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ASSISTED REPRODUCTION TECHNOLOGIES

Impact of vaginal microecological differences on pregnancy outcomes and endometrial microbiota in frozen embryo transfer cycles

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Abstract

Purpose This prospective study investigates the correlation between vaginal microecology and pregnancy outcomes and explores their impact on endometrial microbiota composition during frozen embryo transfer (FET) cycles. Additionally, the impact of transvaginal *Lactobacillus* supplementation on reproductive outcomes in patients with previous failed cycles was assessed.

Methods A total of 379 patients undergoing FET at a reproductive medicine center were categorized into clinical pregnancy (CP), miscarriage (MISC), and non-pregnant (NP) groups. Vaginal specimens were collected for microecological evaluation prior to embryo transfer. Endometrial microbiota samples were obtained during embryo transfer for 16S rRNA gene sequencing analysis to assess endometrial microbiota composition. Vaginal microecological indicators, including pH, *Lactobacillus* dominance, and leukocyte esterase activity, were measured. Transvaginal *Lactobacillus* supplementation was investigated in 60 patients with previous failed cycles.

Results Vaginal microecology significantly correlated with pregnancy outcomes, with normal microecology associated with a higher clinical pregnancy rate. Vaginal pH and leukocyte esterase activity were significantly associated with clinical pregnancy. Furthermore, vaginal microecological differences significantly impacted endometrial microbiota composition. However, no significant differences were observed in endometrial microbiota composition among the CP, MISC, and NP groups. Notably, transvaginal *Lactobacillus* supplementation increased the clinical pregnancy rate without affecting the miscarriage rate.

Conclusion This study highlights that normal vaginal microecology, characterized by lower pH and leukocyte esterase negativity, is associated with a higher likelihood of clinical pregnancy following FET. Importantly, vaginal microecological differences influence endometrial microbiota composition. Moreover, transvaginal *Lactobacillus* supplementation appears promising in improving clinical pregnancy rates in patients with previous failed cycles. These findings contribute to a better understanding of the interplay between vaginal and endometrial microbiota and offer potential interventions to enhance reproductive success in assisted reproductive technologies.

Keywords Frozen embryo transfer · Vaginal microecology · Clinical pregnancy · Endometrial microbiota · Assisted reproductive technologies

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Introduction

Assisted reproductive technologies (ART) have significantly improved the management of infertility, offering hope to numerous couples worldwide. Among these technologies, frozen embryo transfer (FET) has emerged as a prominent approach due to its favorable outcomes, reduced risks, and increased success rates compared to fresh embryo transfer [1, 2]. Despite the progress in ART, the success of FET remains influenced by various factors, including the endometrial receptivity and microbiota, which play pivotal roles in the complex process of embryo implantation and early pregnancy establishment [3, 4].

In the context of reproductive health, the concept of vaginal microecology has gained increasing attention over the past decade. Traditional vaginal secretion testing methods have been limited by challenges such as precise timing of specimen collection, specimen quality, and the expertise of the examiner, resulting in relatively low accuracy and efficiency in diagnosing pathogenic infections and vaginitis. However, recent advancements in female vaginal microecological detection systems have demonstrated significant improvements, offering rapid, user-friendly, and comprehensive results, including clear morphological staining and functional assessments [5]. This enhanced approach enables a more accurate and comprehensive examination of pathogenic microorganisms, thereby providing valuable insights into the overall vaginal health.

In recent years, studies have indicated that vaginal microecological differences may exert substantial influence on endometrial microbiota, prompting further investigation into their potential impact on pregnancy outcomes in FET cycles. The establishment of a stable, *Lactobacillus*-dominated vaginal microbiota has been associated with a favorable reproductive environment, characterized by an acidic pH that inhibits harmful pathogens and supports embryonic development [6]. Conversely, vaginal dysbiosis, characterized by an imbalanced microbial composition, has been linked to adverse reproductive consequences, including infertility and an increased risk of preterm birth [7, 8].

In this study, we sought to investigate the correlation between vaginal microecological differences and pregnancy outcomes in FET cycles. Additionally, we aimed to examine the influence of vaginal microecological indicators on the composition of endometrial microbiota. Understanding these relationships could offer valuable insights into optimizing both vaginal and endometrial microecology for improved reproductive success in FET cycles, potentially enhancing the overall efficacy of ART procedures.

