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Vaginal and endometrial microbiome dysbiosis associated with adverse embryo transfer outcomes

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Abstract

Background Assisted reproductive technology (ART) is the most effective method to treat infertility and the pathogenesis of implantation failure after in vitro fertilization-embryo transfer (IVF-ET) is a challenging filed in infertility. Microbes in the female reproductive tract are considered to be associated with gynecological and obstetric diseases. However, its effects on embryo implantation failure are unsured.

Purpose This study aimed to investigate reproductive tract dysbiosis, identify different bacteria in reproductive tract as potential biomarkers of embryo implantation failure and demonstrate the pathogenesis through metabolites analysis.

Methods We compared the data from 16S rRNA gene and metagenome in reproductive tracts through QIIME2 and HUMAnN2 by the times of embryo implantation failure on 239 infertile patients and 17 healthy women.

Results Our study revealed a strong positive correlation between *Lactobacillus* abundance and embryo implantation success (IS) after IVF-ET. The microbial community composition and structure in reproductive tract showed substantially difference between the embryo implantation failure (IF) and healthy control. Moreover, we established a diagnostic model through receiver operating characteristic (ROC) with 0.913 area under curve (AUC) in IS and multiple implantation failures (MIF), verified its effectiveness with an AUC = 0.784 demonstrating microbial community alterations could efficiently discriminate MIF patients. Metagenome functional analyses of vaginal samples from another independent infertile patients after IVF-ET revealed the L-lysine synthesis pathway enriched in IF patients, along with ascended vaginal pH and decreased *Lactobacillus* abundance.

Conclusions This study clarifies several independent relationships of bacteria in vagina and endometrial fluid on embryo implantation failure and undoubtedly broadens the understanding about female reproductive health.

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Keywords Diagnostic model, Infertility, In vitro fertilization-embryo transfer, Implantation failure, *Lactobacillus*, Microbiome, Reproductive tract

Introduction

Infertility is a reproductive disease that affects 10–15% of couples worldwide [1]. Assisted reproductive technology (ART) is the most effective method to treat infertility [2]. Among more than 900,000 ART cycles in 39 European countries, only 34.6% of patients in a single transfer cycle achieved clinical pregnancy and the estimated cumulative delivery rate based on all transfers resulting from one oocyte pick-up was 30.8% [3]. Failure after in vitro fertilization-embryo transfer (IVF-ET) has become a substantial challenge with increasing occurrence among infertility patients and constitutes a huge financial and emotional burden for patients [4]. Despite advances in IVF and embryo transfer for ART, the pregnancy rate after ART intervention remains low closely related to implantation failure. During the implantation process, embryos are transferred to uterine cavity, where the endometrium undergoes significant changes in order to receive them [5]. The endometrium thus has critical roles in embryo implantation.

In recent years, there has been increasing evidence that cells in the human body are accompanied by numerous symbiotic microbes that are tightly bond with human health [6]. Microbes in the female reproductive tract have received considerable attention and may provide important insights regarding gynecological and obstetric diseases [7, 8], such as microbial imbalances in the vagina are associated with diverse reproductive diseases (e.g., premature labor, vaginosis, and miscarriage) [9–12]. To our knowledge, a few studies have investigated the microbial community existing in the upper reproductive tract [13, 14]. We speculated dysbiosis in the reproductive tract including vagina and uterine may lead to endometrium alterations and influence embryo transfer outcomes.

Various *Lactobacillus* species are present as the dominant vaginal microbes in healthy women of reproductive age and produce lactic acid that helps to maintain a low pH in the vaginal environment [15]. When the concentration of lactic acid decreases and vaginal pH greatly increases, the environment becomes more hospitable for harmful species such as *Gardnerella* and *Prevotella*; this change leads to dysbiosis and increases risks of various diseases, including bacterial vaginosis and preterm birth [15, 16]. Similarly, alterations of the inuterine microbiome are closely associated with various intrauterine diseases, such as endometriosis, endometrial polyps, and endometrial cancer [17–19]. The preservation of ecological stability in the vaginal and inuterine microbiomes is critical for ensuring female reproductive health. Although there is a proposed association between embryo implantation failure and the genital tract microbiome, which has key roles in embryo implantation, specific mechanisms that affect embryo transfer outcomes have not been elucidated [20–23].

To address the questions above, we collect vagina, cervix and endometrial fluid samples from healthy and infertile women underwent IVF-ET and conduct 16S rRNA gene and metagenomic sequencing data excavating of the reproductive tract microbiome. Through a variety of analyses, we reveal the effect of reproductive tract microbiome in embryo implantation failure, set a diagnostic model to identify multiple implantation failures and firstly presume the L-lysine–BAs–*Lactobacillus* axis regulating the vaginal microbiome. These results explain the independent effect of reproductive tract microbiome on embryo implantation failure which will increase our understanding about the importance of microbiome health in successful implantation.

Materials and methods

Participants

All participants were recruited at the Second Affiliated Hospital of Wenzhou Medical University. Clinical information was gathered in accordance with standard procedures. Participants including infertile and healthy control were excluded if they had abnormal leucorrhea, cervicitis, TCT- or HPV-positive, malignant tumors, abnormal levels of sex hormones, autoimmune disease, severe metabolic diseases, or mental disorders and received probiotics, antibiotics or antifungal treatment within the previous 4 weeks and partook in sexual activity within 7 days before the hospital visit. We recruited healthy control from physical examination center without any aforesaid abnormalities in medical reports and meeting the criterion:1) under 40 years old; 2) natural conception and childbirth in the past 2 years. The infertile women undergoing IVF-ET for bilateral fallopian tube occlusion or male factor and using the same controlled ovarian hyperstimulation (The Prolonged Protocol) were assembled from the reproductive center. Based on the outcomes of embryo transfer in infertile women, those with gestational sac in the uterus were categorized into the implantation success group (IS, embryo implantation failure time=0) and those without any gestational sac were categorized into the implantation failure (IF) group. The IF group was further divided into single implantation failure group (SIF, the first embryo transfer but failure and embryo implantation failure time=1) and multiple implantation failures group (MIF, never

