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TECHNOLOGICAL INNOVATIONS

Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16S ribosomal subunit

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Abstract
Purpose Characterization of the human microbiome has become more precise with the application of powerful molecular tools utilizing the unique 16S ribosomal subunit's hypervariable regions to greatly increase sensitivity. The microbiome of the lower genital tract can prognosticate obstetrical outcome while the upper reproductive tract remains poorly characterized. Here, the endometrial microbiome at the time of single embryo transfer (SET) is characterized by reproductive outcome.
Methods Consecutive patients undergoing euploid, SET was included in the analysis. After embryo transfer, performed as per routine, the most distal 5-mm portion of the transfer catheter was sterilely placed in a DNA free PCR tube. Next-generation sequencing of the bacteria specific 16S ribosome gene was performed, allowing genus and species calls for microorganisms.
Results Taxonomy assignments were made on 35 samples from 33 patients and 2 *Escherichia coli* controls. Of the 33 patients, 18 had ongoing pregnancies and 15 did not. There were a total of 278 different genus calls present across patient samples. The microbiome at time of transfer for those patients with ongoing pregnancy vs. those without ongoing pregnancy was characterized by top genera by sum fraction. *Lactobacillus* was the top species call for both outcomes.
Conclusions The data presented here show the microbiome at the time of embryo transfer can successfully be characterized without altering standard clinical practice. This novel approach, both in specimen collection and analysis, is the first step toward the goal of determining physiologic from pathophysiologic microbiota. Further studies will help delineate if differences in the microbiome at the time of embryo transfer have a reliable impact on pregnancy outcome.

Keywords Microbiome, embryo transfer · Next-generation sequencing · 16S ribosomal subunit

Capsule The microbiome at the time of embryo transfer can successfully be characterized without altering standard clinical practice utilizing next-generation sequencing of the 16S ribosomal subunit.

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Introduction

The human body is colonized with many more bacteria than there are human cells in the body [1]. The need to fully characterize the human microbiome was recognized in 2001 at the time the human genome was published [2] after which a "second human genome project" was proposed that would investigate the normal microbiome colonies at various sites in order to understand the synergistic interactions between the microbiome and its host [3, 4]. The National Institutes of Health (NIH) launched the Human Microbiome Project (HMP) in 2007 which utilized high-throughput sequencing technologies to characterize the human microbiome in 250 normal, healthy volunteers at several different body sites which included the vagina in women [1].

It is important to note that published microbiome data are procured utilizing one of two major technologies: culture based or sequencing based. Much of the early work describing

the microbiome as it relates to reproduction comes from culture-based approaches followed by identification. This includes growth in different culture systems, analysis of substances secreted by the bacteria, or characterization of conserved genes in the 16S ribosomal RNA (rRNA) to identify organisms in mixtures [5, 6]. However, data from the vaginal microbiome show that many organisms are not identified with culture-based paradigms, and the requirement of successful culture prior to analysis results in an underestimate of the diversity of organisms when describing their relation to health and disease [7, 8]. Thus, culture-based data, while still informative, must be interpreted within the limits imposed by those paradigms. More recent data in reproduction and the HMP rely upon 16S RNA gene sequencing, specifically the hypervariable regions within the gene which serves as a molecular fingerprint down to the genus and species level [9, 10].

Some culture-based data exists investigating the link between the vaginal microbiome and ART. A study done prospectively in 152 women using culture-based technology investigated the microbiome at the vagina, cervix, and embryo transfer catheter tip [11]. The investigators found that specific species, *Enterobacteriaceae* and *Staphylococcus,* were significantly less likely to be found in patients who went on to be pregnant. Given the limitations of culture-based technology, only four major species were reported and the rest categorized as other. Thus, while this provides some information, it may not accurately characterize the robustness of the microbiome when analyzing species which do not grow in culture well. In another study using 16S sequencing technology, the vaginal microbiome was analyzed at stimulation baseline, time of oocyte retrieval, at time of embryo transfer, and, for those who became pregnant, at 6–8 weeks gestation [12]. They were able to analyze 30 patients with 99 vaginal swabs and showed a change in the species diversity index across stimulation time points and between women who had a live birth and those that did not. The authors indicated that relatively small sample size and varied stimulation approaches required subsequent larger, well-controlled studies to extend these findings.