Materials and methods

Patient population

Patients who underwent FET in a hormone replacement therapy (HRT) cycle at the Reproductive Medicine Center of the Second Hospital of Chongqing Medical University between October 2020 and November 2021 were included in this study. For vaginal microecology analysis, a total of 379 patients met the inclusion and exclusion criteria. The inclusion criteria were as follows: non-smoking, aged between 20 and 40 years, having 2 good quality cleavage stage embryos, and having a normal uterus. The exclusion criteria were endometrial carcinoma, uterine adhesions, severe immune diseases, poorly controlled endocrine diseases, current reproductive tuberculosis (pelvic tuberculosis, endometrial tuberculosis), history of recurrent miscarriage and repeated embryo transfer failure, and reproductive tract infection with *Neisseria gonorrhoeae*, *Chlamydia*, *Syphilis spirochetes*, HPV, and HIV. No patients reported any symptoms in our study. Clinical pregnancy was defined as a intrauterine pregnancy up to 12 weeks of gestation. Miscarriage was defined as the loss of a pregnancy before the completion of 12 weeks of gestation.

For investigating the impact of transvaginal *Lactobacillus* supplementation, we recruited 60 patients with previous failed cycles randomly assigned to control and treatment groups. Inclusion and exclusion criteria mirrored those mentioned earlier, excluding the history of recurrent miscarriage and repeated embryo transfer failure.

Vaginal secretion collection and microecological evaluation

Vaginal specimens were collected on the day of embryo transfer before the procedure. A sterile dry swab was used to gently rotate on the upper 1/3 of the lateral wall of the vagina collecting vaginal secretions for vaginal microecological assessment. Gram staining was performed to evaluate bacterial density, species of bacteria, dominant bacteria, leukocytes, and epithelial cells under a light microscope. Vaginal cleanliness and Nugent scores were determined following established protocols [5, 9].

Endometrial preparation and microbiota sampling

Endometrial microbiota sampling was performed during a hormone replacement therapy-frozen embryo transfer (HRT-FET) cycle. Prior to use, the transfer catheter, dish, and EP tube from the same lot number were tested for DNA contamination. After appropriate priming with oral estradiol (Femoston/estradiol, 1 mg bid) for 10–14 days, a trilaminar

endometrium was achieved with a thickness ≥ 8 mm. When the appropriate hormonal status was confirmed, oral progesterone (Duphaston, 10 mg bid) was administered for 3 days. Before embryo transfer, the perineum was cleaned using cotton swabs soaked in iodophor solution while the patient was in the lithotomy position. A vaginal speculum was inserted, and vaginal secretions were removed using cotton swabs soaked in saline solution. Embryo transfer was performed with a double-lumen embryo transfer catheter (T-1731511, Pacific Contrast Scientific Instruments Co. Ltd., Jinan, Shandong, China) in an operating room with a ceiling air filtration system. To minimize the risk of cervicovaginal contamination, the outer sheath of the catheter was inserted into the endocervix without contacting the vaginal wall after removal of cervical mucus. Subsequently, the inner catheter containing the embryos at the top was inserted into the sheath and advanced into the uterine cavity. After the transfer, the inner catheter was re-sheathed, and both the sheath and catheter were withdrawn from the uterine cavity. The distal 1-cm portion of the inner catheter was then sterilely cut and placed in a DNA-free tube as described in a previous study [10]. The remaining transfer media served as a negative control.

16S rRNA gene amplification and sequencing

After cell lysis with Sodium Phosphate Buffer (Thermo Scientific, Waltham, MA, USA) and MT Buffer (Thermo Scientific), DNA extraction was performed using FastDNA® Spin Kit (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. To eliminate potential bacterial contamination during DNA processing and library preparation, one blank DNA extraction was processed as a background negative control for each batch of extractions. The V3-V4 region of the 16S rRNA gene was amplified via PCR using barcode-index primers 338F (5′-ACTCCTACG GGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGG TWTCTAAT-3′) using a TransStart FastPfu DNA polymerase (TransGen Biotech, Beijing, China) on a GeneAmp 9700 thermocycler (Applied Biosystems, Wakefield, RI, USA) as described previously [11]. The PCR amplification conditions were as follows: pre-denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 45 °C for 30 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min.

Purification of amplified DNA products was performed using AxyPrep DNA Gel Extraction Kit. DNA sequences were read using Illumina's MiSeq PE300. Quality control, sequence splicing, and noise reduction were carried out using the fastp, FLASH, and DADA2 plug-in in Qiime2, respectively. Species taxonomy analysis of ASVs (Amplicon Sequence Variants) was performed using the Naive Bayes classifier in Qiime2 based on the Silva 16S rRNA gene database (v 138).

Transvaginal *Lactobacillus* supplementation treatment

A live *Lactobacillus* capsule for vaginal use (Dingjunsheng, Inner Mongolia ShuangQi Pharmaceutical Co., Ltd.) was administered intravaginally for 30 consecutive days before the FET cycle initiation. The probiotic treatment with the live *Lactobacillus* capsule for women with gestational diabetes has been reported to improve maternal and infant health outcomes [12].

