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Adverse effect of lactobacilli-depauperate cervicovaginal microbiota on pregnancy outcomes in women undergoing frozen–thawed embryo transfer

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Abstract

Purpose: The cervicovaginal microbiota is essential for maintaining the health of the female reproductive tract. However, whether cervicovaginal microbiota status prior to frozen embryo transfer (FET) associates with pregnancy outcomes is largely unexplored.

Methods: Cervical mucus from 29 women who had undergone FET was collected. Microbial composition was analyzed using 16S rRNA gene sequence to assess the correlation to the pregnancy outcomes.

Results: CST-categorized *Lactobacillus* was the most dominant (41.71%) in the pregnant group, while CST-IV-based and BV-related *Gardnerella* (34.96%) prevailed in the non-pregnant group. The average abundance of *Gardnerella* compared non-pregnant to pregnant women was the highest (34.96% vs. 4.22%, $p = 0.0015$) among other CST-IV indicator bacteria. Multivariate analysis revealed that CST-IV-related bacteria have a significantly adverse effect on ongoing pregnancy outcomes (odds ratio, 0.083; 95% confidence index, 0.012–0.589, $p = 0.013^*$).

Conclusions: The study found that the CST-IV microbiota, with significantly increasing *Gardnerella* and the loss of *Lactobacilli* as the dominant bacteria, can potentially contribute to pregnancy failure. Therefore, dysbiotic microbiota may be a risk factor in women undergoing FET. Assessing the health of the cervicovaginal microbiota prior to FET would enable couples to make a more thoughtful decision on the timing and might improve pregnancy outcomes.

KEYWORDS
cervicovaginal microbiota, community state types, frozen–thawed embryo transfer, *Gardnerella*, pregnancy

1 | INTRODUCTION

The microbial community is essential for the female reproductive tract.[1] Dysbiotic change of the microbiota can cause inflammation, induce host–innate immune responses, and further contribute to diseases such as bacterial vaginosis (BV), chorioamnionitis, and premature delivery.[2–4] The newly developed high-throughput sequencing approaches have helped us examine the microbial community

and composition before, during, and after disease states for linking microbiota change to human diseases. Along with the microbial composition, microbial metabolism, and their interactions with the host can help improve disease conditions, promote better health, and facilitate clinical management.

The healthy cervicovaginal microbiota is generally dominated by *Lactobacillus spp.*, including *L. crispatus, L. gasseri, L. iners, or L. jensenii*, forming four community state types (CST-I, II, III, and V) based on large-scale 16 S rRNA and cultivation studies.[5] These *Lactobacillus spp.* can produce lactic acid, generating an acidic environment to protect the cervicovaginal area from pathogen infection. However, many factors may interfere and shape its composition, such as vaginal hygiene, sexual activity, lifestyle, and most importantly, cyclical ups and downs of estrogen levels throughout the menstrual cycle.[6] High estrogen levels maintain the dominance of *Lactobacillus spp.*, which is supported by vaginal epithelia by promoting glycogen deposition and lactic acid production, with a vaginal pH shift to an acid environment and preventing colonization by other pathogenic bacterial.[7] A loss of the *Lactobacillus* dominance, often categorized into CST-IV microbiota or sometimes called microbial dysbiosis, may increase the colonization of BV-related bacterial, including *Gardnerella, Prevotella, Escherichia*, and *Shigella* genera, resulting in an increased chance of pathogenic infection.[8] Microbial dysbiosis may further activate the maternal immune response, triggering proinflammatory cytokines production, prostaglandin synthesis, and potentially preterm birth.[9] More importantly, studies have confirmed that microbial dysbiosis can be related to adverse pregnancy outcomes in pregnant women, such as premature membrane rupture, premature delivery, and chorioamnionitis.[10,11] Therefore, microbial community change in the reproductive tract, characterized by the gain and loss of *Lactobacillus spp* dominance and the rise of facultative/anaerobic bacteria, can impact the health of the female reproductive tract and pregnancy outcomes.

Frozen embryo transfer (FET) allows the optimal endometrial preparation and identification of the receptive window of the endometrium and the embryos into a more physiologic uterine environment and have drawn much attention in recent years.[12,13] A previous study has reported that during fresh embryo transfer, the dominance of *Lactobacillus* in the vagina may have better reproductive outcomes; conversely, the presence of a *Lactobacillus*–depauperate microbiota in a receptive endometrium may be associated with poor embryo implantation rate.[14,15] However, unlike the case of fresh embryo transfer, we wondered in the FET cycle if the balance of cervicovaginal microbiota also affected pregnancy success. It is worth investigating whether the composition and the health of the cervicovaginal microbiota would associate with the success rate of embryo implantation during the FET cycle.

Our study aimed to explore the impact of cervicovaginal microbial dysbiosis on pregnancy outcomes of the FET cycle. We hypothesized that the unfavorable cervicovaginal microbiota might be the leading indicator of overall reproductive dysbiosis and interfere with the embryo implantation of FET. We examined the microbial composition by 16s rRNA gene sequencing of cervical mucus swab sample from the FET procedure and investigated the bacteria associated with the pregnancy failure. Our study would profile the potential pregnancy failure-associated bacteria and, therefore, can be a potential predictor for evaluating the timing for FET.

2 | MATERIALS AND METHODS

2.1 | Study design and patients

This observational, prospective study was performed at the Reproductive Center of the Kaohsiung Veterans General Hospital in Taiwan from January 2020 to December 2020. Ethical approval (IRB No. VGHKS19-CT12-13) was obtained from the Institutional Review Board at the Kaohsiung Veterans General Hospital. All participants provided written informed consent. Patients enrolled in an oocyte donation or gestational surrogacy program were excluded. The demographics, cycle characteristics, and clinical and laboratory data from participants were extracted from electronic medical records. Sample size calculation reveals that 15 participants in each study group were optimal for analysis with a power of 80% and a *p*-value of 0.05 based on the average embryo implantation rate of about 40% for women at our infertility center.