As for the upper genital tract, colonization with microbes has been originally assumed to be due to pathologic ascension of organisms from the vagina through the cervical canal. Early studies utilized culture-based technology at the time of hysterectomy [13]. More recent studies using quantitative PCR specific to limited species have found colonization rates by one or more species in 95 % of cases at the time of hysterectomy. Data showed evidence of both *Lactobacillus* as well as non-*Lactobacillus* species presence. Importantly, those results are in the context of a limited number of species specific probes [14]. In addition to these data, research on biofilms in the reproductive tract has provided further insight into the upper genital tract microbiome. These biofilms are routinely present in the vagina and have been shown to extend up into the endometrial cavity and even the fallopian tubes [15].

To date, the ability to characterize the endometrial microbiome at the time of embryo transfer without altering clinical practice has not been demonstrated. Furthermore, no outcome data exist in IVF using comprehensive 16S rRNA subunit sequencing techniques for the uterine microbiome. The present study reports on microbiome sequencing-based data on fluid from the embryo transfer tip after single embryo transfer of euploid embryos.

Methods

Patient population

The study sought to characterize the microbiome at the time of embryo transfer utilizing molecular-based technology in the form of 16S ribosomal subunit hypervariable region analysis with next-generation sequencing (NGS). All patient samples were collected under approval from the Internal Review Board (IRB), and all patients were undergoing treatment at a single infertility center.

In order to clearly characterize the microbiome in ongoing and non-ongoing gestations while controlling to the extent possible for embryonic components, we recruited consecutive patients who were undergoing a single embryo transfer with a euploid blastocyst as determined by comprehensive chromosome screening. The methods have been described previously [16–18]. Stimulation protocols were designated at the discretion of the patient's providers and not dictated by the study.

Transfer catheter tip specimens

The clinicians and embryologists involved in patient care followed strict guidelines and standards to prevent transmission of disease as set forth by the Center for Disease Control and Prevention (CDC) as well as the American Society for Reproductive Medicine (ASRM) and the Society for Assisted Reproductive Technology (SART). All media was certified to be free of endotoxins per manufacturer's specifications. Embryo transfer was performed with a Wallace Classic soft tip catheter (Smiths Medical, Dublin, OH, USA) with the formable outer sheath advanced under ultrasound guidance. After embryo transfer, the distal 5-mm portion of the transfer catheter was sterilely placed in a DNA free PCR tube.

Pregnancy outcomes were ongoing, designated as positive fetal cardiac activity at time of discharge from infertility care at 8 weeks, or not ongoing. Outcomes were further determined to be chemical pregnancies, presence of hcg at +16 blood draw but no further ultrasound findings; clinical pregnancies,

visualization of gestational sac and yolk sac within the uterine cavity; or ongoing, as defined above.

16S ribosomal hypervariable region sequencing

Cell lysis was first performed on the sample. Nuclease-free water was added for a final volume of 8 μL, and 1 μL of KOH lysis buffer was then added. After vortex and centrifugation, this solution was incubated at 65 °C for 10 min. One microliter of neutralizing buffer was then added, and the specimen was vortexed, centrifuged, and snap-frozen in liquid nitrogen and stored at −20 °C.

Following lysis, DNA purification was performed. Twenty microliters of sterile water is added. The Agencourt® AMPure® XP Reagent is re-suspended and 54 μL is added to each 30 μL sample. After incubation at room temperature for 5 min, the plate is placed in the DynaMag™-96 Bottom Magnet for 3 min ensuring the solution is clear. The supernatant is discarded. Then, 100 μL of 70 % ethanol is added and incubated for 30 s. Once the solution clears, the supernatant is discarded. A second wash is performed as before. The beads are air-dried for 4 min ensuring the pellet does not dry out completely. The plate is removed from the magnetic rack and 15 μL of nuclease-free water directly to the pellet to disperse the beads. The plate is placed back in the magnetic rack for at least 1 min until the solution clears. The supernatant which now contains the eluted DNA is transferred to a new PCR plate without disturbing the pellet.