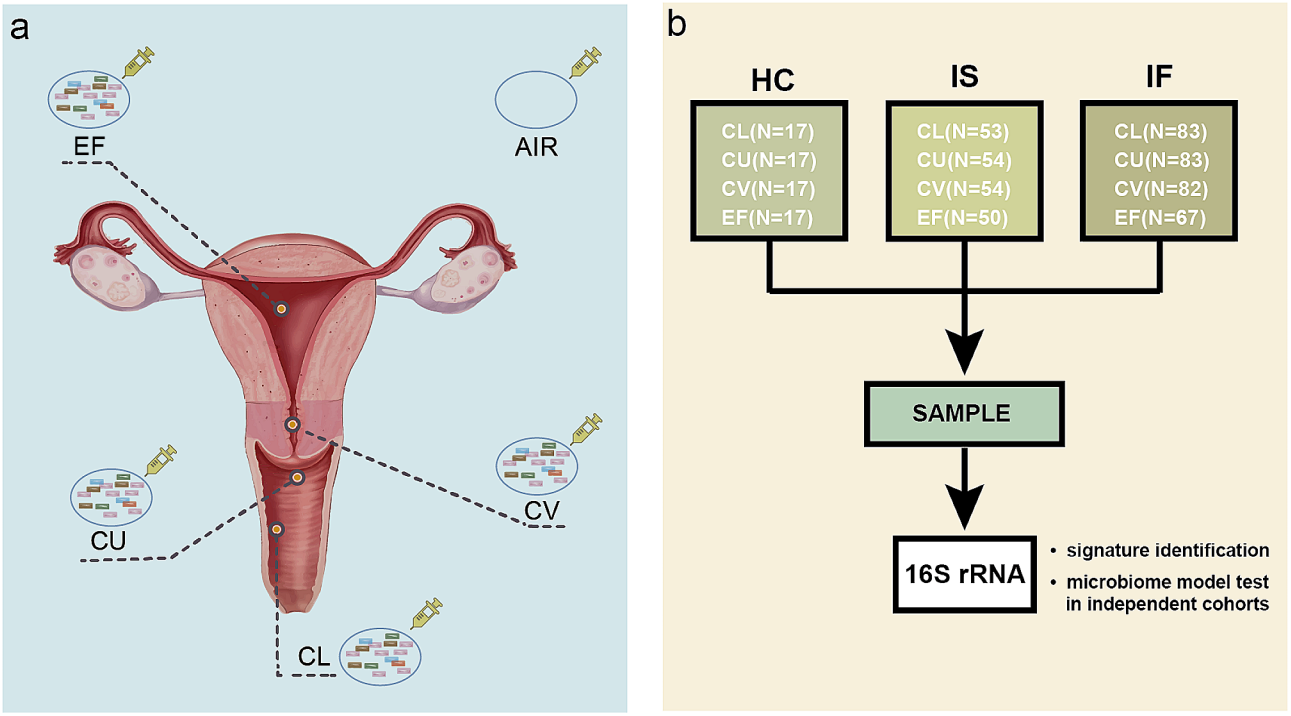

Fig. 1 Study design and flow diagram. **a** Samples were collected from CL, CU, CV, and EF sites during embryo transfer; control group was set in the air of lab. **b** In total, 594 samples were collected from 154 participants, including 17 HC (17 CL, 17 CU, 17 CV, and 17 EF samples), 54 infertile women with IS (53 CL, 54 CU, 54 CV, and 50 EF samples), and 83 infertile women with IF (83 CL, 83 CU, 82 CV, and 67 EF samples). All samples were subjected to 16S rRNA sequencing to characterize the microbiome and construct diagnostic models. HC, healthy control; IS, implantation success; IF, implantation failure; CL, the lower third of the vagina; CU, the posterior fornix; CV, cervical mucus drawn from the cervical canal; EF, endometrial fluid

achieved pregnancy in prior IVF cycles and embryo implantation failure time ≥ 2).

The study was approved by the ethics committee of the Second Affiliated Hospital of Wenzhou Medical University, and all participants provided consent for inclusion in the study.

Sample collection and DNA extraction

Sample collection was conducted by experienced professionals under strict sterile conditions. Vaginal and endometrial secretions from women undergoing IVF-ET were collected at the time of embryo transfer and health women in the mid-secretory phase (LH+7). The level of pH in vagina were assessed using color-changing pH test paper. Vaginal samples that included the lower third of the vagina (CL), the posterior fornix (CU), and cervical mucus drawn from the cervical canal (CV) were collected via swabbing (ClassicSwabs, Copan), which comprised 3–4 wipes of the epithelium in each sample area under direct visualization. Endometrial fluid (EF) was sampled when embryo transferred using a double-lumen Pipelle catheter (Embryo Transfer Catheter, Cook) through rigorous operating procedure to avoid cross contamination [24, 25]. Meanwhile, two empty sampling tubes put in laboratory environment were also prepared for sequencing as negative controls (Fig. 1a). All specimens were placed in sterile tubes, immediately frozen in liquid nitrogen, and stored at −80 °C until total DNA extraction for subsequent sequencing. Bacterial DNA was extracted at G-BIO Bioinformatics Technology Co., Ltd. using a TIANGEN kit (DP316, China), in accordance with the manufacturer's recommendations.

16S ribosomal RNA gene sequencing

For each sample, we amplified variable regions 3 and 4 (V3–V4) of the 16S rRNA gene using modified primers, including 341F (5'-CCACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'). Polymerase chain reactions were performed using the following conditions: denaturation at 95 °C for 3 min; eight cycles of denaturation at 94 °C for 55 s, annealing at 50 °C for 30 s, elongation at 72 °C for 45 s; and final extension at 72 °C for 5 min. Negative controls had been introduced during the amplification process and no positive was shown in the negative controls by electrophoresis. Purified positive amplicons with different index sequences were pooled in equimolar amounts, then sequenced on a MiSeq PE300 platform (Illumina, San Diego, CA, USA).

16S rRNA sequence analysis

Raw sequencing reads of the 16S rRNA gene sequences were quality-filtered and analyzed through pandaseq

(version 2.9) and QIIME2 (version 2020.1). The criteria for high-quality reads were length > 300 bp, amplification with polymerase chain reaction primers, mean quality score ≥ Q20, no non-spliceable sequences (pandaseq overlap ≥ 10 bp), and no ambiguous N bases. The plugin dada2 within QIIME2 was utilized for denoising and produce Feature tables. To avoid contamination and false positives, a BLAST against the database of human genome of NCBI was implemented, as well as singletons and chimeras were removed. α diversity and β diversity were calculated for microbiota analyses. The taxonomy annotation was classified by the pretrained naive Bayes classifier (https://docs.qiime2.org/2022.2/plugins/available/feature-classifier/fit-classifier-naive-bayes/) based on the Greengenes (http://greengenes.secondgenome.com/) database with an identity cutoff of 99% [26, 27]. The taxonomic compositions of microbial communities were analyzed and visualized using R packages.

Alpha/Beta diversity and rarefaction curve analyses

To estimate sample richness, the within-sample (alpha) diversity was carried out in QIIME2 software, and community richness and diversity were determined by Shannon index and observed Operational Taxonomic Units (OTUs). A Wilcoxon rank sum test was used to verify significance. Beta diversity, which represents the differences in microbial community structures among groups were evaluated using principal-coordinate analysis (PCoA), and the differences between groups were analyzed by permutational multivariate analysis of variance (PERMANOVA). Rarefaction curves, draw by R software, were performed to assess the OTU abundance in CL, CV, CU, and EF samples.