2.2 | Timing and grade of the transferred embryos

Study patients who underwent FET were prescribed hormone therapy, starting with 6 mg estradiol (E2) valerate (Progynova; Schering, Germany) orally per day for 14 days. The endometrial thickness was evaluated on the 13th to 15th day of oral E2 treatment. If the endometrial thickness was ≥8 mm, luteal and progesterone support with oral dydrogesterone (20 mg/d; Duphaston, Abbott Health care, USA) and crinone vaginal gel (Merck Serono, Switzerland) 90 mg/d were administered to the patients. Embryo scoring was performed prior to transfer according to standard validated morphological characteristics. Each Day 3, embryo was morphologically scored by combining the number and size of blastomeres, the degree of fragmentation, and the cleavage rate. On Day 3, good quality embryos were defined as embryos with at least 6 cells and < 20% fragmentation.[16,17] On Day 5, embryo quality was scored, according to Gardner and Schoolcraft criteria, including blastocyst expansion and stage of hatching (score 1–5), inner cell mass (A–D), and trophectoderm (A–D). The embryos were then divided into four categories: excellent, good, moderate, and poor quality.[18,19] Transfer of one or two high-quality embryos was performed on Days 3 or 5. Once pregnancy was achieved, exogenous estrogen and progesterone supplementation were continued until 8 weeks of gestation. The patient was followed up with ultrasonography to determine fetal viability until approximately 8 weeks. Ongoing pregnancy was defined as a clinical pregnancy continuing past 12 weeks of gestation.

2.3 | Sample collections and DNA preparations

Cervicovaginal secretions were collected carefully during the embryo transfer procedure. A sterile sample collection kit (FLOQSwabs; COPAN, Murrieta, CA) was used in the sample collection. The samples were immediately placed in an ice box and then transferred to a −80°C freezer for DNA extraction and 16S rRNA gene sequencing.

DNA from the cervicovaginal secretions samples was isolated by phenol/chloroform extraction.[20] The isolated and purified DNA was stored at −20°C for further analysis. The phylogenetic analysis of the bacterial community was performed using Illumina sequencing. For the sequencing, the 16S rRNA gene, V3–V4 hypervariable regions were selected. PCR amplification was carried out in 25uL reactions with 0.5uL of KAPA High-Fidelity PCR Master Mix (Kapa biosystems), 0.5uM of forward and reverse primers. Reaction conditions as recommended by the manufacturer (95°C for 3 min, 25 cycles of 95°C for 20s, 57.5°C for 20s, 72°C for 20s and, after the last cycle, 72°C for 3 min) with region-specific (341F and 805R) primers. The primer sequence was as follows: (forward primer: 5' CCTAC GGGNGGCWGCAG 3′, reverse primer: 5'GACTACHVGGGTATCTA ATCC 3′). Sequencing libraries were generated using Celero DNA-Seq System (NuGEN, USA) and index codes were added. The library quality was assessed on the QuantiFluor® dsDNA System (Promega Corporation, USA). At last, the library was sequenced on an Illumina Miseq platform, generating 300bp paired-end reads.

2.4 | Sequencing data analysis

Adapters, primers and low-quality sequences were removed from the raw reads using Quantitative Insights Into Microbial Ecology 2 (QIIME2) package (https://qiime2.org). Amplicon sequence variants (ASVs) were inferred from raw reads using the QIIME2-DADA2 pipeline. Taxonomy was assigned to ASVs with the SILVA Database. MicrobiomeAnalyst[21,22] was used for composition bar plot, Alpha-diversity analysis, and Principal Coordinate Analysis (PCoA). To evaluate Alpha-diversity, Chao1 and Shannon index were used. PCoA was assessed by Bray-Curtis index and evaluated using Analysis of Similarities (ANOSIM).

2.5 | Functions prediction of microbial community

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States II (PICRUSt II) is a bioinformatics software package designed to predict functional abundances based on marker gene sequences (16S rRNA in this study).[23] PICRUSt II was used to identify Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) abundances. The KOs were categorized by KEGG pathway database, and the increasing level reveals more specific pathways of specific metabolites. Differences in the metabolic pathway between non-pregnant and pregnant groups were assessed for statistical significance using Student's t-test.

2.6 | Statistical analysis

The outcome measures associated with clinical variables and the analysis of bacterial abundances were performed using Pearson's chi-squared test or Student's t-test as appropriate. Univariate linear regression analysis was used to assess the relationship between pregnancy outcomes and the cervicovaginal microbiota. A p-value ≤0.05 was considered significant in all the analyses. The Statistical Package for Social Sciences (SPSS, Chicago, IL) and GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA) were used for statistical analysis.

3 | RESULTS

3.1 | Women in the pregnant and non-pregnant groups had similar clinical parameters prior to hormone-prepared FET

In total, 32 women were included with a cervicovaginal swab. Two samples were discarded due to inadequate sampling, and one was discarded due to sampling error. Thus, data from a total of 29 women were available for analysis, including 14 pregnant and 15 non-pregnant women. The clinical, demographic characteristics data, and pregnant outcomes of women included in this study are summarized in Table 1. The primary characteristics of the participants, including age, serum anti-Mullerian hormone (AMH) level, body mass index

TABLE 1 Clinical characteristics of the study participants

	Non-pregnant (n = 15)	Pregnant (n = 14)	p
Age (y)	37.1±2.48	35.1±2.80	0.053
AMH (ng/ml)	4.3±3.43	5.0±2.67	0.507
BMI (kg/m²)	22.9±4.38	21.3±1.62	0.187
Primary infertility, n (%)	8 (53.3%)	10 (71.4%)	0.316
Duration of infertility (years)	3.3±2.48	3.4±2.07	0.880
No. of high-quality embryo transferred (n)	1.6±0.48	1.5±0.64	0.660
Endometrial thickness while FET (mm)	10.6±1.18	11.5±1.29	0.061
Infertility cause, n (%)			0.295
Tubal factor	3 (20.0)	1(7.1)	
Endometriosis/ovulatory disorder	1 (6.7)	5(35.7)	
Male factor	5 (33.3)	2(14.3)	
Multiple factors	2 (13.3)	2(14.3)	
Unexplained	4 (26.7)	4(28.6)	

Note: Values are mean±SD or n (%).
Abbreviations: AMH, anti-Mullerian hormones; BMI, body mass index.

(BMI), duration of infertility, number of high-quality embryo transfers, endometrial thickness while FET, the percentage of Day 3 embryos, and cause of infertility were comparable between the two groups with no significant differences (Table 1).