Next-generation sequencing of the bacteria specific 16S ribosome gene was performed utilizing the Ion 16S Metagenomics Kit (Ion Torrent by Life Technologies, Grand Island, NY, USA). The Ion 16S™ Metagenomics Kit includes two primer sets that selectively amplify the corresponding hypervariable regions of the 16S ribosomal subunit unique to bacteria. One primer set amplified V2–4–8 and the other V3–6, 7–9. The amplified fragments were sequenced on the Ion PGM™ system, and results were analyzed using the Ion Reporter™ software according to the manufacturer's guidelines. This dual primer pool allows for sequence-based identification of a broad range of bacteria within a mixed population.

Positive controls utilizing *Escherichia coli* along with negative controls were run to detect any contamination from reagents.

Data analysis

Sequence reads were assigned to Operational Taxonomic Units (OTUs) with modified 16S Metagenomics beta workflows in Ion Reporter™ software version 4.4 to analyze each amplicon region separately. Taxonomy assignments of these OTUs were carried out using the RDP classifier version 2.2 [19] with a confidence cutoff of 0.8 within the Quantitative Insights Into Microbial Ecology (QIIME) package [20]. The total genus count and the fractions of reads that support each genus were calculated for each sample overall as well as for different amplicon regions separately. To characterize the samples, two alpha diversity metrics were utilized (separately for each amplicon region): the Shannon diversity index (SDI) and chao1. These alpha diversity metrics were calculated with the QIIME package after picking OTUs with uclust [21] with "rev_strand_match" enabled. The Greengenes database version 13_8 was used for OTU reference and taxonomy assignments for analysis within QIIME [22, 23]. The alpha diversity metrics and read and taxonomy analysis results constitute a set of factors that can be tested for association to the ongoing status of patients.

Statistical tests were carried out between the binary patient ongoing status and different individual factors using nested logistic regression models in R [24]. A p value is derived based on the likelihood ratio test statistic for each factor of interest. To evaluate the family-wise error rate, 100 random permutations of ongoing status were obtained and the most significant p value from the original tests was adjusted with permutation-based test results.

Results

Demographics

A total of 33 patients were included in the study. All patients underwent transfer of a single, euploid blastocyst. The average age of the patients was 35.9 (range 22.5–43.0). Of the patients in the study, 26 (79 %) self-identified their ethnicity as Caucasian, 5 (15 %) as Asian, 1 (3 %) as African American, and 1 (3 %) as Hispanic.

All of the patients achieved a peak endometrial thickness of greater than 7 mm (mean 9.4 mm; range 7.3–13 mm) with a trilaminar appearance of ultrasound prior to initiating progesterone. The patients' peak estradiol during transfer cycle was documented at a standard morning phlebotomy assessment. The average peak estradiol was 907.5 pg/dL (range 136–2733 pg/dL).

Of the 33 patients, 18 individuals had embryo transfers resulting in ongoing pregnancies and 15 did not.

Microbiome characterization

There were a total of 278 different genus calls present across patient samples. An example of the plots generated supporting each of the 278 genus calls is shown in Fig. 1. In this figure, the plots of *Acinetobacter* and *Pseudomonas* are shown as examples where differences existed between the groups prior to multiple correction analysis. The plots of *Lactobacillus* and *Flavobacterium* are included as they