Random forest model

A random forest classifier was constructed using the randomForest package (version 4.6-7) in R to evaluate several species as potential markers in discriminating participants with embryo implantation failure from implantation success. The classifier was trained on 70% of the data, then tested on the remaining 30% of the data. Bootstrapping was conducted 1000 times. For each bootstrap result, each species' contribution to the model was recorded, along with the diagnostic score for each participant.

The diagnostic capacity of the model for distinguishing patients with different pregnancy outcomes was evaluated according to the area under the receiver operating characteristic (ROC) curve (AUC) using the ROCR package (version 1.0–6) in R. The model with the best diagnostic capacity was selected; it was used for ROC analysis and data verification. Another 16S rRNA validation datapoints from infertile women we reassembled were used to test the model effectiveness and the results were also visualized using an ROC curve.

Metagenomic sequencing and gene catalog construction

All samples were sequenced using an Illumina NovaSeq 6000 sequencing platform (strategy PE150) at Novogene Bioinformatics Technology Co. The raw data were preprocessed using Readfq (version 8, https://github.com/cjfields/readfq) to acquire clean data for analysis. Preprocessing was performed as follows. First, reads were removed if they contained ≥ 40 low-quality bases (quality threshold value ≤ 38); second, reads were removed if they contained ≥ 10 N bases; reads were removed if they overlapped with the adapter sequence for ≥ 15 bp. Considering the possibility of host contamination, reads were aligned to the human genome using Bowtie2 (version 2.4; parameters: --end-to-end, --sensitive, -I 200, -X 400) [28, 29]; reads successfully matching with the human genome were removed.

Taxonomic and functional profiling

Species-level abundances were estimated using MetaPhlAn2 with the default parameters [30]. MetaPhlAn2 utilizes clade-specific marker genes to estimate the abundances of organisms present in a sample. Phylum, class, order, family, genus, and species abundances were calculated and compared using R software (version 3.6.3); comparisons were made among the groups within CL and EF samples. The metabolic functional profile was estimated using HUMAnN2 with the full UniRef90 database [31, 32]. HUMAnN2 constructs a reference database for each sample, then compares the sample reads against these databases to calculate gene abundances in each sample. UniRef90 protein abundances were merged with MetaCyc pathway abundances by a parsimony approach (MinPath) [33, 34]. Of the output files generated by HUMAnN2, we focused on pathway abundance to obtain comprehensive quantitative insights into the functional aspects of each microbial community.

Correlation coefficient analysis

The relative abundances of genera and species were calculated for each sample type by comparing both mean and median relative abundances between CL and EF groups. For the correlation coefficient analysis, spearman correlation was used to assess the bacterial correlations between the CL and EF samples based on the relative abundance of each genus and species, using R software (R version 3.6.3). The correlation coefficient output of the EF microbiome was visualized using the corrplot and pheatmap packages (version 3.6.3) in R.

Statistical analysis

Differences between two groups were compared using the Wilcoxon rank-sum test for continuous variables, while differences among three groups were assessed using the χ^2 test or Fisher's exact test for categorical variables. Statistical analyses were performed using the ggpubr package (version 0.4.0) in R or using SPSS for Windows (version 24.0.0.0).

Alpha and beta diversities of 16S rRNA sequencing results were calculated by QIIME2 and visualized using the ggplot2 package (version 3.3.2) in R. Alpha diversities were compared using the Wilcoxon rank-sum test and beta diversities using PERMANOVA in QIIME2. Beta diversities of metagenomic sequencing results were assessed using the vegan package (version 2-5.6) in R to quantify Canberra Distance; they were visualized using PCoA plots. PERMANOVA was used to test sample clustering in beta diversity analyses with 10,000 replicate permutations; this test was performed using the Adonis function in the vegan package (version 2-5.6) in R.

Results

We recruited a total of 256 participants for 16S rRNA gene sequencing and metagenomic sequencing. 594 samples from multiple body sites of 154 participants, including 17 HC (17 CL, 17 CU, 17 CV, and 17 EF samples), 54 infertile women with IS (53 CL, 54 CU, 54 CV, and 50 EF samples), and 83 infertile women with IF (83 CL, 83 CU, 82 CV, and 67 EF samples) were collected (Fig. 1b; Table S1). In total, 22.5 million read counts were obtained, with a mean of 37,177 counts per sample after demultiplexing via QIIME2. Rarefaction curve in each site was near saturation, suggesting that the sequencing data were sufficient for detection of rare OTUs (Fig. S1a). Another 43 CL (13 IS, 12 SIF and 18 MIF) samples recollected from infertile women for metagenomic sequencing generated more than 38 million PE reads (2×125 bp).

In our study, we found no differences in alpha and beta diversity were observed among CL, CU, and CV samples (Fig. S1b-c). Comparative analysis of OTU types among body sites revealed that there were 671 unique OTUs in EF, but only 24, 4, and 8 in CL, CU, and CV, respectively (Fig. S1d). The composition of microbiota at genus level and species level was also exhibited greater abundances in EF samples than in other body sites (Fig. S1e-f). As noted above, vagina samples had similar microbiomes and CL is the most common sampling site for clinical examinations; thus, we selected CL and EF as representative sites for downstream analysis.

Pairwise comparison identified distinct microbial discrepancy in IF

To examine the impacts of bacterial community changes on embryo implantation outcomes, we analyzed the uterine and vaginal microbiomes in IS and IF groups (Fig. 2a). Given that aging and BMI in women result in embryo transfer outcome and human microbiome environment including that of the reproductive system, we compared the age and BMI between IS and IF and found no difference (Table S2). And faith's PD index (a measure of alpha diversity) based on OTU profiles, considering the evolutionary relationships between bacteria in CL and EF, was used to assess changes in the microbiome after IVF-ET treatment. We found that alpha diversity was much higher at CL in IS and IF groups than in the HC group (P=9.91e-3, IS vs. HC; P=0.047, IF vs. HC; P=0.22, IS vs. IF; PERMANOVA, Fig. 2b), whereas it was lower at EF in IS and IF groups than in the HC group (P=8.73e-3, IS vs. HC; P=0.034, IF vs. HC; P=0.72, IS vs. IF; PERMANOVA, Fig. 2b). Moreover, the community composition was unique in the IF group. Using Bray–Curtis distances calculated with normalized OTU abundances, the distributions at both CL and EF were considerably different between IF and HC groups (Fig. 2c). The community distances within samples obtained from HC-IS, as expected, were lower than HC-IF in both CL and EF (CL: P=1.8e-9, EF: P=0.008, HC–IS vs. HC–IF; Fig. 2d, Fig. S2a), indicating that the IF group had distinct microbial communities in both the endometrium and vagina.