3.2 | The composition and diversity of cervicovaginal microbiota between pregnant and non-pregnant group

The collected cervical mucus underwent microbial DNA extraction followed by 16s rRNA gene sequencing, and the sequences were compared between the pregnant and non-pregnant groups. We hypothesized that the compositional differences of cervicovaginal microbiota might be associated with pregnancy outcomes. At the phylum level, we found that three dominant groups—*Bacillota*, *Actinobacteria* and *Pseudomonadota* accounted for more than 97.46% of the total sequences in the non-pregnant group and more than 98.94% in the pregnant group. However, the microbial composition between the two groups was quite different. In the pregnant group, *Bacillota* (47.60%) and *Pseudomonadota* (46.99%) were the majority, while *Actinobacteria* (4.35%) was the minority; conversely, in the non-pregnant group, *Actinobacteria* (40.71%) was the largest, followed by *Bacillota* (40.55%), and *Pseudomonadota* (16.20%) (Figure 1A). In genus level, *Lactobacillus* was the most dominant (41.71%) in the pregnant group, followed by *Burkholderia-Caballeronia-Paraburkholderia* (24.84%), a normal bacterial flora. However, in the non-pregnant group, *Gardnerella* (34.96%) was the most dominant; *Lactobacillus* still exists in the vaginal flora (30.05%) in the non-pregnant group but showed a significant decrease as compared to the pregnant group (Figure 1B). Moreover, *Streptococcus, Atopobium, and Sneathia spp.* were presented in the non-pregnant compared with the pregnant group. To further investigate the species level of *Lactobacillus* within these two groups, web-based BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed on each *Lactobacillus* sequence. We found *L. iners* and *L. jensenii* contributed the most to the *Lactobacillus* genus in pregnant group, whereas *L. iners* and *L. crispatus* in the non-pregnant group (Figure 1C).

To then determine whether the compositional difference is significant, the diversity analysis regarding bacterial richness, abundance, and evenness of the microbial community from each individual was examined (Figure 2). In Alpha-diversity, we found that the pregnant group had a significantly higher Chao1 index (Figure 2A). However, we found an insignificant level of the Shannon index in the pregnant group (Figure 2B). In Beta-diversity, Principal Coordinates Analysis (PCoA) analysis was performed to examine the similarity between groups. In general, we found a significant difference in similarity between the pregnant and non-pregnant groups (Figure 2C). We also found a relatively more diverse microbial composition in the pregnant group as the range of dot distribution was comprehensive. These data suggest that the non-pregnant group, compared with the pregnant group, may have a more diverse but even microbial composition that can be associated with pregnancy outcomes.

3.3 | The abundance of CST-IV indicator bacteria was significantly higher in the non-pregnant group prior to hormone-prepared FET

CST-IV microbiota is characterized by the loss of *Lactobacillus* dominance together with increased diverse facultative/anaerobic bacteria.[14] To further confirm that the microbial composition varies between the two groups, the Linear discriminant analysis Effect Size (LEfSe) analysis was performed to show the unique niche of the bacteria between pregnant and non-pregnant groups. We found a unique niche of *Gardnerella* and *Dialister*, two indicator bacteria genera in CST-IV microbiota, in the non-pregnant group (Figure 3, Red), whereas a more diverse bacteria composition was distinguished in the pregnant group (Figure 3, Blue). The data reiterate the analysis of diversity differences and consolidate the link between non-pregnant outcomes to the CST-IV microbiota in FET.

To further determine whether CST-IV microbiota associates with the non-pregnant group, each CST-IV indicator bacteria genera in the pregnant- and the non-pregnant group were pooled and compared (Figure 4). Among the comparisons, we found a trend of increased indicator bacteria genera. However, *Gardnerella*, one most representative bacteria genera in CST-IV and BV, was significantly higher in the non-pregnant group. The data suggest that CST-IV microbiota is potentially associated with pregnancy failure in FET individuals.

3.4 | The abundance of CST-IV bacteria associated with the pregnancy failure

To further determine whether the microbiota, other than common factors regulating the pregnancy, plays an essential role in pregnancy success in FET. We performed logistic regression analysis with ongoing pregnancy outcome as the dependent variable factor and CST-IV bacteria, age, AMH level, BMI, and endometrial thickness while FET as the covariates. Univariate logistic regression analysis revealed that CST-IV bacteria (odds ratio, 0.061; 95% confidence index, 0.009–0.399, $p = 0.004^*$) and age (odds ratio, 0.726; 95% confidence index, 0.540–0.976, $p = 0.034^*$) showed a statistically significant correlated with pregnancy outcomes (Table 2). Multivariate analysis still indicated that CST-IV bacteria had a significantly adverse effect on the pregnancy outcomes (odds ratio, 0.083; 95% confidence index, 0.012–0.589, $p = 0.013^*$). Therefore, the data suggest that the abundance of CST-IV bacteria indeed have an adverse impact on pregnancy outcomes within the hormone-prepared FET cycle, accordingly, the deteriorating effect of *Gardnerella*.

3.5 | Predicted function difference in microbiota between pregnant and non-pregnant group

