilli-dominated vaginal microbiota during early pregnancy in
women with a first conception may increase the likelihood of bacterial migration into the uterus and interfere
with immune regulatory mechanisms essential for proper embryo intrauterine implantation. Unfortunately, In
the present study we do not have information on whether or not the reported abortions preceded a successful
pregnancy in all of our subjects. Further investigations are needed to identify biomarkers that can pinpoint the
subset of women with an *L. iners*-dominated vaginal microbiota who are truly at risk for spontaneous abortion.

It should be stressed that the great majority of women in the present study delivered a healthy baby at term
following an uneventful gestation, regardless of which bacteria were numerically most abundant in their vaginal
microbiota. Thus, while differences in the composition of the vaginal microbiome may influence susceptibility to
an adverse pregnancy outcome in a subset of women, these variations do not predispose the majority of women to
a pathological event. There clearly remains an unmet need to identify the genetic, environmental, immunological
and physiological factors that, when combined with the presence of a specific vaginal microbiota, increase the
likelihood of an adverse outcome in individual pregnant women.

Advantages of our study include the relatively high number of subjects evaluated, collection of all samples by
a single investigator and utilization of state of the art techniques for analysis of vaginal communities. A limitation
is that since all subjects were from a single institution in New York City it remains uncertain if the findings will be
similar in women from other geographical locations and racial groups. It is also possible that an unknown num-
ber of women in the first conception group had a prior pregnancy that ended in an early stage abortion without
the woman's awareness and that their designation as having no prior exposure to the products of conception is
incorrect. Therefore, we cannot rigorously exclude the possibility that this group includes some women with a
very early stage pregnancy loss. In addition, it remains unknown whether previous pregnancy history influences
the composition of the vaginal microbiota in later pregnancy stages or postpartum. Exposure to the male partner's
semen can elicit immunity to paternal antigens prior to conception. The extent of induction of pre-conception
anti-paternal immunity may be expected to vary depending on the length of semen exposure as well as to indi-
vidual variations in the couple's genetic, immune and physiological capacity. It would, therefore, be of interest to
determine the influence of length of exposure to semen from a specific male partner on the composition of his
partner's vaginal microbiome.

While it appears unlikely to us based on the totality of experimental data, the possibility still remains that
differences in unidentified demographic characteristics between the study groups may have contributed, at least
in part, to the observed variations in the vaginal microbiota. Further investigation of different study populations
should help to more firmly resolve the validity of our data interpretation.

In conclusion, the composition of the vaginal microbiome in women in their first trimester of pregnancy
varied depending upon whether they have been previously exposed to the products of conception. This suggests
that the influence of specific bacteria or groups of bacteria in the vagina that promote fetal well-being and devel-
opment in early gestation may differ between primiparous and multiparous women.

**Methods**

**Subjects.** This prospective study consisted of 155 women who were seen at the outpatient obstetrical service
at Weill Cornell Medicine between September 2013 and November 2014 and who were between 8–12 weeks
gestation. Selection was based solely on the clinician's ability to obtain informed consent, collect the requisite
samples and transfer them to the laboratory in a timely manner for processing. Each woman's prior pregnancy
history - first conception, prior spontaneous pregnancy loss, previous delivery - was not known at the time of
sample collection. This information as well as outcome of the index pregnancy were obtained by chart review
only after completion of all laboratory studies. Exclusion criteria from participation were signs or symptoms of
a gynecological disorder or infection at the time of examination, multifetal gestation, presence of an endocrine
disturbance, steroid or antibiotic treatment in the previous four weeks, vaginal bleeding or the inability to give
informed consent. The study was approved by the Institutional Review Board at Weill Cornell Medicine and
each participant gave informed written consent. The methods were carried out in accordance with the relevant
guidelines and regulations.

**Sample collection.** During a routine speculum-based examination secretion and epithelial cells were
obtained from the posterior vaginal fornix with cotton swabs. Each swab was vigorously shaken into 1 ml ster-
ile phosphate-buffered saline, centrifuged and the supernatant and epithelial cell pellet fractions immediately
 aliquoted and stored at −80 °C. An additional sample was collected from the vaginal wall using the Copan
Diagnostics ESwab sample collection tubes (Fisher Healthcare, Houston, TX). These samples were immediately
frozen at −80 °C until shipped on dry ice to the Forney lab at the University of Idaho for microbiome analysis. All
samples utilized in this study were collected from pregnant women by the same investigator (AB).