The results showed that a decreasing trend for the ratio of *Lactobacillus* and an increasing trend for the ratio of *Gardnerella* from the HC and IS groups to the IF group and metagenomic sequence got the similar consequences in IS and IF group (Fig. 2e; Fig. S2b). The IF group had a smaller proportion of >90% *Lactobacillus* samples and a larger proportion of <70% samples, while almost all samples in HC and IS groups had a *Lactobacillus* abundance of >90% (P=0.027; Fisher's exact test, Fig. 2f). To identify characteristics of health and disease statuses, we used genera with accumulated relative abundance higher than 0.01 and found 13 discriminant bacterial taxa in the endometrial microbiome by LEfSe (Fig. 2g). We reconfirmed a greater abundance of *Lactobacillus* was observed in the HC group compared with IS and IF. Several genera such as *Curvibacter*, *Bradyrhizobium*, *Acinetobacter* were enriched in IS and *Sphingobium*, *Corynebacterium*, *Ralstonia*, *Enterobacter*, and *Enterococcus* were discriminatory bacteria and concentrated in IF. Additionally, in the specie level, *Lactobacillus. iners* was predominant in CL and we found the relative abundance of *Lactobacillu. helveticus* was greater in IF than in HC and IS groups, when *L. iners* was enriched in IS and HC groups, albeit without statistical significance (Fig. S3).

Bacterial variety was associated with number of failures

To further explore whether bacterial diversity was associated with the number of embryo transfer attempts, we

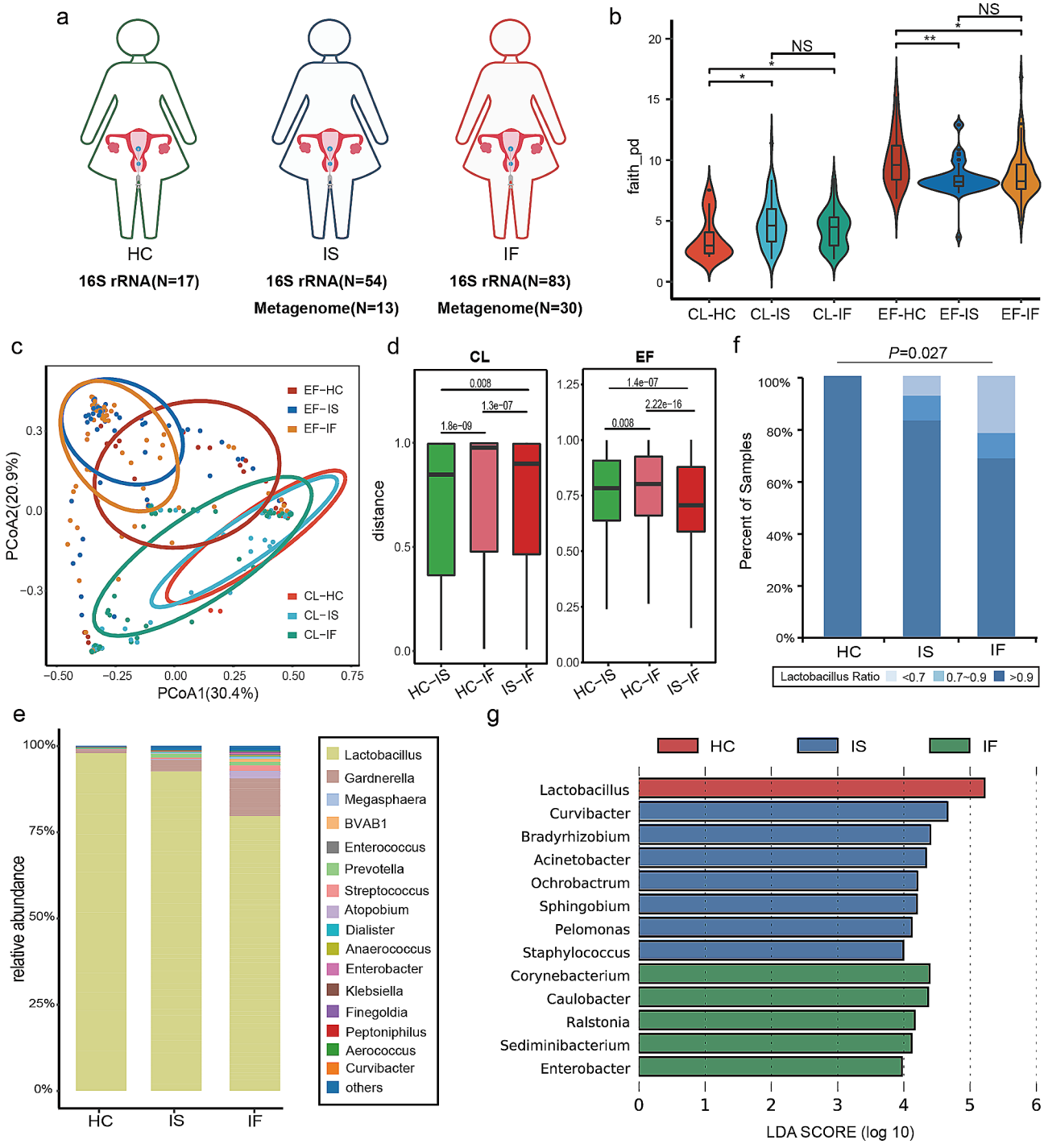

Fig. 2 Endometrial and vaginal microbiomes in healthy and infertile women. **a** The numbers of samples in each group in this study. **b** Comparisons of microbial alpha diversity (determined by Faith's PD index) in CL and EF samples based on OTU profiles among the three groups. HC, healthy control; IS, implantation success; IF, implantation failure. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. *P*-values were calculated by Wilcoxon rank-sum test. **c** PCoA of Bray–Curtis distances in CL and EF samples. **d** Bacterial community dissimilarities for each group of pairwise comparisons in CL and EF samples. **e** Relative abundances of vaginal bacterial genera in HC, IS, and IF groups. Bars on the left show the mean relative abundances of each group. **f** Abundances of *Lactobacillus* in HC, IS, and IF groups. $P=0.027$ across groups; χ^2 test. **g** LEfSe was performed to identify genera in endometrial fluid that significantly differed among HC, IS, and IF groups; genera are ranked according to effect size