The change in the microbial composition may reflect the change in microbial function in metabolism. As a result, the metabolic change
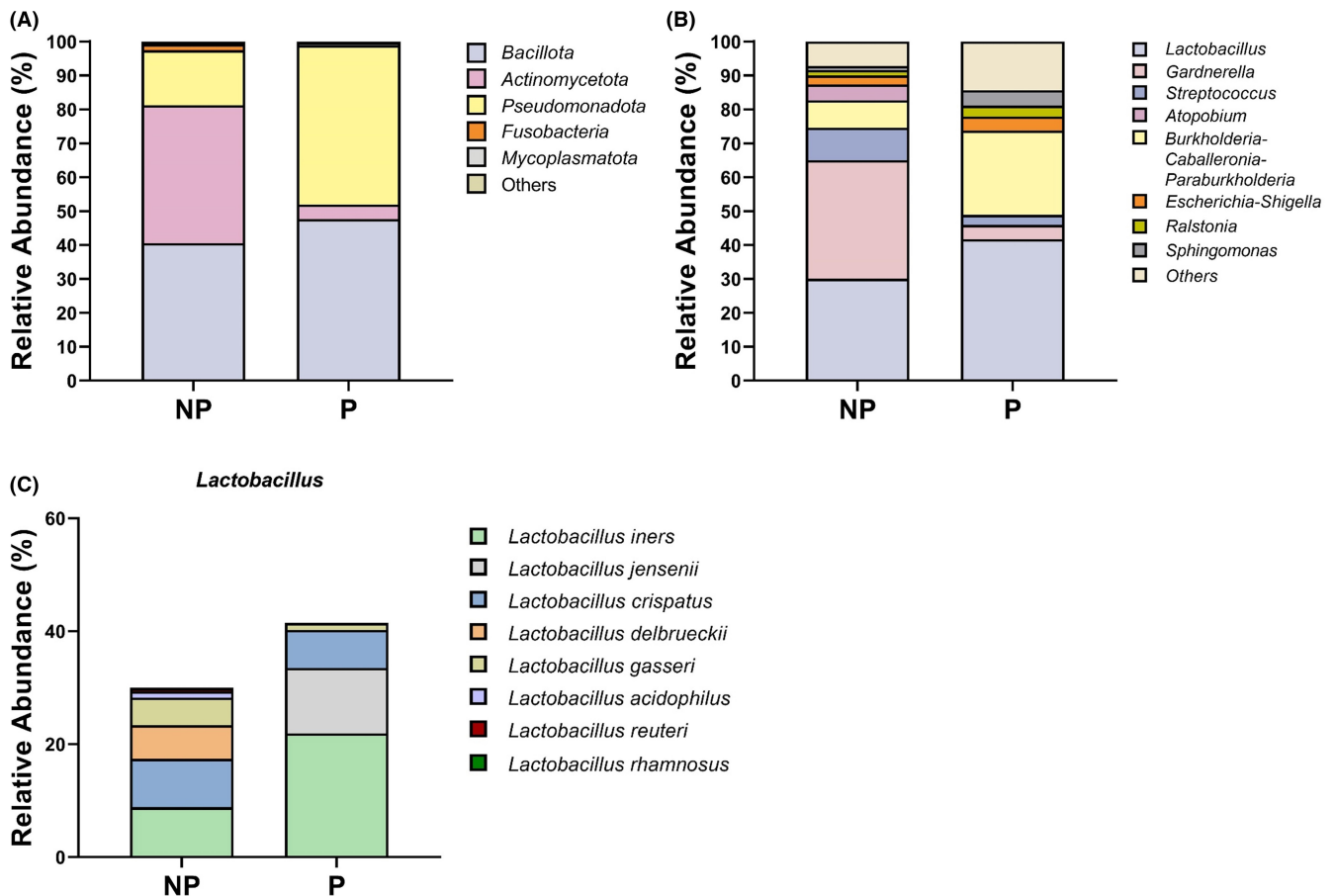
FIGURE 1 Compositional differences of cervicovaginal microbiota were found between pregnant and non-pregnant women. Relative abundances of cervicovaginal microbiota between pregnant (P) and non-pregnant (NP) women were shown at (A) the phylum level, (B) the genus level, and (C) the abundance of *Lactobacillus* at BLASTn-based species level

in total determines the health status of the reproductive environment.[24] To evaluate whether the functional change occurred in the microbiota between the pregnant and non-pregnant group, we used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt II) to predict the metabolic capacity and function output and compared the two groups. We found significant changes in five categories, including carbohydrate, amino acid, glycan, nucleotide, and energy metabolism in level 2 (Figure 5A). However, the most significant change was found in the increased secondary metabolites biosynthesis and starch/sucrose metabolism, amino acid biosynthesis, and purine metabolism in the non-pregnant group in level 3 (Figure 5B). These data suggest that a metabolic change existed from the microbial compositional change between the two groups. These data also imply that microbial composition change, as well as the following metabolic function change, can be associated with the pregnancy outcome.

4 | DISCUSSION

Cervicovaginal microbiota has been implied to play an essential role in reproductive health.[6,25] Whether cervicovaginal microbiota

is associated with the pregnancy outcomes of FET is largely unknown, we explored this association by examining cervicovaginal mucus microbiota prior to FET related to the pregnancy outcome. We found that the increased CST-IV-based bacteria and the loss of dominant *Lactobacillus* in cervicovaginal microbiota universally existed in non-pregnant participants. Further comparison of CST-IV indicator bacteria showed the pregnancy failure might be attributed to the increased *Gardnerella*, in which the microbial composition and metabolic function can also change accordingly. Thus, our results indicate that CST-IV microbiota, particularly *Gardnerella*, potentially adversely affect pregnancy outcomes of FET. The microbiota profile might serve as one parameter for couples to determine the timing of FET.