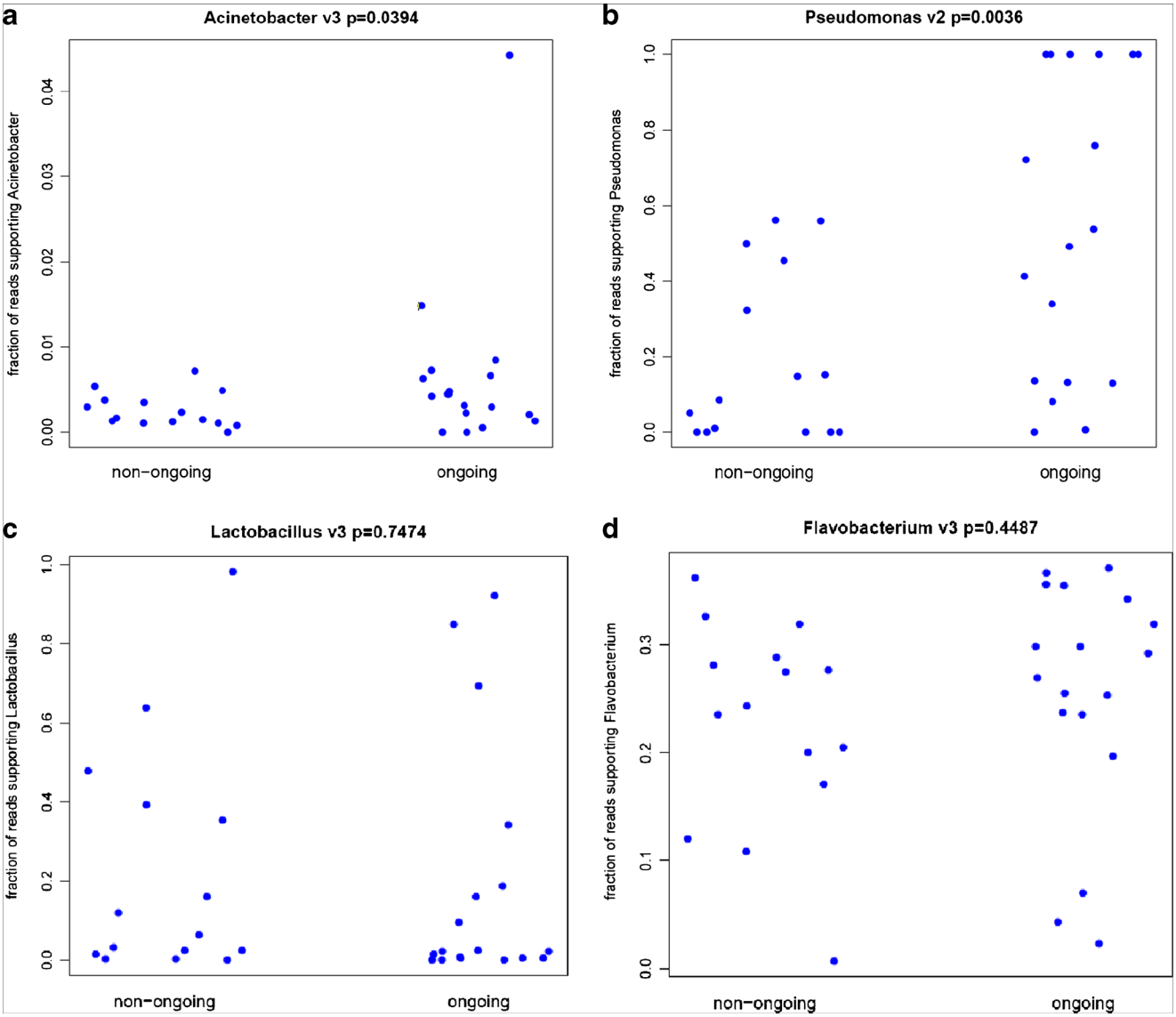

Fig. 1 Examples of the overall fraction of reads supporting the genera are shown for *Acinetobacter* (**a**), *Pseudomonas* (**b**), *Lactobacillus* (**c**), and *Flavobacterium* (**d**) in the non-ongoing and ongoing categories. Uncorrected *p* values are shown

were the most prevalent genus calls in both groups. The positive and negative controls for the study protocol were performed as expected.

The microbiome at time of transfer for those patients with ongoing pregnancy vs. those without ongoing pregnancy was also characterized by top genera. Results are summarized in Fig. 2. The genus calls are listed and the heat map represents the fraction of reads assigned to that genus compared to the total reads assigned. Several dominant species were present in both as would be expected; however, there were major species which appear to vary by outcome, although the differences did not reach statistical significance. *Flavobacterium* and *Lactobacillus* represent the majority of the bacterium seen in both groups.

To characterize the diversity of the samples, two alpha diversity metrics were utilized: the Shannon diversity index (SDI) and chao1. These diversity indices for hypervariable region 3 are shown in Fig. 3. The SDI gives an estimation of community richness as it combines both species number as well as abundance. Chao1 is a marker of species richness and is based upon the number of rare classes of operational taxonomic units. The diversity indices between the two groups appear similar, although the overall diversity index measures are high in both groups.