firstly divided the IF group into SIF and MIF with the IS group as a control (Fig. 3a). Among 42 individuals who underwent embryo retransplantation in the SIF group, 23 patients were pregnant (pregnancy rate=54.7%, Table S3). The first principal component (PC1) of PCoA based on OTUs in CL samples showed a decreasing trend in both SIF and MIF groups, compared with the IS group, although the difference between IS and SIF groups was not statistically significant (P=0.356, IS vs. SIF; P=8.1e-3, IS vs. MIF; P=0.044, SIF vs. MIF; Wilcoxon rank-sum test, Fig. 3b and Fig. S4a). A similar trend in EF samples was also observed, such that the PC1 of PCoA significantly differed between the MIF and IS groups (P=0.032, IS vs. MIF; P=0.199, SIF vs. MIF; P=0.869, IS vs. SIF; PERMANOVA, Fig. 3c). Notably, we made the comparisons of bacterial abundance among these groups to examine variations in CL samples and 18 genera were differentially enriched in IS, SIF and MIF groups (P<0.1 among groups, Wilcoxon rank-sum test, Fig. 3d). We found several genera were enhanced in the IS group (e.g., *Bifidobacterium* and *Anaerococcus*), but the abundance of *Corynebacterium* was decreased in the IS and SIF groups and increased in the MIF group.

Considering that the vaginal microbiome was substantially altered in patients with embryo implantation failure compared with pregnant women, we constructed a random forest classifier and evaluated its performance in discriminating IS and IF participants. ROC evaluation with 1000 rounds of bootstrapping showed that a species-based model accurately described deviations between the IS and MIF groups with an AUC=0.913 (Fig. 3e). A diagnostic model was also constructed to distinguish the SIF and MIF groups with an AUC=0.905 (Fig. S4b). Another 16S rRNA validation datapoints we recollected from 59 IVF-ET women (including 19 IS, 20 SIF and 20 MIF) were used to remeasure the model effectiveness with 0.784 AUC in IS-MIF (Fig. 3f) and 0.632 in SIF-MIF (Fig. S4c). Moreover, the key species made major contributions to the diagnostic model with MeanDecreaseGini>2. There were five species mostly deviated both between SIF and IS, MIF and IS groups (Fig. S4d–e), such as the relative abundance of *L.helveticus* in CL samples was enriched in the MIF group(P=0.160, MIF vs. IS), while the abundance of *L. iners* was statistically decreased (P=0.0086, MIF vs. IS, Fig. S5a–c).

Function profiles of metagenomes in patients with SIF and MIF

Overall, 330 organism-specific pathways were identified by HUMAnN2 based on the MetaCyc database. General

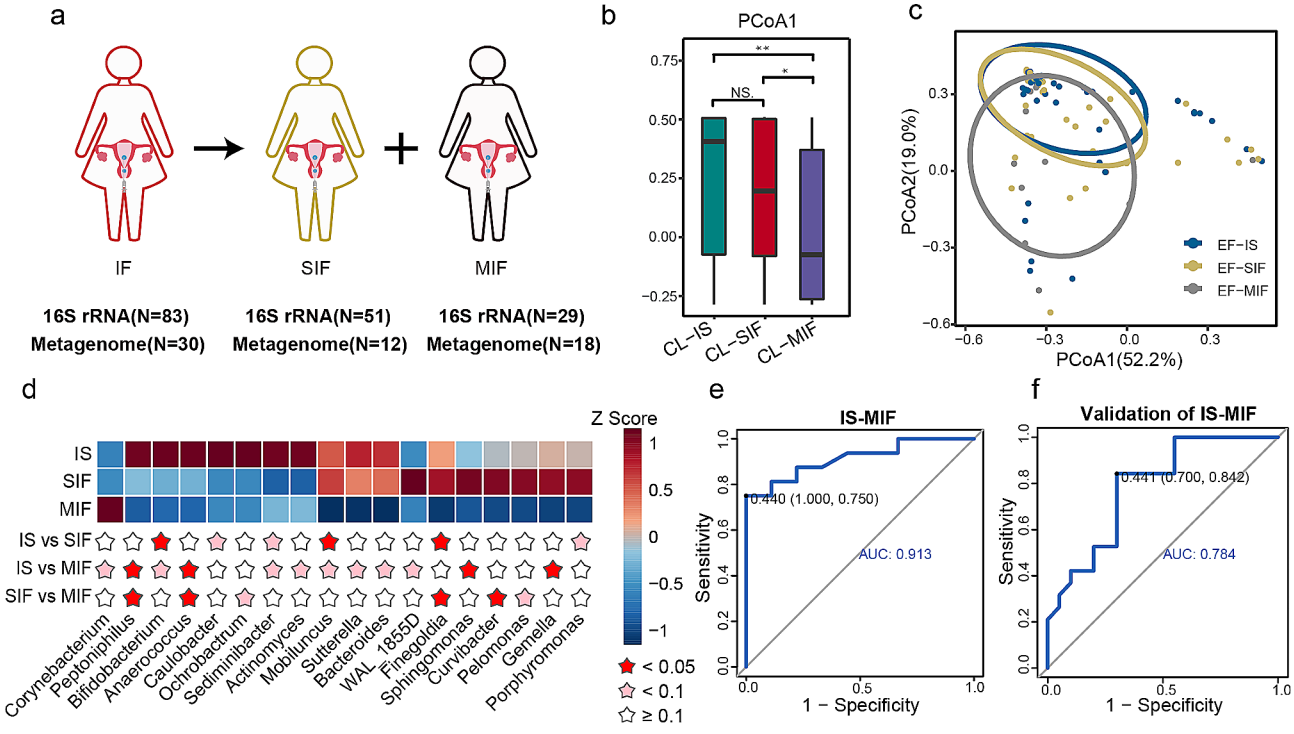

Fig. 3 Microbial differences associated with number of failures. **a** The numbers of samples in each group after regrouping. **b** PC1 based on OTU distribution in CL microbial communities. IS: implantation success; SIF: single implantation failure; MIF: multiple implantation failures. **c** PCoA of EF microbial communities. P=0.032, IS vs. MIF; P=0.199, SIF vs. MIF; P=0.869, IS vs. SIF; PERMANOVA. Each circle shows a 70% confidence interval for the indicated group. **d** Z-scores in the heatmap according to relative abundances of 18 genera in CL (across groups). Genera with P<0.05 are marked with red stars, P<0.1 with pink stars, and P≥0.1 with white stars. **e** ROC analysis of performance of the IS–MIF diagnostic model. **f** Validation of IS–MIF diagnostic model by another fifty-nine samples

pathway differences between groups were evaluated according to Bray–Curtis dissimilarity and visualized in PCoA plots. The total diversities identified in PC1 and PC2 were 18.78% and 9.16%, respectively (Fig. 4a). Significant differences were observed between MIF and IS, but not between SIF and IS or SIF and MIF ($P=0.049$, IS vs. MIF; $P=0.350$, SIF vs. MIF; $P=0.406$, IS vs. SIF; PERMANOVA, Fig. 4a). To determine key functional pathways, we identified 25 bacteria-specific metabolic pathways using the Kruskal–Wallis test ($P<0.1$; Fig. 4b). Among these pathways, we found that pathways associated with pyrimidine deoxyribonucleoside salvage were more abundant in IS than in SIF or MIF (Fig. 4b). Pyrimidine metabolism is reportedly one of the most enriched metabolic pathways in progesterone and the progesterone level were greater in IS group than SIF and MIF groups in our study($P=0.230$, IS vs. SIF; $P=0.023$, IS vs. MIF, Fig. 4c).