The microbiota varies across the different locations in the female reproductive tract.[26] In a recent large-scale study in China, the microbiota at the lower reproductive tract from the ectocervix to the vagina represents a similar composition of 95% *Lactobacilli*. In contrast, the microbiota in the upper reproductive tract from the uterus to fallopian tube varies and possesses less in number.[26] It is suggested that the abundance of *Lactobacilli* is the crucial health regulator, which occupies most of the microbial composition in the lower reproductive tract.[27] However, we found that the ratio of *Lactobacilli*
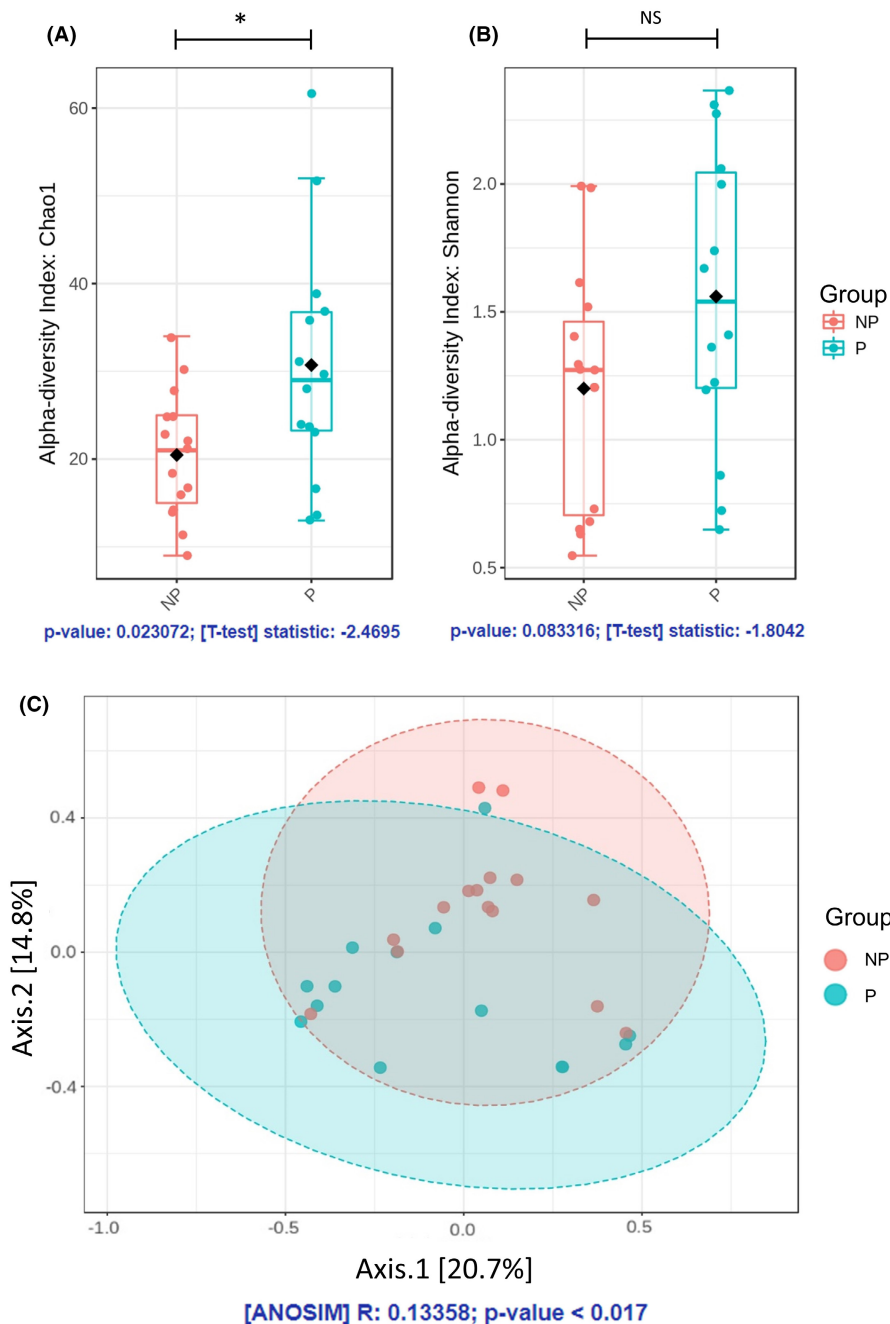
FIGURE 2 Alpha diversity and Beta diversity of cervicovaginal microbiota between the pregnant and non-pregnant group. (A) Chao1 and (B) Shannon Alpha-diversity indices. Statistical significance was determined by Student's t-test at a p-value of <0.05. (C) In Beta diversity, Principal Coordinate Analysis (PCoA) was performed at the ASVs level based on Bray–Curtis dissimilarity and showed the distribution of the bacterial composition of individuals in each group (p < 0.017 by ANOSIM test)

to other bacteria in our study was not as dominant as previous studies reported. The possible reason is that the participants are infertile women, not the general population; therefore, the microbiota in the samples in this study may not represent the microbiota in the healthy reproductive tract by large. A related study conducted on mostly Caucasian participants found that the *Lactobacilli* can only occupy up to 60%–80% of vaginal microbiota in participants under in vitro fertilization study,[28] showing the microbiota variation within this population compared with the general. Moreover, a recent retrospective study combining 51 reports showed that the vaginal *Lactobacillus* only occupies 30% of the microbiota.[29] The actual abundance of *Lactobacillus* may vary and fluctuate from person to person. Following Koedooder's study, ethnicity has also been

shown to impact the selection of reproductive microbiota, especially in *Lactobacilli*. Ravel et al. have reported that the CST-categorized *Lactobacilli* dominancy can be different between Asians as *L.iners*-dominated CST-III and other ethnic groups in other *Lactobacilli* CST types.[5] While Zhou et al. mentioned that Japanese women possess a similar ratio of CST types to other Black and White women of North America,[5,30] our data has shown a *L. iners*-dominated CST-III type in the participants, matching with Koedooder and Ravel's observation. However, since the microbiota can vary, the CST type and the microbial composition before participants initiated the FET process and in each stage of FET were unknown. Multiple comparisons between general population and FET group in the future will shed some light on the microbiota uniqueness of women needing FET.