Statistical analysis

Statistical analysis was performed using R (version 4.0.0) and SPSS (version 24.0) software package (SPSS Inc., Chicago, IL, USA) to compare the differences between groups. Continuous variables were tested for normality, and parametric or non-parametric tests were used accordingly (Student's *t*-test for normal distribution and Mann–Whitney *U* test for non-normal distribution). Continuous variables with normal distribution were presented as mean ± SD, while non-normally distributed continuous variables were expressed as median (25th percentile, 75th percentile). Categorical data were expressed as frequencies or percentages and compared between groups using the chi-square test. Differences were considered statistically significant at $P < 0.05$.

Results

Patients' clinical characteristic

The study enrolled a total of 379 patients who underwent FET in a HRT cycle at the Reproductive Medicine Center of the Second Hospital of Chongqing Medical University. The patients' mean age was 31.5 ± 3.8 years. The participants were categorized into three groups: clinical pregnancy group (CP group) ($n = 194$), miscarriage group (MISC group) ($n = 39$), and non-pregnant group (NP group) ($n = 146$). Comparison among the three groups revealed no statistically significant differences in baseline characteristics and cycle-associated parameters ($P > 0.05$, Table 1). Multivariate logistic regression was applied to assess the impact of patient characteristics and cycle-associated parameters on pregnancy outcomes, revealing no significant effects ($P > 0.05$, Table 2).

Correlation of vaginal microecological differences with pregnancy outcomes

To investigate the association between vaginal microecology and pregnancy outcomes, the microecological evaluations were compared among the CP, MISC, and NP groups. The chi-square test revealed a statistically

Table 1 Patients' clinical characteristic and cycle-associated parameters for vaginal microecology analysis

Variables	CP group ($n=194$)	MISC group ($n=39$)	NP group ($n=146$)	P value
Age (years)	31 (29, 34)	32 (29, 33)	32 (28, 34)	0.92[a]
BMI (kg/m^2)	21.5 (20.0, 23.4)	21.8 (20.3, 23.3)	21.0 (19.2, 22.6)	0.06[a]
Duration of infertility (years)	3 (2, 5)	3 (1, 4)	2 (2, 4)	0.44[a]
Infertility cause				0.77[b]
Female factor	143 (73.7%)	33 (84.6%)	111 (76.0%)	
Male factor	21 (10.8%)	4 (10.3%)	16 (11.0%)	
Both factor	21 (10.8%)	1 (2.6%)	13 (8.9%)	
Unexplained factor	9 (4.6%)	1 (2.6%)	6 (4.1%)	
Previous conception	0 (0, 0)	0 (0, 0)	0 (0, 0)	0.51[a]
Embryo transfer attempts	0 (0, 0.2)	0 (0, 1)	0 (0, 1)	0.75[a]
AMH (mIU/ml)	2.7 (1.8, 4.5)	2.9 (1.7, 5.0)	3.0 (1.8, 4.9)	0.86[a]
Basal FSH (mIU/ml)	6.7 (5.6, 8.3)	7.5 (6.0, 8.5)	6.7 (5.7, 8.1)	0.65[a]
Basal LH (mIU/ml)	4.4 (3.2, 6.0)	4.2 (3.4, 7.3)	4.5 (3.2, 5.8)	0.91[a]
Basal E2 (pg/ml)	43.3 (29.7, 61.2)	42.3 (27.1, 56.6)	42.1 (30.3, 60.6)	0.96[a]
Basal P (ng/ml)	0.2 (0.1, 0.4)	0.3 (0.2, 0.4)	0.3 (0.2, 0.4)	0.08[a]
Basal PRL (μg/l)	15.7 (12.0, 20.8)	16.8 (12.0, 29.2)	14.9 (10.7, 20.8)	0.15[a]
Retrieved oocytes	10 (6, 14)	11 (6, 16)	10 (7, 15)	0.70[a]
D3 embryos	6.2 ± 3.7	5.9 ± 4.4	6.2 ± 3.5	0.87[c]
D3 high-quality embryos	3.8 ± 2.6	3.7 ± 2.9	3.8 ± 2.7	0.96[c]
Blastocyst rate (%)	0.8 (0.6, 1.0)	0.7 (0.6, 0.8)	0.7 (0.6, 0.9)	0.11[a]

FSH follicle-stimulating hormone, *LH* luteinizing hormone, *E2* estrogen, *PRL* prolactin, *AMH* anti-Mullerian hormone

[a]Mann-Whitney *U* test. The data were presented as the median (25th percentile, 75th percentile)

[b]Chi-square test. The data were presented as the rate (%)

Table 2 Multivariate logistic regression of patient characteristics and cycle-associated parameters for pregnancy outcomes