Notably, enrichments of pathways associated with L-lysine biosynthesis (including L-lysine biosynthesis II/III/VI) were observed in both SIF and MIF groups (Fig. 4b, d). We wondered whether any element present in vagina could establish a connection with the L-lysine biosynthesis and bacteria. Accordingly, we measured the vaginal pH increasing in SIF and MIF compared to IS group ($P=0.037$, IS vs. SIF; $P=0.005$, IS vs. MIF, Fig. 4e)

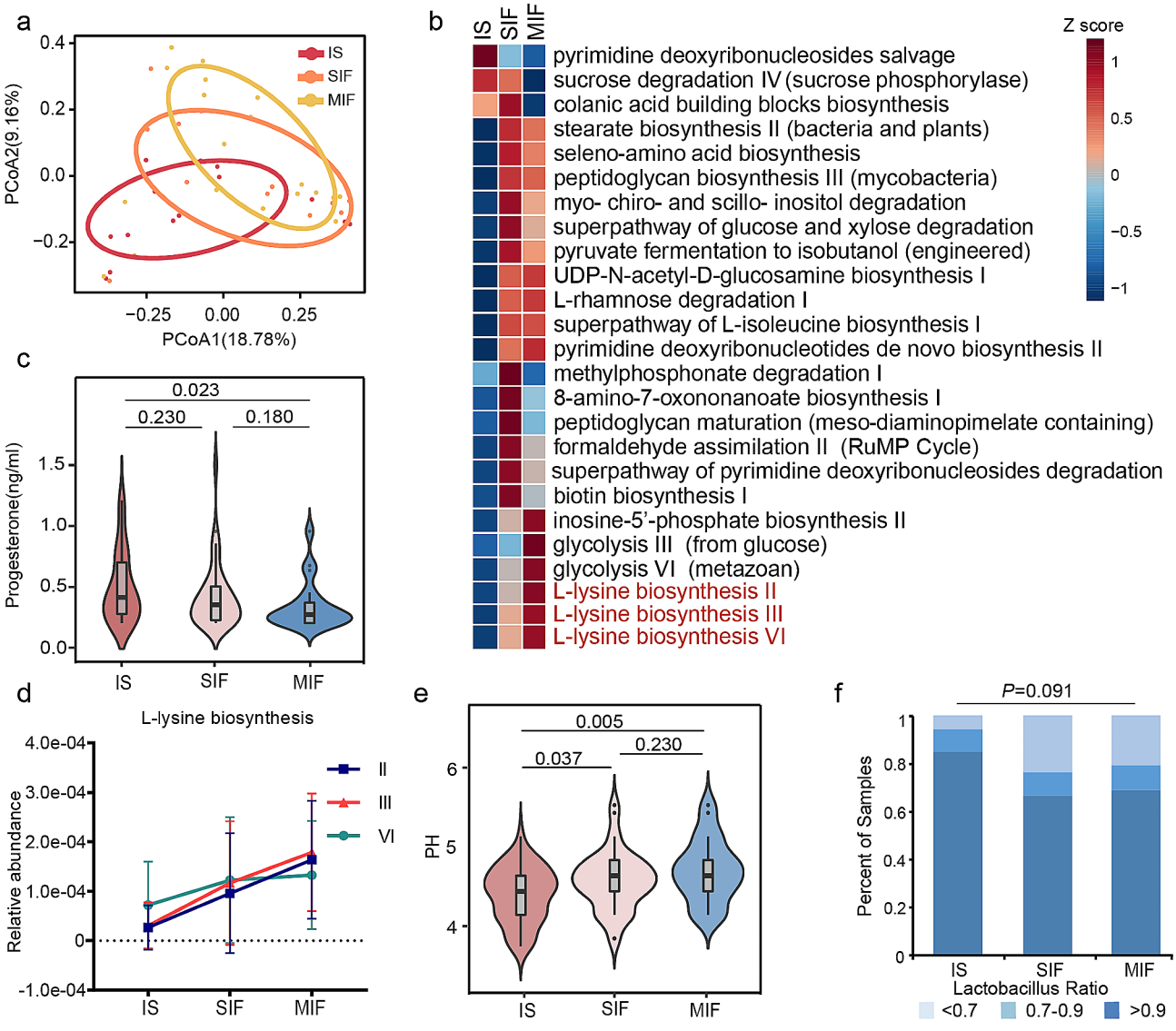

Fig. 4 Metagenomic pathway variations in the vaginal microbiome and their correlations with clinical factors. **a** PCoA based on metabolic pathway distribution. $P=0.049$, IS vs. MIF; $P=0.350$, SIF vs. MIF; $P=0.406$, IS vs. SIF. Each circle shows a 60% confidence interval for the indicated group. **b** Heatmap comparing relative abundances of metabolic pathways among IS, SIF, and MIF groups. Red, more abundant; blue, less abundant. **c** Serum progesterone concentrations in patients undergoing IVF-ET. **d** Line chart of L-lysine biosynthesis II/III/VI. Points and short lines represent the means and 95% confidence intervals. **e** Vaginal pH values in patients undergoing IVF-ET. **f** Abundances of *Lactobacillus* in IS, SIF, and MIF groups. $P=0.091$ across groups; χ^2 test