FIGURE 3 LEfSe analysis of cervicovaginal microbiota composition between pregnant (P) and non-pregnant (NP) women. LEfSe identified LDA bar graphs of genera that are differentially abundant between pregnant (blue) and non-pregnant (red). Taxa in graph were statistically significant ($p < 0.05$) and had LDA score greater than ±2.0, considered significant effect size using Kruskal–Wallis test
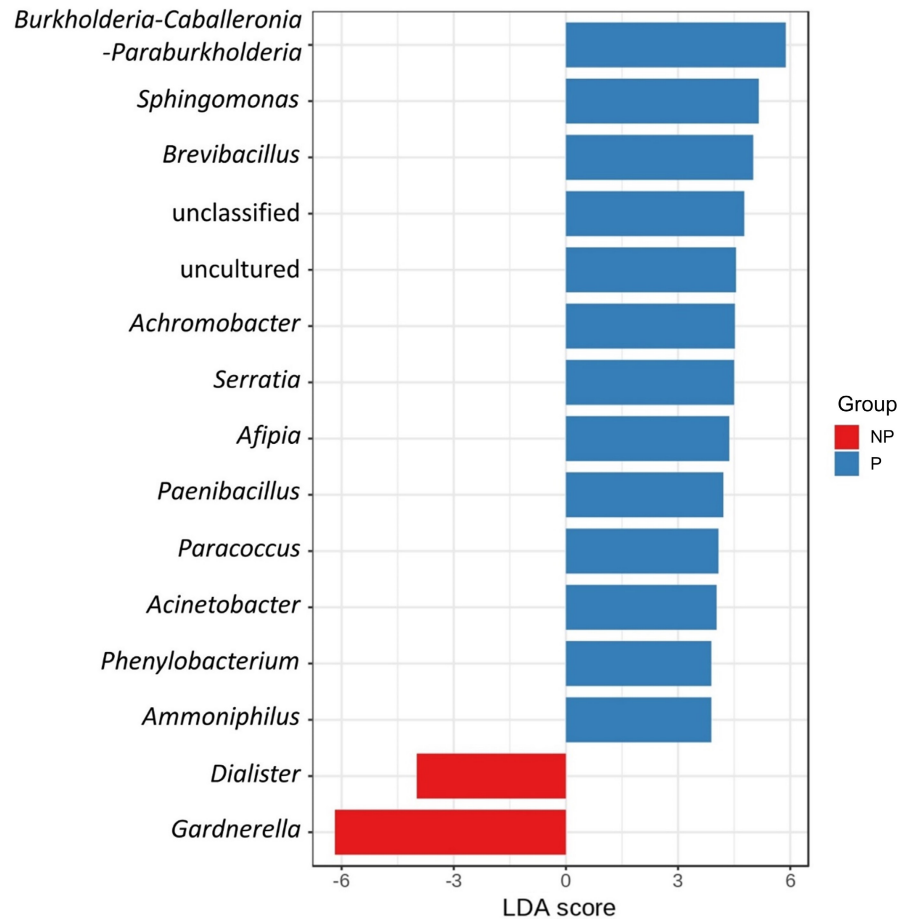
Frozen–thawed embryo transfer with the programmed cycle for endometrial preparation has become the widely used technique in assisted reproductive technology, not only reducing the risk of ovarian hyperstimulation syndrome but also a cost-effective method to increase cumulative pregnancy rates per oocyte retrieval.[31] However, a programmed cycle modulated by measures such as a high hormone dose for endometrial preparation might interfere with the balanced microbiota. Current studies found that hormone level can be one of the significant factors interfering with the microbiota, and *Lactobacillus* dominance is closely correlated with hormone level.[32] With this premise, a progesterone vaginal suppository may alter the cervicovaginal microbiota. Progesterone suppositories are clinically beneficial and easy to use in relieving injection pain for patients.[31,33] Nevertheless, the excessive remaining residue left in the cervicovaginal area can alter the composition of the cervicovaginal microbiota along with the pH change.[34] In our FET settings, we also observed irritations in different degrees with vaginal tab supply as hormone suppositories in our study participants. The complaints of itching and burning sensations and unpleasant-malodor discharge may imply the alteration of the microbiota that is clinically prone to unfavored bacteria growth. Since microbial dysbiosis can ultimately lead to pelvic inflammatory disease, tubal factor infertility, and adverse pregnancy outcomes,[35–37] assessing the composition of the cervicovaginal microbiota before and through each stage of

FET will be needed to not only track how the microbiota changes but also maintain the favored microbiota for FET success.

The impact of pathogenic bacteria on FET can be dated back to two decades ago when studies from Fanchin et al. and Egbase et al. found a significantly lower implantation rate in the presence of pathogenic bacteria in the endometrium by the culturing method.[38,39] Using similar methods, Salim et al. showed that the presence of pathogenic bacteria in the cervical canal also correlated with a lower pregnancy rate.[40] At the time, the most defined pathogenic bacteria were *Streptococcus*, *Gardnerella*, *Staphylococcus*, and *Enterococcus*. Several studies have been then conducted using the next-generation sequencing platforms to examine how reproductive microbiota impact embryo implantation. One study focused on the microbiota composition of the endometrium and confirmed that the abundance of *Lactobacillus* could indeed increase the rate of successful embryo implantation.[14] It also, in particular, suggested the potential adverse impact of the abundant vaginal genus *Gardnerella* and *Streptococcus* but not the diversity of microbiota on FET failure.[41,42] In recent studies, Fu et al. has established the linkage of the recurrent implantation failure to vaginal microbiota and metabolites from patients and indicated an increased richness and evenness of CST-IV bacteria such as *Gardnerella*, *Atopobium*, *Prevotella*, and *Streptococcus* along with changing metabolites were associated with recurrent implantation failure.[43] Our study, aligned with the studies above, found similar results that the abundance of CST-IV-based and BV-related
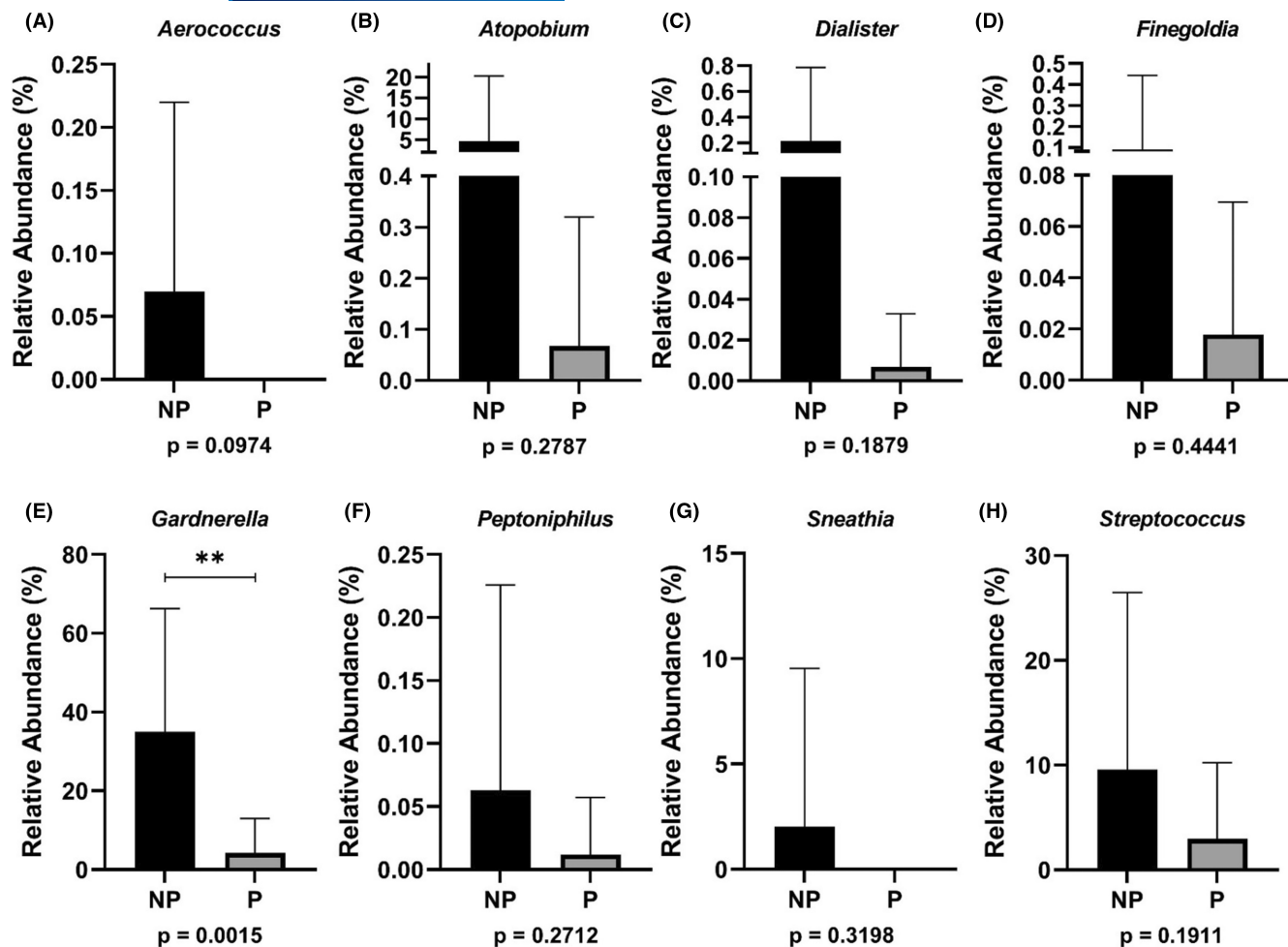
FIGURE 4 Abundance of CST-IV bacteria in women underwent FET in pregnancy outcomes. (A–H) Relative abundances of the 8 genera differentially enriched between pregnant (P) and non-pregnant (NP) group. *p*-values to Student's t-test is designated on the figure (**, *p* < 0.01)