Variables	OR	95% CI	P value
Age (years)	0.98	0.92–1.04	0.51
BMI (kg/m^2)	1.17	1.00–1.35	0.06
AMH	1.00	0.92–1.08	0.96
Retrieved oocytes	1.00	0.95–1.06	0.82
D3 embryos	1.03	0.93–1.16	0.49
D3 high-quality embryos	0.98	0.86–1.12	0.83
Blastocyst rate (%)	2.71	0.85–8.56	0.08

significant correlation between vaginal microecology and pregnancy outcomes ($\chi^2=17.344$, $P<0.001$, Table 3). Among the participants with normal vaginal microecology, 64.3% belonged to the CP group, 9.8% to the MISC group, and 25.9% to the NP group. In contrast, individuals with dysbiosis exhibited different proportions, with 43.2% in the CP group, 10.6% in the MISC group, and 46.2% in the NP group. The scoring values of vaginal microecological indicators were further analyzed (Table 4). Specifically, the vaginal pH was significantly lower in the CP group (53.6%) than in the MISC group (48.7%) and the NP group (33.6%) ($P<0.001$). The leukocyte esterase negative rate was significantly better in the CP group (92.3%) and MISC group (94.9%) compared to the NP group (84.2%) ($P=0.03$). Nevertheless, no significant differences were observed between the groups in terms of cleanliness degree, presence of pathogens, bacterial density, bacterial diversity, *Lactobacillus*-dominant microbiota, Nugent scores, *Lactobacillus* classification, catalase, and sialidase ($P>0.05$).

Table 3 Correlation between vaginal microecology and pregnancy outcomes

Vaginal microecology	Total	CP group ($n=194$)	MISC group ($n=39$)	NP group ($n=146$)	χ^2	P value
Normal	143	92 (64.3%)	14 (9.8%)	37 (25.9%)	17.344	< 0.001
Dysbiosis	236	102 (43.2%)	25 (10.6%)	109 (46.2%)		

Table 4 Comparison of vaginal microecology in the CP, MISC, and NP groups

Variables	CP group ($n=194$)	MISC group ($n=39$)	NP group ($n=146$)	χ^2	P value
Cleanliness				3.043	0.218
Degree I–II	189 (97.4%)	38 (97.4%)	137 (93.8%)		
Degree III	5 (2.6%)	1 (2.6%)	9 (6.2%)		
Vaginal pH				13.710	0.001
< 4.6	104 (53.6%)	19 (48.7%)	49 (33.6%)		
> 4.6	90 (46.4%)	20 (51.3%)	97 (66.4%)		
Pathogens				3.208	0.201
No	183 (94.3%)	39 (100%)	135 (92.5%)		
Yes	11 (5.7%)	0	11 (7.5%)		
Bacterial density[a]				0.956	0.620
Grade II–III	181 (93.3%)	36 (92.3%)	132 (90.4%)		
Grade I or IV	13 (6.7%)	3 (7.7%)	14 (9.6%)		
Bacterial diversity[b]				0.245	0.885
Grade II–III	178 (91.8%)	36 (92.3%)	132 (90.4%)		
Grade I or IV	16 (8.2%)	3 (7.7%)	14 (9.6%)		
Lactobacillus-dominant microbiota				4.305	0.116
Lactobacillus	117 (60.3%)	19 (48.7%)	73 (50.0%)		
Other flora	77 (39.7%)	20 (51.3%)	73 (50.0%)		
Nugent scores				5.574	0.233
(1–3)	179 (92.3%)	34 (87.2%)	127 (87.0%)		
(4–6)	14 (7.2%)	5 (12.8%)	15 (10.3%)		
(> 6)	1 (0.5%)	0	4 (2.7%)		
Lactobacillus classification[c]				3.890	0.143
Grade I–IIa	115 (59.3%)	19 (48.7%)	72 (49.3%)		
Grade IIb-III	79 (40.7%)	20 (51.3%)	74 (50.7%)		
Catalase				3.941	0.139
Negative	118 (60.8%)	19 (48.7%)	75 (51.4%)		
Positive	76 (39.2%)	20 (51.3%)	71 (48.6%)		
Leukocyte esterase				7.034	0.030
Negative	179 (92.3)	37 (94.9%)	123 (84.2%)		
Positive	15 (7.7%)	2 (5.1%)	23 (15.8%)		
Sialidase				1.725	0.442
Negative	190 (97.9%)	39 (100%)	141 (96.6%)		
Positive	4 (2.1%)	0	5 (3.4%)		

[a]The grading of the bacterial density was expressed as follows: 1–9/average number of bacteria per field of view observed at 10 × 100 × was graded I, 10–99/PFV was graded II, > 100/PFV was graded III, bacteria gathering in clusters was graded IV

[b]The grading of the bacterial diversity was expressed as follows: 1–3 species was graded I, 4–6 species was graded II, 7–9 species was graded III, > 10 species was graded IV