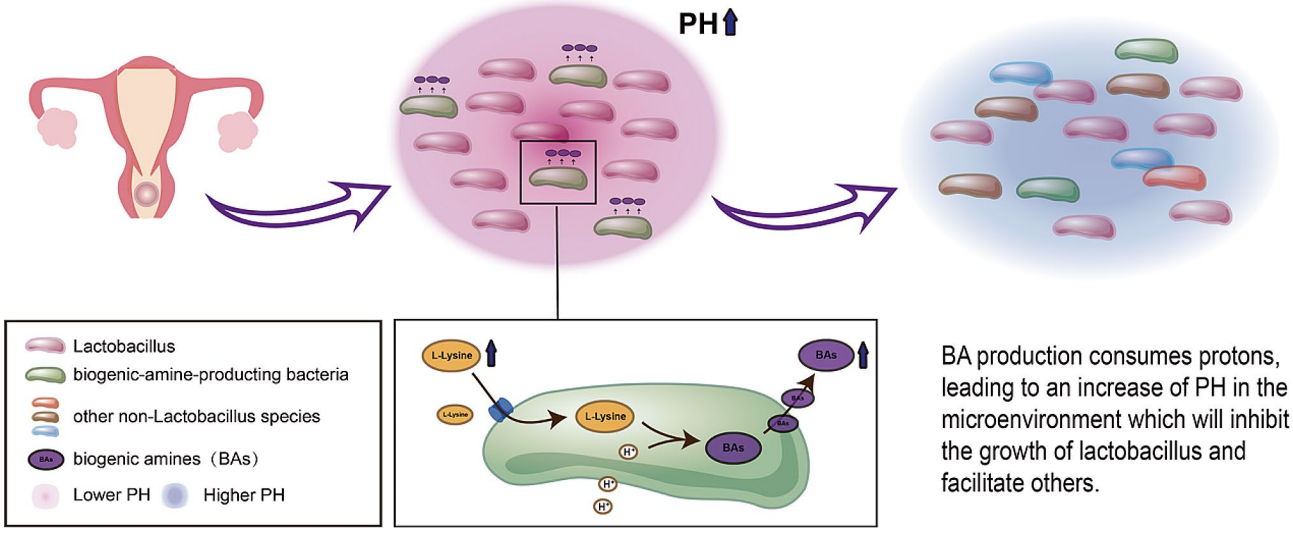

Fig. 5 Schematic of hypotheses concerning mechanisms by which the L-lysine–BAs–*Lactobacillus* axis regulates the vaginal microbiome

with the abundance of *Lactobacillus* declined ($P=0.091$; Fisher's exact test, Fig. 4f, Fig. S5d). Similar findings were also observed between the IS and IF groups (Fig. S6a–b).

Discussion

To our knowledge, few studies have demonstrated direct associations between the endometrial microbiome and embryo transfer outcomes. There remain considerable knowledge gaps in this area, particularly concerning the microbiome profiles of patients with infertility in clinical trials, the microbial biomarkers for detection of embryo implantation failure and the biosynthesis signature profiles of embryo implantation failure. We used a strategy based on 16S rRNA gene and metagenomic sequencing methods. This study sequenced the total bacterial DNA from several sites including CL, CU, CV, and EF collected from a cohort of 256 Chinese individuals. Importantly, most reproductive tract samples in our study were gathered during embryo transfer, which allowed us to directly evaluate relationships between the reproductive tract microbiome and embryo transfer outcomes.

Our 16S rRNA gene sequencing data eliminating pollution detected clear bacterial signals from endometrial fluid samples; these samples exhibited higher species richness and distinct microbiome composition, compared with vaginal and cervical samples, revealing microorganisms inhibit in endometrium and differ from other sites [35]. Now that the bacteria in vagina and cervix bespoke tremendously resemble, we selected CL from lower reproductive tract and EF from upper reproductive tract as representative sites for analysis. In this study, we demonstrated that microbiome diversity and specific bacterial taxa were present in the IF group. Analyses of Bray–Curtis dissimilarity demonstrated that the microbiomes in both CL and EF samples were distinct between the IF and HC groups. Additionally, CL samples exhibited higher abundance of *Lactobacillus* and lower abundance of *Gardnerella* in the HC and IS groups, compared with the IF group. Similarly, *Lactobacillus* abundance in EF samples was enriched from the HC group, declined in that from the IF group. Previous studies have suggested that decreased *Lactobacillus* abundance and increased *Gardnerella* abundance in the reproductive tract microbiome contribute to dysbiosis that drives infertility and vaginitis [36, 37]. Based on these findings, we had the reason to evaluate the direct impact of reproductive tract composition in the IF group, with the expectation that changes in *Lactobacillus* and *Gardnerella* could lead to embryo implantation failure. Moreover, several genera (e.g., *Sphingobium*, *Corynebacterium*, *Ralstonia*, *Enterobacter*, and *Enterococcus*) were identified as candidates for the onset of reproductive tract dysbiosis in patients with embryo implantation failure. These findings indicated that dysbiosis in the reproductive tract microbiome may contribute to the pathogenesis of embryo implantation failure.

For the first time, we provided important insights concerning the relationships between dysbiosis in the reproductive tract microbiome and the number of embryo implantation failure times. Both vaginal and endometrial microbiota in the multiple implantation failures (MIF) patients were significantly different from implantation success (IS) and single implantation failure (SIF). We found in both IF and MIF groups, an enhanced *Corynebacterium* abundance in vagina. The enrichment of *Corynebacterium* could have a negative effect for reproductive health and is associated with Human papillomavirus-infection and preterm birth [38, 39]. This may be partially responsible for the occurrence of embryo implantation failure by disrupting the female reproductive tract

microbiota. Additionally, we creatively constructed a random forest classifier from 16S rRNA sequence data in vagina and evaluated its performance, with the high level of 0.913 and 0.905 diagnostic power in IS-MIF and SIF-MIF. These results encourage us to pay attention to the reproductive health, ponder the possibility of bacterial composition from reproductive tract to predict embryo transfer outcomes in clinic.

The dominant genus *Lactobacillus* in the vagina of childbearing age women constitutes the first line of defence against pathogens and is a protective bacterium for vaginal microbiome [40]. *L. iners* the smallest lactobacillus presenting only in vagina constitutes an important biomarker of vaginal microbial health; its abundance has been used to predict ART outcome [41, 42]. In our study, *L. iners* was the most abundant species in vagina and contributed much to the diagnostic model [43]. Nowadays, research on *L. iners* is still debatable and its exact function in female reproductive health is indefinite. Several studies pointed *L. iners* is a beneficial species in vagina and enriched in normal pregnant than embryo arrest women and could play a positive role in follicle maturation [44, 45]. It also could contribute to be a negative factor of female pregnancy and was associated with a high risk of adverse pregnancy outcomes [46–49]. Our research showed *L. iners* may have protective effects on embryo implantation and inspired us to consider external supply of *L. iners* as a choice of clinical invention to raise pregnancy rate.