TABLE 2 Adjusted odds ratios of ongoing pregnancy outcomes from the logistic regression analyses

Adjusted	Univariate		Multivariate	
	OR (95% CI)	*p*	OR (95% CI)	*p*
Microbial dysbiosis with CST-IV bacteria	0.061 (0.009–0.399)	0.004*	0.083 (0.012–0.589)	0.013*
Age	0.726 (0.540–0.976)	0.034*	0.773 (0.545–1.097)	0.149
AMH	1.089 (0.851–1.395)	0.497		
BMI	0.836 (0.631–1.106)	0.210		
Endometrial thickness while FET	1.822 (0.959–3.461)	0.067		

Abbreviations: 95% CI, 95% confidence interval; BMI, body mass index; FET, frozen embryo transfer; OR, odds ratio.

microbiota, especially *Gardnerella*, adversely affected the success rate of FET. Besides, we also observed the increase of *Atopobium*, *Streptococcus*, *Dialister*, and *Sneathia* in trend. Nonetheless, unlike previous studies, we found that the pregnant group has a higher microbial diversity than the non-pregnant group. One reason related to this contradicted observation is the assumption of recurrent FET in both groups, which gives both groups different and diverse microbiota. This can also be shown in Figure 2, where the diversity can be
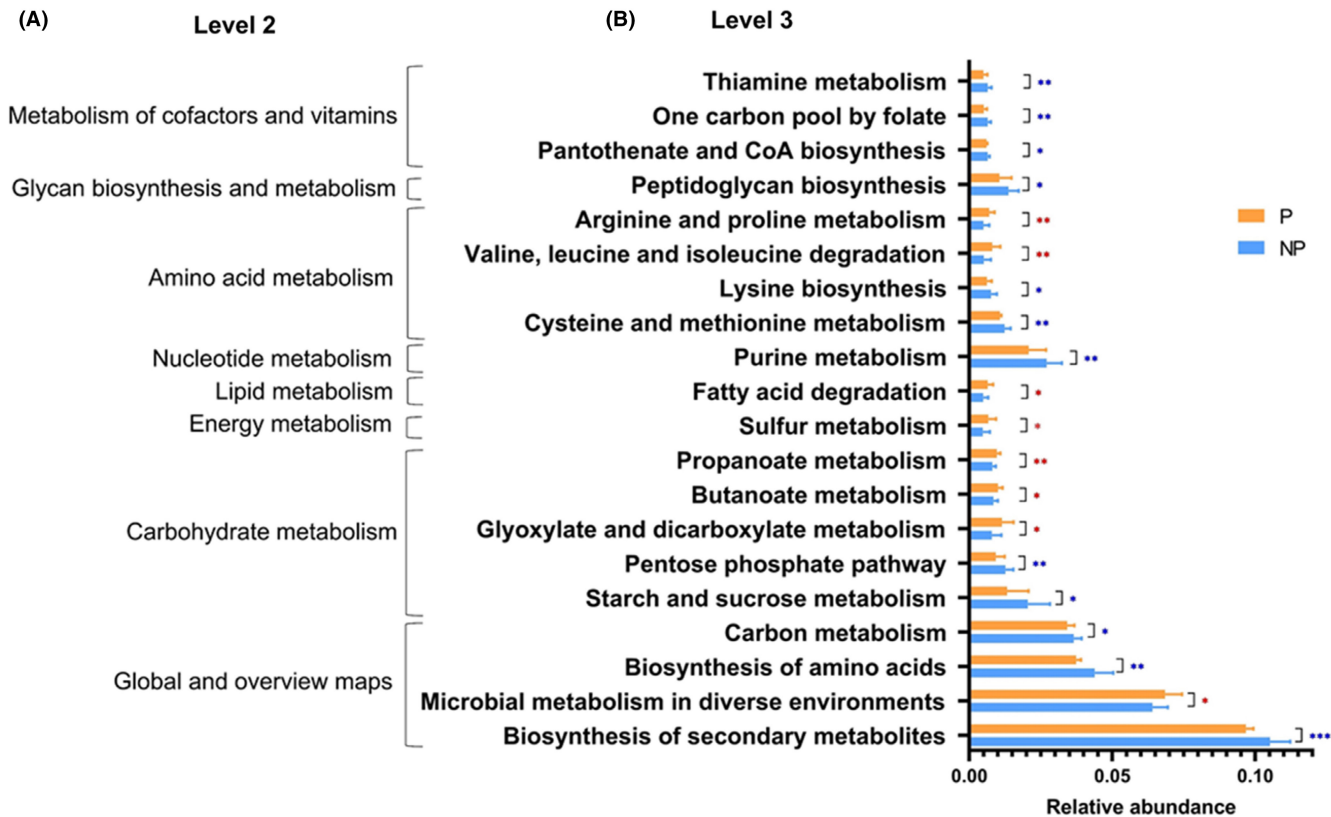
FIGURE 5 Functional and metabolic capacity of microbiota in women underwent FET. Differences in bacterial metabolism function was shown at KEGG pathways in (A) level 2 and (B) level 3 between NP and P using PICRUSt II. The increased KEGG pathway level represents a more specific or detailed metabolic process in the pathways. The level of significance is displayed with *p*-value (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$). Red and blue asterisks indicate significantly higher metabolism in P and NP, respectively