**Purification of genomic DNA and 16SrRNA amplicon production.** A validated method was used to
extract and purify total genomic DNA from vaginal swabs as described in Supplemental Material. Amplicons of
the V-1 to V-3 region of bacterial 16S rRNA genes in samples were produced by two consecutive rounds of PCR
as described in Supplementary Information. The primers used in the first round were those developed by Frank et
al. that better maintain the rRNA gene ratio of Lactobacillus to Gardnerella present in the original sample. The
second round of PCR attached sample specific barcodes and Illumina sequencing adapters. The concentrations of
amplicons were determined using a Pico Green assay (Promega Inc.) and a SpectraMax Gemini XPS fluorometer (Molecular Devices, Sunnyvale, CA), then equal amounts (~100 ng) were pooled in a single tube. The amplicon pool was cleaned to remove short undesirable fragments using the following procedure. First the pool was size selected with using AMPure beads (Beckman Coulter Inc., Pasadena, CA), the product was then run on a 1% gel, excised from the gel, column purified using a Qiagen MinElute PCR purification kit and size selected again with AMPure beads (Beckman Coulter, Indianapolis, IN). To determine the quality of the amplicons, the pool was PCR amplified with Illumina adaptor specific primers followed by size selection using a DNA1000 chip and an Agilent 2100 Bioanalyzer. The cleaned amplicon pool was then quantified using the KAPA Illumina library quantification kit (KAPA Biosciences) and the Applied Biosystems StepOne plus real-time PCR system. Finally, sequences were obtained using an Illumina MiSeq. 300 paired-end protocol (Illumina, Inc., San Diego, CA) as described in the Supplementary Information.

**Classification of 16S rRNA gene amplicons.** Raw DNA sequence reads from the Illumina MiSeq were assigned to samples and classified as described in Supplementary Material. Reads belonging to the genus Lactobacillus were analyzed further to identify which species of this genus were present in the samples. Blastn was used in BLAST + (Camacho et al., 2009) and each read was compared to a database composed of Lactobacillus 16S rRNA gene sequences longer than 1000 bp that were downloaded from NCBI in February 2014). The identity of the different species reported as the best match for each read was recorded, the number of reads assigned to each species counted and their relative abundance in the different samples calculated. Phylotype rank abundance data were used to include only samples with ≥3,000 reads such that the depth of coverage for each community was sufficient to detect taxa with a relative abundance of at least 0.01 (1%). For constructing phylotype relative abundance tables we used a simple heuristic rule: to be included in a table a phylotype had to either (a) be present in more than one sample at an abundance of 1% or more, or (b) constitute more than 5% of a single community. Phylotypes that did not meet either of these criteria were aggregated into an “Other” category.

**Statistical analyses.** Statistical analysis was performed with the GraphPad (Graphpad Software Inc, San Diego, CA) and SPSS v24 (IBM Corp, Armonk, NY) statistical packages. Distribution of categorical variables was compared with the chi-square test or Fisher’s exact test if found with low frequency. Normality of distribution of continuous variables was examined with the Shapiro-Wilk test. Given that all variables were non-normally distributed Kruskall-Wallis and paired Mann-Whitney U tests were used. All p values were 2-sided and the alpha level of statistical significance was set at 0.05.

**Data availability statement.** The amplicon sequences will be deposited in the NCBI Short Read Archive (http://www.ncbi.nlm.nih.gov/sra/).

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**Author Contributions**

D.N., S.S.W. and L.J.F. conceived the original idea and study design. A.B., M.S. and J.S. obtained and processed the patient samples and collected the clinical data. G.M.S., K.G., L.J.F. and M.F. performed and analyzed the microbiome data. D.N., S.S.W. and L.J.F. interpreted the data and wrote the initial manuscript. All authors contributed to the final version of the manuscript.

**Additional Information**

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