The *p* values for all comparison groups are plotted along with their max mean fraction within status group in Fig. 4. Given the large number of variables tested for association with patient ongoing status in this initial study,

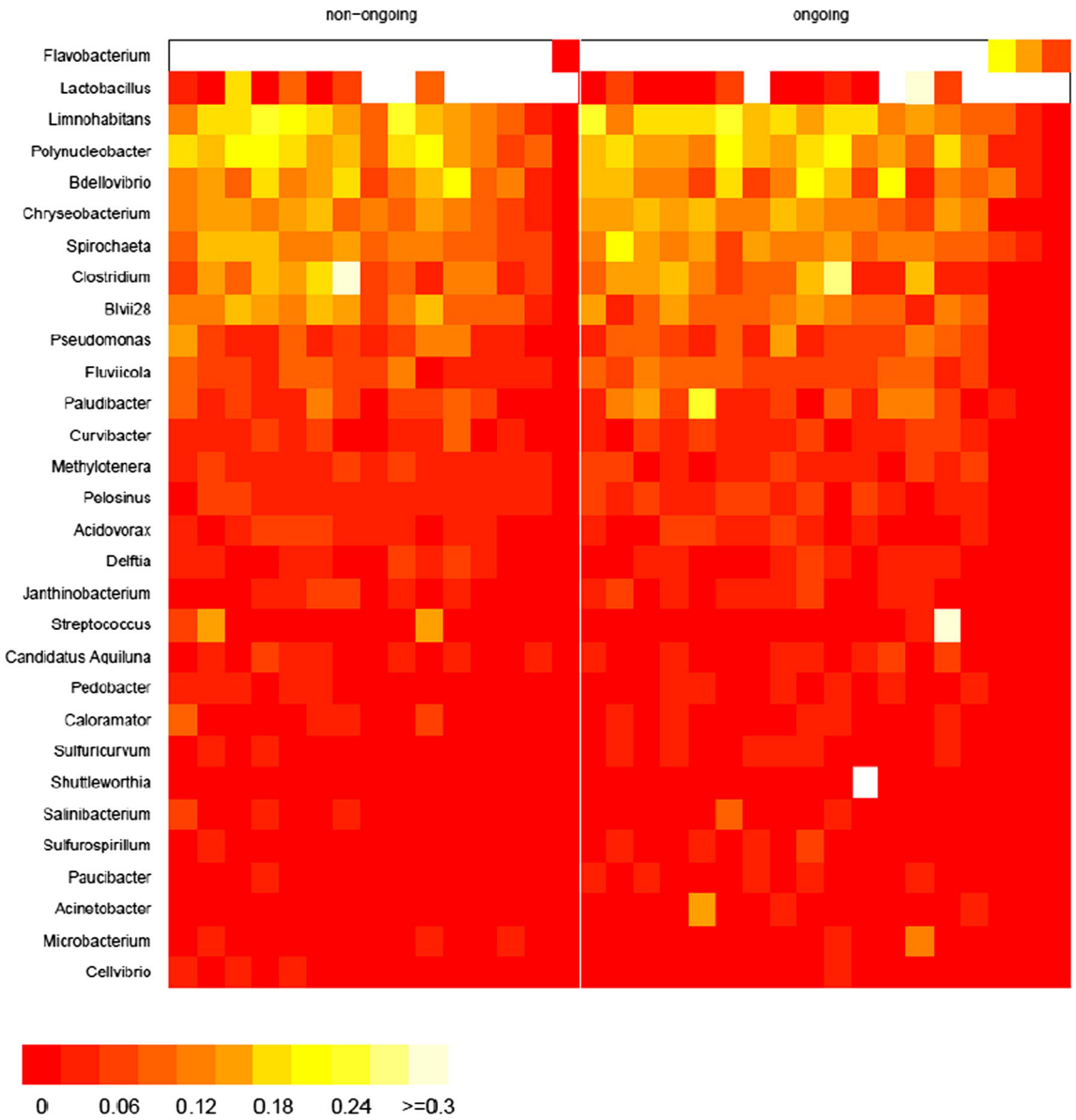

Fig. 2 The microbiome at time of transfer for those patients with ongoing pregnancy vs. those without ongoing pregnancy characterized by top genera. *Key* represents fraction of reads given to a genus among all reads assigned

no differences between the two status groups were large enough to survive multiple test corrections (corrected *p* value =0.59).