In addition to the altered microbial composition in vaginal samples, we observed disruptions of bacterial biosynthesis and tried to explain the occurrence of reproductive disorder. In this study, the pathways associated with pyrimidine metabolism were enriched in the IS group. Pyrimidine the form of lives consisting of DNA and RNA has been identified to play an important role in conceptus development and was in wake of progesterone [50]. We compared progesterone level and found it increased in IS groups, which may stimulate pyrimidine metabolism and facilitate female to be pregnant. In addition, we presumed the L-lysine–BAs–*Lactobacillus* axis acting to regulate the vaginal microbiome (Fig. 5). Our study indicated that the increased biosynthesis of lysine metagenomes in the SIF and MIF groups were observed. Biogenic amines (BAs) as downstream products of lysine through specific amino acid decarboxylation pathways have adverse effects on the growth rate and lactic acid production of *Lactobacillus* [51–53]. As the dominant genus in the vaginal microbiome, *Lactobacillus* species inhibit other bacteria by maintaining a high concentration of lactic acid, which reduces the surrounding pH [54]. Accordingly, we found increasing vaginal pH and declining of the abundance of *Lactobacillus* were in both SIF and MIF groups compared to IS group. Hence, we hypothesized that the L-lysine–BAs–*Lactobacillus* axis with the enhanced lysine biosynthesis indirectly resulting to changes in vaginal pH through BA production, thereby inhibiting *Lactobacillus*, stimulating the growth of other bacteria and destabilizing reproductive health.

As we know, this is the first observational study to analyse both vaginal and endometrial microbiome with advanced embryo implantation failure time in infertile women undergoing IVF-ET and presume the L-lysine–BAs–*Lactobacillus* axis acting to regulate the vaginal microbiome. However, the limitations of this retrospective study should be considered when interpreting the results. There was some lacking data for CL and EF in IS and CV and EF in IF group. The deletion samples from vagina ($n=2$) were operation error and discarded. The loss EF samples ($n=20$) without sufficient bacteria content to sequence were considered to the low-biomass in uterine and a more sensitive sequence technology is in great request to be explored in the future. Our study considered about the antibiotics effects on the microbiome, but we just cut the time of no-antibiotics in 4 weeks which is not strict enough and may result in the bias of some samples. Moreover, although our study provided noteworthy biomarkers discovered in the cohort of patients with pregnancy and those with embryo implantation failure, there still need different sizes of multicenter and a larger external validation based on this work to confirm the results from this research.

Conclusion

We have described disordered microbiome profiles and metabolism in vagina and uterine were associated with infertile patients especially underwent multiple embryo implantation failures and had destructive effects on reproductive health. Accordingly, we offered a promising approach to predict embryo transfer outcomes and proposed a new hypothesis aiming to expound the mechanism of embryo implantation failure which may facilitate external microbial interventions to improve the rate of embryo implantation success in clinic.

Abbreviations

IVF	in vitro fertilization
ET	embryo transfer
ART	Assisted reproductive technology
CL	the lower third of the vagina
CU	the posterior fornix
CV	cervical mucus drawn from the cervical canal
EF	endometrial fluid
OTU	Operational Taxonomic Unit
PCoA	principal-coordinate analysis
ROC	receiver operating characteristic
AUC	under the ROC curve
BA	biogenic amine

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12958-024-01274-y.

Supplementary Material 1: Fig. S1. Variations in reproductive tract microbiomes among body sites. **a** Rarefaction analysis between the number of samples and number of OTUs. **b** Comparisons of microbial observed OTUs and alpha diversity (as accessed by Shannon index) based on OTU profiles in four body sites. For observed OTUs: $P = 2.25e\text{-}47$, CL vs. EF; $P = 1.48e\text{-}17$, CU vs. EF; $P = 1.44e\text{-}45$, CV vs. EF. For alpha diversity: $P = 2.25e\text{-}47$, CL vs. EF; $P = 1.48e\text{-}17$, CU vs. EF; $P = 1.44e\text{-}45$, CV vs. EF. *P*-values were calculated by PERMANOVA. **c** Principal Coordinate Analysis (PCoA) of the Bray–Curtis distance in CL, CU, CV, and EF samples. **d** Venn diagram displaying overlaps among body sites. **e-f** Relative abundances of bacterial genera (**e**) and species (**f**) in CL, CU, CV, and EF samples

Supplementary Material 2: Fig. S2. The correlation between individuals within different groups in different body sites and vaginal microbiomes in infertile women. **a** Bray–Curtis distances of microbial communities among individuals within different groups and between different body sites. **b** Relative abundances of vaginal bacterial genera in the IS, and IF groups by metagenomic sequence

Supplementary Material 3: Fig. S3. Relative abundances of vaginal bacterial species in the HC, IS, and IF groups

Supplementary Material 4: Fig. S4. Diagnostic model and feature importance predicted by random forest. **a** PCoA of EF microbial communities based on OTU distribution. **b** The SIF–MIF diagnostic model. **c** ROC curve of the SIF-MIF validation cohort. **d** The ten most important features in the random forest classifier of IS–MIF groups, indicated by MeanDecreaseGini values. **e** Feature importance plot of SIF and MIF groups

Supplementary Material 5: Fig. S5. Relative abundances of species and genera enriched in various groups. **a** Relative abundance of one species enriched in IS and SIF or only in MIF. The top portion of the circle indicates the three groups, while the bottom portion indicates the relative species abundances. A larger area indicates higher relative abundance. **b** Relative abundances of *Lactobacillus. iners* in IS, SIF, and MIF groups. **c** Log-transformed relative abundances of *Lactobacillus. helveticus*. **d** Relative abundances of vaginal bacterial genera in IS, SIF, and MIF groups

Supplementary Material 6: Fig. S6. Metabolic pathways and vaginal pH values in patients between IS and IF groups. **a** Heatmap showing relative abundances of metabolic pathways in each sample in IS and IF groups. Pathways with $P < 0.1$ (calculated by Wilcoxon rank-sum test) were selected for analysis. Colors indicate relative abundances. Pathway names are labeled on the right side of the heatmap. **b** Vaginal pH values in patients undergoing IVF-ET. The violin plot shows the medians and interquartile ranges (IQRs) of the pH values in IS and IF groups; the width of the violin represents the density distribution of the indices

Supplementary Material 7: Table S1. Clinical characteristics of 137 infertile women and 17 healthy women.

Supplementary Material 8: Table S2. The compare of age and BMI in 54 implantation success (IS) and 83 implantation failure (IF) women.

Supplementary Material 9: Table S3. Outcomes of another or embryo transfer accepted by those participants with single implantation failure.

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Author contributions

SW, ZH, XH and ZJ contributed to the study design. ZH and GC interpreted the patient data. SW and XH analyzed the data. SW and YH wrote the original manuscript. CY, FX and JJ participated in revising the manuscript. All authors read and approved the final manuscript.

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Data availability

The sequence data in our study have been uploaded in the NCBI SRA database with under accession number PRJNA815812 for metagenome and PRJNA814891 for 16S rRNA.

Declarations

Ethics approval and consent to participate

The study was approved by the ethics committee of the Second Affiliated Hospital of Wenzhou Medical University (No.LCKY2020-214). All procedures involving human participants were performed in accordance with the ethical standards of the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University.

Consent for publication

An informed written consent was signed by the participant.

Competing interests

The authors declare no competing interests.

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