[c]The grading of *Lactobacillus* classification expressed as follows: Grade I was defined as vaginal discharge dominated by *Lactobacillus* and no other flora, Grade IIa was defined as vaginal discharge with mixed flora, but mainly *Lactobacillus*, Grade IIb was defined as mixed flora in vaginal secretions, but predominantly abnormal flora, with a marked decrease in the proportion of *Lactobacillus*, and Grade III was defined as severe reduction or absence of *Lactobacillus* and overgrowth of other types of flora

Vaginal microecological indicators affect endometrial microbiota composition

In contrast to the previous work [3], our endometrial samples contained a low proportion of *Lactobacillus* (2.7%) (Fig. 1A). The top three predominant microbiota in the endometrial samples were *Rhodococcus* (23.7%), *Pseudomonas* (4.9%), and *Achromobacter* (4.1%). No statistically significant differences were found among the three groups in terms of endometrial microbiota composition (Fig. 1B, C) ($P=0.09$). However, the abundance of *Achromobacter* was positively associated with clinical pregnancy, while the abundance of *Romboutsia*, *Psychrobacter*, *Roseiflexaceae*, and *Chryseobacterium* displayed

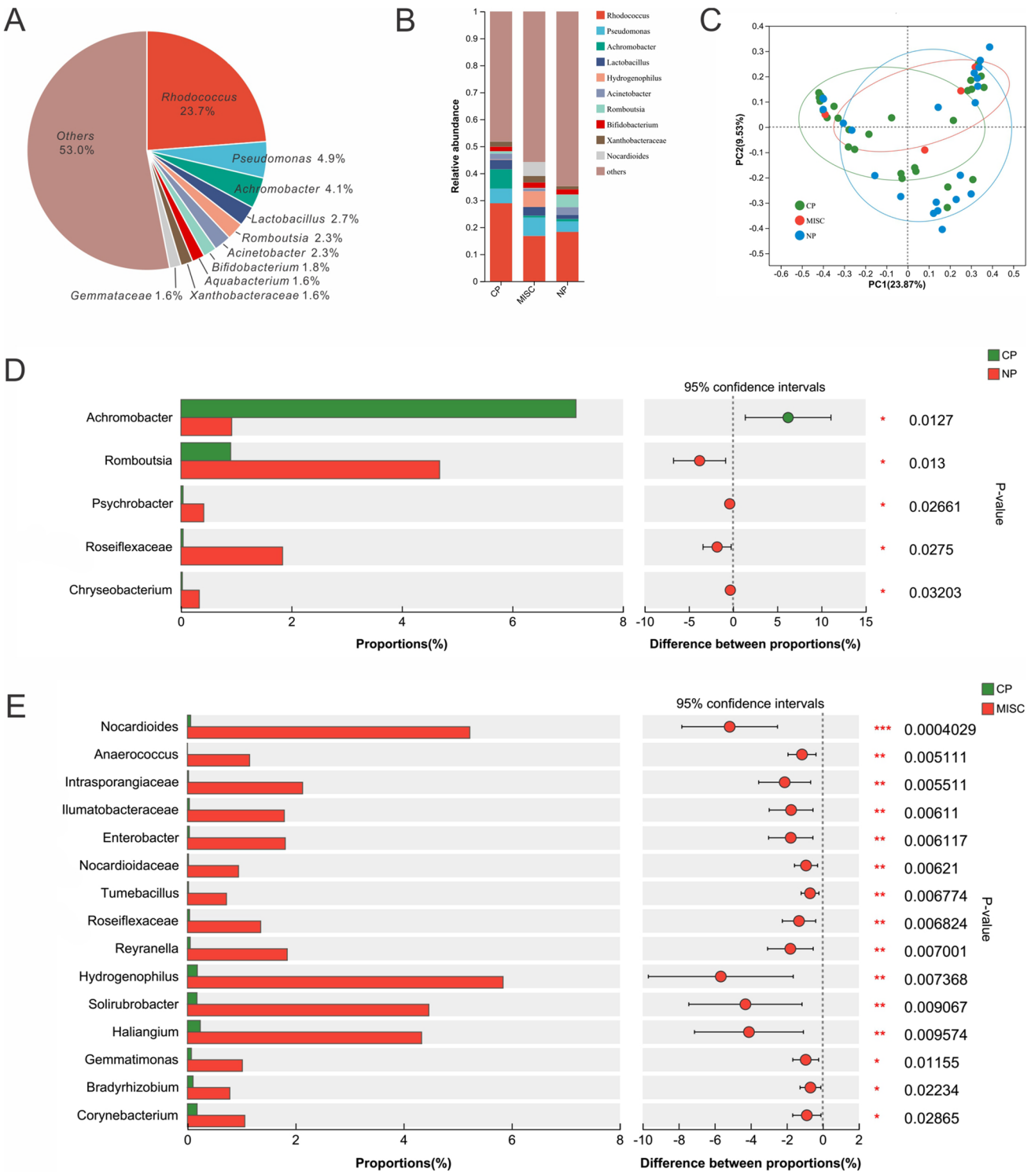

Fig. 1 Endometrial microbiota composition among clinical pregnancy (CP), miscarriage (MISC), and non-pregnant (NP) groups. **A** Pie chart for the microbial genera showing mean values of 10 most abundant genera in all endometrial samples. **B** Bar charts showing mean values of 10 most abundant genera. **C** Principal coordinate analysis (PCoA) plots indicating microbial similarity ($P = 0.09$) based on Bray–Curtis distances at the genus level. **D** Bar plots indicating the differences at the genus level between CP and NP groups using Student's *t*-test ($P < 0.05$). **E** Bar plots indicating the differences at the genus level between CP and MISC groups using Student's *t* test ($P < 0.05$)

negative correlations with clinical pregnancy (Fig. 1D). Fifteen microbiota including *Nocardioides*, *Enterobacter*, *Roseiflexaceae*, and *Corynebacterium* were associated with miscarriage (Fig. 1E).