Discussion

The human microbiome has been termed the second genome, and its importance in reproductive success and failure has yet to be fully appreciated. To date, much of the scientific literature has focused on culture-based technology. The Human Microbiome Project has focused on the field of sequencing-based technologies which allow for a more complete understanding of the diversity which exists. To date, there has not been a characterization of the microbiome using sequence-based technology at the time of embryo transfer.

The present study serves to characterize and describe the microbiome at the time of embryo transfer in women undergoing single embryo transfer of a euploid blastocyst. The genus of bacteria is described in those with ongoing pregnancies and those without ongoing pregnancies. *Flavobacterium* and *Lactobacillus* represent the majority of the bacterium seen in both groups. By comparison, Mitchell et al. reported on the upper genital tract colonization by performing PCR assays for 12 bacterial species, including three separate types of *Lactobacillus*. When utilizing just the 12 assays, they found 95 % colonization [14]. They found the most prevalent

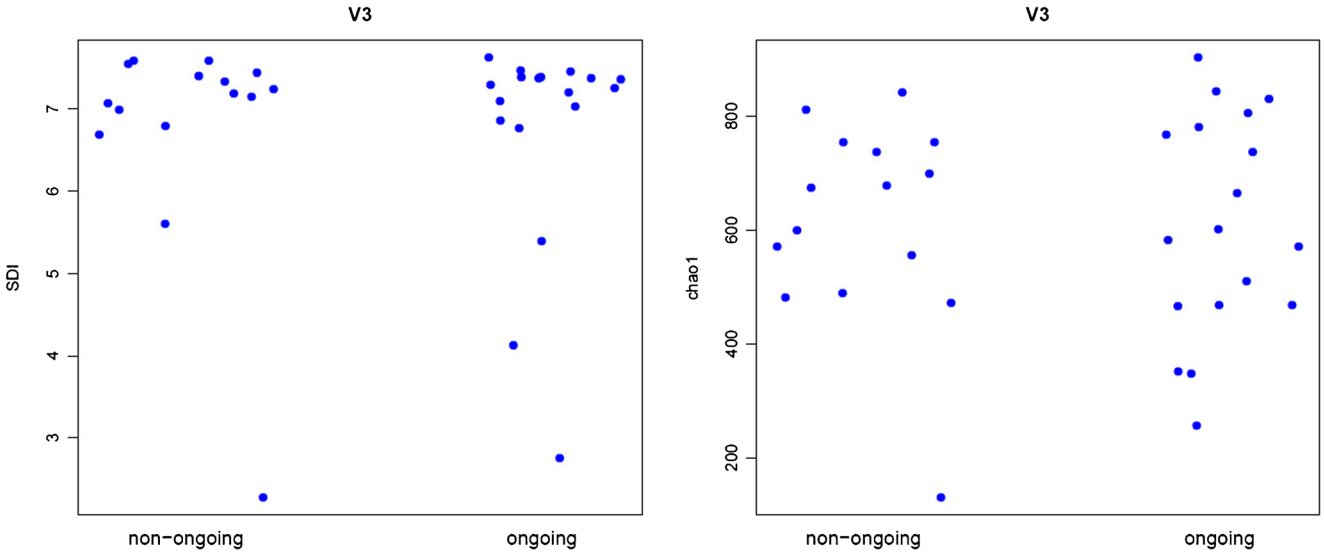

Fig. 3 To characterize the diversity of the samples, two alpha diversity metrics were utilized: the Shannon diversity index (SDI) and chao1. The indices for the V3 hypervariable region are shown here for the samples obtained from the ongoing vs. non-ongoing pregnancy outcomes

organisms to be *Lactobacillus*, similar to the findings of this study. They did not test for *Flavobacterium* among the chosen probes; however, *Flavobacterium* has been reported to be associated with the reproductive tract for some time [12, 25].