higher in control, but the evenness remains similar in both groups. If this is the case, it emphasizes the importance of the *Gardnerella* abundance as an indicator of FET success rate without considering recurrent FET. Even though CST-IV-based FET pregnancy can still happen,[28] assessing the status of cervicovaginal microbiota prior to FET and ruling out the possibility of BV-related bacterial infection should then potentially improve the successful embryo implantation rate of FET.

Bacterial metabolites or secondary metabolites in the lower reproductive tract may affect human cell function, cause inflammation, and further contribute to disease.[43] Since BV is suggested to be linked with pregnancy health, previous studies mainly focused on metabolite differences comparing BV and non-BV women. Mass spectrometry-based studies linking metabolic markers to BV have conclusively shown 1. Higher concentrations of amino acid catabolites, 2. Higher short fatty acids, and 3. Highly associated with the composition of microbiota as the balance of *Lactobacillus* and CST-IV indicator bacteria species.[24,44–46] However, these studies also showed that the most significant metabolic difference exists between BV/CST-IV to CST-I (*L. crispatus*) and II (*L. gasseri*) type microbiota, whereas the slightest metabolic difference occurs between BV/CST-IV to CST-III (*L. iners*) type. Therefore, our study with *L. iners* CST-III-based microbiota in the pregnant group was expected to show less difference than *Gardnerella* CST-IV-based

microbiota. Indeed, in our study, the predicted metabolic analysis showed that the bacterial metabolites changed with only a few categories between the pregnant and non-pregnant groups. However, significance in amino acid, lipid, and carbohydrate/glycan metabolism was still found. Our results showed significantly less amino acid biosynthesis in the pregnant group. This observation may result from the fact that *L. iners* possessed by the pregnant group lacks several De novo amino acid synthesis genes.[47] Consistent with the dominance of *L. iners*, our results also showed arginine and proline metabolism higher in the pregnant group, similar to the observation by Oliver et al.[24] Similar observation in lower fatty acid degradation in the pregnant group aligned with the reported fatty acid increase in BV-related microbiota. While carbohydrate metabolism was still largely unexplored, it relates the details of simple sugar metabolism to the lower level of simple sugars with BV-related microbiota.[24] Two notable functional changes, microbial metabolism in diverse environments and biosynthesis of secondary metabolites, were shown to emphasize the difference between the two groups associated with microbiota. Although less significant change can be found by comparing the metabolic profile of CST-III and CST-IV microbiota, *L. iners*-based CST-III microbiota still possesses a more diverse metabolic capacity. However, the CST-IV microbiota, though less diverse, can utilize host-derived molecules for metabolism.[24] These studies

support the prediction of the microbial metabolic capacity between the pregnant and non-pregnant groups. Even though the cause-and-effect relationship between metabolic change and pregnancy is just emerging and still under investigation, metabolites in accumulating studies can serve as a potential marker for evaluating the chance of pregnancy. In the future, combining 16 S rRNA sequence analysis and mass spectroscopy would be necessary to determine and confirm links between vaginal flora and the corresponding metabolites to the pregnancy for making these metabolic markers meaningful.

The strength of our study is that we provide a reproductive-relevant clinical report within Taiwan. Our sampling time was just before implantation at the cervicovaginal region. Such sampling time point and location can represent the status of fertilization-related microbiota at the time of embryo implantation and the association with uterus–vaginal bridged cervicovaginal microbiota. Moreover, this is one of the few studies reporting the associated female reproductive microbiota in FET participants, which shed lights on the role of microbiota composition in FET pregnancy since no definite conclusion was drawn. Since ethnicity, age, and dietary culture can affect female reproduction, our study can serve as an initiation of FET-based female reproductive microbiota report correlated to the surrounding southeast Asian region for future development in FET. The limitation of our study was the relatively small study sample size. Additional extensive prospective studies with large and accumulated sizes are required. In addition, our study was designed for only FET participants. Future studies can compare the cervicovaginal microbiota between the natural cycle and hormone cycle of FET, as well as monitor the effect of the cervicovaginal microbiota changes before and after pregnancy outcomes, and we should be able to obtain more evidence for better assessment of FET timing.

5 | CONCLUSION

In conclusion, the study shed some light on the association of cervicovaginal microbiota to pregnancy outcomes of FET. The CST-IV microbiota, increasing *Gardnerella*, and the loss of *Lactobacilli* as the dominant bacteria can potentially contribute to pregnancy failure, therefore, serving as a risk factor in women undergoing FET. Assessing the health of the cervicovaginal microbiota before performing FET would enable couples to make a more considerate decision on the FET timing and might thus improve pregnancy outcomes.

CONFLICT OF INTEREST
The authors declare no conflicts of interest related to the subject matter or materials discussed in this article.

ETHICAL APPROVAL
Ethical approval (IRB No. VGHKS19-CT12-13) was obtained from the Institutional Review Board at the Kaohsiung Veterans General Hospital.

HUMAN RIGHTS STATEMENTS AND INFORMED CONSENT
All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all patients to be included in the study.

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