Variance inflation factor analysis identified pH, LDM, *Lactobacillus* classification, catalase, and leukocyte esterase as the primary factors influencing endometrial microbiota composition (Table 5). Subsequently, using Bray–Curtis based dbRDA, we showed that microbial variation in endometrial samples was significantly explained by LDM ($P=0.01$), *Lactobacillus* classification ($P=0.03$), catalase ($P=0.008$), and leukocyte esterase ($P=0.001$) (Fig. 2A). Specifically, pH was correlated with the relative abundance of *Lactobacillus* and *Bifidobacterium* in the endometrial microbiota; *Lactobacillus* classification was related to the relative abundance of *Rhizobiaceae*, while leukocyte esterase was related to the relative abundance of *Romboutsia* and *Roseiflexaceae* (Fig. 2B).

Table 5 Vaginal environmental factors after VIF screening

Variables	Vaginal pH	LDM*	*Lactobacillus* classification	Catalase	Leukocyte esterase
VIF value	1.62	2.41	3.61	1.80	1.13

*LDM *Lactobacillus*-dominated microbiota

Transvaginal Lactobacillus supplementation improves pregnancy outcomes in patients with previous failed cycles

To investigate the impact of transvaginal *Lactobacillus* supplementation on reproductive outcomes, 60 patients with previous failed cycles were recruited, including 30 control patients and 30 patients with transvaginal *Lactobacillus* supplementation. The clinical characteristics of the participants are presented in Table 6. Transvaginal *Lactobacillus* supplementation significantly increased the clinical pregnancy rate, while the miscarriage rate showed no difference between the two groups (Table 7).

Discussion

In this prospective study, we aimed to explore the correlation between vaginal microecological differences and pregnancy outcomes in patients undergoing FET in an HRT cycle. Our results revealed that patients with normal vaginal microecology had a significantly higher frequency of clinical pregnancies compared to those with dysbiosis. The importance of a balanced and healthy vaginal microenvironment in supporting successful pregnancy outcomes has been recognized in previous studies [11, 13, 14]. Our findings support the notion that maintaining a stable and favorable vaginal microecology may be crucial for implantation and successful embryo development.