The findings of a complex microbiome at the time of embryo transfer lead to an interesting possibility of an equally complex immune environment during this critical time. Indeed, an important facet of embryo implantation that is possibly influenced by the vaginal and uterine microbiomes is the immune and cytokine environment during conception. It is known that a number of cytokines are involved in both endometrial receptivity as well as embryo development and are influenced by infection and inflammation [26], and thus the microbiome. These factors include the expression of Toll-like receptors (TLRs) which are expressed extensively in the reproductive tract. When TLRs are bound, they cause expression of a number of cytokines, including tumor necrosis factor alpha (TNFA), interleukin 6 (IL6), granulocyte-macrophage colony stimulating factor (GM-SIF), granulocyte-colony stimulating factor (GSCF), and interleukin 1B (IL1B) [27–29]. The most well characterized is the binding of TLR4 to a Gram-negative mimetic lipopolysaccharide which initiates a cytokine induced inflammatory cascade [30]. Given the microbiome characterized on embryo transfer catheter tips, this is of interest. Indeed, mouse studies have shown TLR4 activation results in reduced embryo viability and cellularity, pregnancy rates, and decreased fetal and placental weights [26]. The interaction between the endometrial microbiome and immune milieu is one that remains to be fully characterized but is of great interest.

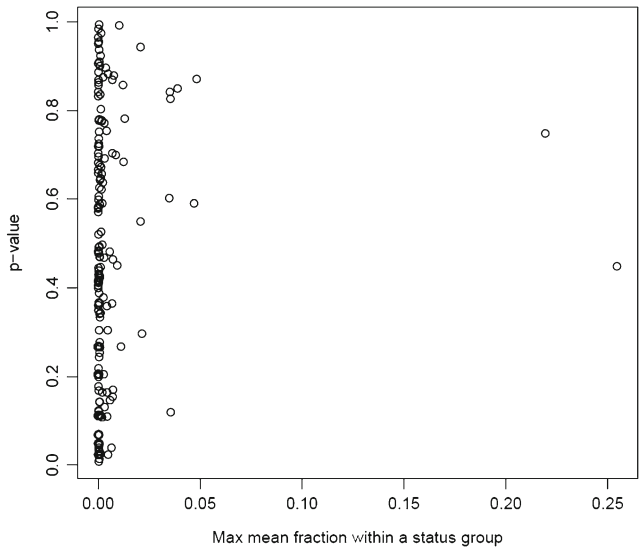

Fig. 4 The *p* values for all comparison groups are plotted along with their max mean fraction within status group. Given the large number of variables tested for association with patient ongoing status in this initial study, no differences between the two status groups were large enough to survive multiple test corrections

There are several strengths and limitations of the present analysis which bear discussion. The strengths of this study are the use of sequencing-based technology and a focus on the 16S ribosomal subunit hypervariable regions which allow for robust characterization of genera. The proof of concept that a very limited starting material can be amplified from an embryo transfer catheter tip without the need to extract additional fluid at the time of embryo transfer is also novel and may open the door for diagnostic testing once the environment is more rigorously characterized such that physiologic and pathophysiologic can be identified. Given the history of the vaginal microbiome characterized by the Human Microbiome Project

with low alpha, within sample, and beta, between sample, diversity, this remains a strong and realistic possibility.

Limitations of this study primarily involve sample size. Given the large number of data points which require multiple comparison and rigorous statistical correction, the study of the microbiome at the time of embryo transfer will likely require a large patient population to identify what, if any, differences exist which are statistically meaningful and withstand multiple correction analysis.

Other areas of caution exist. While patients are instructed as part of their stimulation protocol not to engage in intercourse, it is possible this occurred and the specimen was in some way influenced by the seminal fluid. Additionally, as an effort was made to characterize the microbiome present at the time of embryo transfer, regardless of origin, the microbiome of the media and catheter tip was not subtracted out from the analysis and this could be an area of future study. As the purpose of the study was to characterize the microbiome present in the endometrium at embryo transfer without altering clinical practice, additional samples from the vagina and cervix were not analyzed. This approach could provide a more robust look at the microbiome of the reproductive tract in the future; however, it does require a deviation from clinical practice.

Further data generated utilizing well-controlled patients who are undergoing euploid single embryo transfers may shed light on how the microbiome as it exists in the uterus at the time of embryo transfer affects reproductive outcomes. Evidence thus far suggests that differences in the vaginal microbiome may have an impact. Further study and characterization of the microbiome at the time of embryo transfer will allow for a better understanding of how this "second genome" impacts reproductive outcomes and may provide additional insight into the interaction between the immune system, the microbiome, biofilms in the reproductive tract, and embryonic success.

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