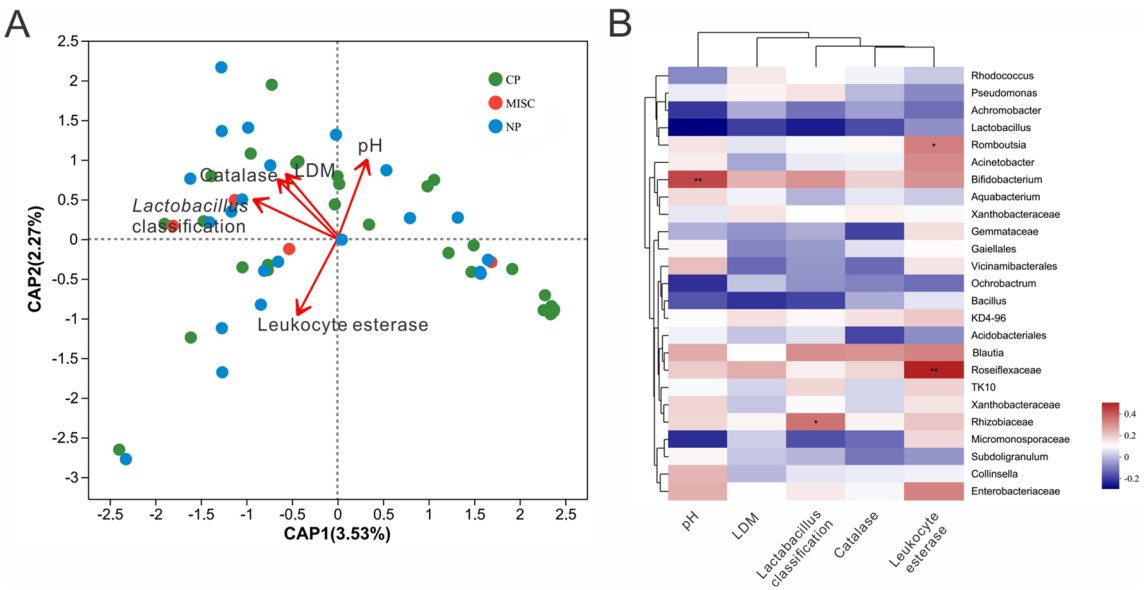

Fig. 2 Correlation between microbial composition and vaginal microecological indicators. **A** Distance-based redundancy analysis (db-RDA) triplot showing the association between microbiota variation and vaginal microecological indicators. **B** Heatmap of correlation between screened vaginal factors and endometrial microbiota. $^{*}P<0.05$, $^{**}P<0.01$

Table 6 Clinical characteristics of participants in *Lactobacillus* supplementation study

Variables	Control group ($n=30$)	Treatment group ($n=30$)	*P* value
Age (years)	30.8 ± 2.3	30.8 ± 1.9	1.00[a]
BMI (kg/m^2)	20.7 ± 1.7	20.9 ± 2.4	0.89[a]
Duration of infertility (years)	3.0 ± 1.2	3.4 ± 0.5	0.53[a]
Previous conception	2 (2, 3)	2 (2, 3)	1.00[b]
AMH (mIU/ml)	2.9 ± 1.9	3.0 ± 0.9	0.93[a]
Basal FSH (mIU/ml)	5.9 ± 1.8	6.2 ± 1.0	0.76[a]
Basal LH (mIU/ml)	5.6 ± 0.6	4.5 ± 1.6	0.24[a]
Basal E2 (pg/ml)	41.3 ± 17.8	36.7 ± 17.4	0.69[a]
Basal P (ng/ml)	0.2 ± 0.1	0.2 ± 0.9	0.38[a]
Basal PRL (μg/l)	18.2 ± 11.0	18.0 ± 9.0	0.97[a]

FSH follicle-stimulating hormone, *LH* luteinizing hormone, *E2* estrogen, *PRL* prolactin, *AMH* anti-Mullerian hormone

[a]Student's *t*-test. The data were presented as the mean ± SD

[b]Mann-Whitney *U* test. The data were presented as the median (25th percentile, 75th percentile)

Table 7 Effects of transvaginal *Lactobacillus* supplementation on pregnancy outcomes

Pregnancy outcomes	Control group ($n=30$)	Treatment group ($n=30$)	*P* value
Clinical pregnancy rate	36.7% (11/30)	66.7% (20/30)	0.04
Miscarriage rate	10.0% (3/30)	13.3% (4/30)	0.63

Interestingly, we observed that certain specific indicators of vaginal microecology were associated with clinical pregnancy. Notably, a lower vaginal pH was significantly associated with a higher rate of clinical pregnancies. A lower vaginal pH is indicative of a more acidic environment, which is considered beneficial for promoting the growth of *Lactobacillus* species. *Lactobacilli*, known for producing lactic acid, hydrogen peroxide, and bacteriocins, contribute to a healthy vaginal microenvironment by preventing the overgrowth of pathogenic microorganisms [15]. Our study found a higher proportion of *Lactobacillus*-dominant microbiota (LDM) in the clinical pregnancy group (60.3%) compared to the miscarriage (48.7%) and non-pregnant (50.0%) groups. Although a higher abundance of *Lactobacillus* has been associated with improved pregnancy outcomes [6], our results indicate a lack of a significant association between *Lactobacillus* dominance and pregnancy rates, raising questions about the role of endometrial biodiversity in fertility. Therefore, a comprehensive evaluation of fertility outcomes should consider the intricate interplay between vaginal microecology and endometrial microbiota, emphasizing the role of factors beyond *Lactobacillus* dominance in successful conception.

The genus *Lactobacillus* is dominated in endometrial microbiota in the majority of the uterine microbiome studies [3, 16–19]. A recent study also showed that women with a live birth within 12 months after a first failed IVF/ICSI cycle exhibited significantly higher *Lactobacillus crispatus* relative abundance compared to those without a live birth [20]. However, contrasting results in some studies revealed a low relative abundance of *Lactobacillus* in endometrial samples [21–23]. Our study also observed a low proportion of *Lactobacillus* in endometrial samples. Population-specific variations in microbiota composition, influenced by factors such as diet, geography, pollution, and genetics, could explain these differences [24]. As our study focused on IVF patients in Chongqing municipality, China, it is plausible that the method of microbiota sampling, regional and genetic variations may explain the distinct endometrial microbiota composition observed in our study. Therefore, it is essential to consider these factors when interpreting and generalizing our findings to other populations. While no significant differences were observed in the endometrial microbiota composition among the CP, MISC, and NP groups, further analysis revealed intriguing associations with specific bacterial abundances. Specifically, the abundance of *Achromobacter* displayed a positive correlation with clinical pregnancy, while the abundance of *Romboutsia*, *Psychrobacter*, *Roseiflexaceae*, and *Chryseobacterium* showed negative correlations. Several pathogenic microbiota including *Nocardioides*, *Enterobacter*, *Roseiflexaceae*, and *Corynebacterium* were associated with miscarriage. These findings provide insights into the complex interactions between vaginal and endometrial microecology, suggesting potential associations between specific bacterial taxa and reproductive outcomes.

In line with previous studies [15, 25], our variance inflation factor analysis highlighted the importance of pH, LDM, *Lactobacillus* classification, catalase, and leukocyte esterase as key factors influencing endometrial microbiota composition. Moreover, using Bray–Curtis based dbRDA, we demonstrated that microbial variation in endometrial fluids was significantly explained by LDM, *Lactobacillus* classification, catalase, and leukocyte esterase. We also revealed that the vaginal microecological indicators were associated with several specific endometrial microbiota. These results emphasize the intricate interplay between specific components of the vaginal and endometrial microecology, influencing overall reproductive success.

Interventions designed to enhance the vaginal microenvironment, such as *Lactobacillus* supplementation, have the potential to exert a positive influence on reproductive outcomes. Nevertheless, the impact of *Lactobacillus* supplementation on pregnancy outcomes is not consistent across studies [26]. Despite this, recent evidence suggests that introducing *Lactobacillus* into the vaginal milieu yields protective effects on the endometrium [27]. In our study, transvaginal *Lactobacillus* supplementation significantly increased the clinical pregnancy rate in patients with previous failed cycles, which aligns with some previous research [28]. The increase in the clinical pregnancy rate underscores the potential benefits of optimizing the vaginal microecology to create a more receptive endometrium for successful implantation and embryo development. Our study adds valuable insights into the practical application of interventions targeting the vaginal microbiota, offering a potential avenue for improving outcomes in patients with previous failed cycles.

Despite the valuable insights provided by our prospective study, certain limitations should be acknowledged. Firstly, the study's specific patient population in Chongqing municipality, China, may limit the generalizability of the results to other regions or populations. Multicenter studies involving diverse patient cohorts and larger sample sizes are warranted to validate and extend our findings to a broader context. Secondly, the study focused on patients undergoing FET with HRT, potentially limiting the applicability of the findings to other infertility treatment modalities or natural conception. Lastly, while we collected comprehensive data on vaginal and endometrial microbiota, other factors such as lifestyle, diet, and hormonal profiles were not extensively explored. The influence of these variables on microbiota composition and pregnancy outcomes warrants further investigation.

Conclusion

In conclusion, our study not only highlights the association between normal vaginal microecology and a higher likelihood of clinical pregnancy following FET, but also introduces the positive impact of transvaginal *Lactobacillus* supplementation on reproductive outcomes in patients with previous failed cycles. Notably, normal vaginal microecology, characterized by lower pH and leukocyte esterase negativity, emerges as a key predictor of a higher likelihood of clinical pregnancy following FET. Moreover, our study demonstrated that vaginal microecological differences can influence the composition of the endometrial microbiota during FET cycles. These findings shed light on the intricate interactions between the vaginal and endometrial microenvironments and their potential impact on successful pregnancy outcomes. Further research is needed to explore the underlying mechanisms and clinical implications of these microbial interactions in the context of assisted reproductive technologies.

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Author contribution QW, LH, and SZ conceived and designed the study. HC, HZ, HZ, and SL recruited the patients. QW, HC, HZ, HZ, SL, JZ, and SZ performed the experiments. QW and SZ analyzed the data and created the figures. QW and SZ drafted and reviewed the manuscript. All authors read and approved the manuscript and all data were generated in-house and that no paper mill was used.

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Data availability The data and materials that support the findings of this study are available from the corresponding author on reasonable request.

Declarations

Ethical approval Ethical approval for the study was obtained from the Ethics Committees of the Second Affiliated Hospital of Chongqing Medical University (2020–29).

Consent to participate Written informed consent was obtained from all participants.

Consent for publication Written informed consent for publication of their clinical details was obtained from the patients. The copies of the consent forms are available for review by the editor of this journal.

Competing interests The authors declare no competing interests.

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