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Original Article

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Comparison of Pregnancy Outcomes and Vaginal Microbiota in Endometriosis Patients Undergoing Frozen Embryo Transfer Using Letrozole Combined HMG Versus Hormone Replacement Therapy with GnRH-a Pretreatment

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Abstract

This study investigated differences in reproductive outcomes and vaginal microbiota profiles between two endometrial preparation protocols—letrozole (LE) combined with human menopausal gonadotropin (HMG) and hormone replacement therapy (HRT) with GnRH-a pretreatment —in women with endometriosis (EMs) undergoing frozen embryo transfer (FET). Following 1∶1 propensity score matching, a total of 770 FET cycles were analyzed. No statistically significant differences were observed in live birth rates or clinical pregnancy rates between the two groups. However, the LE + HMG group showed a lower miscarriage trend (13.7% vs. 19.8%, P = 0.070) and significantly fewer cesarean deliveries (64.9% vs. 75.4%, P = 0.020) and hypertensive disorders of pregnancy (4.8% vs. 10.1%, P = 0.039). Recent evidence suggests that GnRH-a treatment may disrupt reproductive tract microbiota. Given ethical constraints on endometrial sampling during FET, vaginal microbiota was used as a surrogate to explore microbial differences between protocols. In the prospective arm, vaginal samples from 55 women in the LE + HMG group and 50 in the GnRH-a HRT group were analyzed using 16S rRNA sequencing and droplet digital PCR. While no significant differences were observed in *Lactobacillus* or *Gardnerella* abundance, the GnRH-a HRT group exhibited enrichment of potential pathogens, such as *Escherichia-Shigella* and *Staphylococcus*. In conclusion, although both protocols achieved comparable live birth outcomes, the LE + HMG regimen was associated with fewer obstetric complications and a more favorable vaginal microbiota profile compared to GnRH-a HRT.

Keywords: Endometriosis, Letrozole, Gonadotropin-releasing hormone agonist, Frozen embryo transfer, Vaginal Microbiota

Introduction

Endometriosis (EMs) is a common, chronic, estrogen-dependent and inflammatory disease

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characterized by the presence of endometrium-like tissue outside the uterus. It is estimated to impact 10-15% of reproductive women[1], with approximately one-third experiencing infertility[2]. Many of these women require in vitro fertilization (IVF) to achieve pregnancy[3]. In recent years, the global adoption of frozen-thawed embryo transfer (FET) has expanded swiftly, largely due to advancements in vitrification techniques and blastocyst culture.

A key determinant of FET success is the adequacy of endometrial preparation. As an estrogen-dependent condition, EMs is characterized by abnormal aromatase overexpression in both ectopic lesions and the eutopic endometrium, leading to a disrupted estrogenic microenvironment[4]. This imbalance may interfere with embryo-endometrium communication, affecting pregnancy outcomes[5]. This unique pathological feature requires tailored endometrial preparation strategies for EMs patients. However, the European Society of Human Reproduction and Embryology (ESHRE) guidelines offer no specific recommendations for endometrial preparation in EMs patients[3], making this an important area for investigation.

GnRH-a (gonadotropin-releasing hormone agonists) has been shown to suppress local inflammation and reduce oxidative stress, thereby improving endometrial receptivity in EMs patients[6]. Consequently, the hormone replacement therapy (HRT) with GnRH-a pretreatment protocol has become a common choice for FET in EMs patients. However, the latest ESHRE guidelines no longer recommend the use of GnRH-a prior to assisted reproductive technologies (ART) in EMs patients[3]. Emerging evidence suggests that GnRH-a HRT does not offer significant advantages in improving fertility outcomes when compared to HRT alone or natural cycle (NC) protocols[7-8]. It is well known that both HRT and GnRH-a HRT protocols involve excessive supplementation of estradiol and progesterone, which might raise the risk of thromboembolic events[9]. Moreover, lack of corpus luteum (CL) formation in these cycles has been associated with a higher risk of adverse maternal and perinatal outcomes [10]. Although NC protocols are considered safer and more physiological, they lack flexibility in scheduling, requiring frequent ovulation monitoring and facing higher cycle cancellation rates. These limitations underscore the need to explore alternative endometrial preparation strategies that are both effective and patient-friendly for women with EMs undergoing FET.

Letrozole (LE), classified as a third-generation aromatase inhibitor, functions by suppressing estrogen synthesis and facilitating follicular development through negative feedback on the hypothalamic-pituitary axis[11-12]. Importantly, LE-induced ovulation results in the formation of a healthy CL, which reduces the risk of hypertensive disorders of pregnancy (HDP)[13]. Up to now, LE ovarian induction has been increasingly used for endometrial preparation in FET, especially for women with polycystic ovary syndrome[14] and anovulation[15]. Beyond its reproductive applications, LE has also demonstrated efficacy in alleviating EMs-related pain and reducing disease recurrence in both premenopausal and postmenopausal populations[16-17]. Emerging evidence suggests that LE may benefit the endometrium of women with EMs by suppressing the estrogen-inflammatory axis[18], and enhancing integrin αvβ3 expression, which could improve endometrial receptivity and implantation rates[19-20]. Despite its potential advantages, few studies have evaluated the efficiency of LE-based ovarian induction in FET cycles for women with EMs.

Recent microbiota research has revealed that treatment with GnRH-a for 3 to 6 months in EMs patients for gynecological symptom management may led to a marked decline in *Lactobacillaceae* and increased levels of *Streptococcaceae*, *Staphylococcaceae*, and *Enterobacteriaceae* in endometrial samples, suggesting a potential association between GnRH-a use and subclinical intrauterine infections[21]. Despite these findings, the impact of various endometrial preparation regimens on the reproductive tract microbiota during FET cycles remains poorly understood. Importantly, direct sampling of the endometrial microbiota on the day of embryo transfer is not feasible due to both ethical and clinical constraints —performing an endometrial biopsy at this critical time may damage the endometrium, reduce the likelihood of implantation, and raise ethical concerns regarding unnecessary harm. Interestingly, previous studies have demonstrated a continuous gradient in microbial composition extending from the vagina to the pelvic cavity[22]. Moreover, animal experiments have shown that transplantation of vaginal microbiota from patients with chronic endometritis into rats activates the endometrial TLR4/NF-κB pathway[23], indicating that disturbances in the vaginal microbiota may reflect or induce similar changes in the upper reproductive tract. Therefore, in the absence of direct endometrial sampling, analyzing vaginal microbiota provides a representative and ethically acceptable surrogate for assessing microbial impacts during FET.

Accordingly, this study was designed to evaluate and compare pregnancy and perinatal outcomes, as well as vaginal microbiota characteristics, in women with EMs undergoing FET using either LE + HMG or GnRH-a HRT protocols. By integrating clinical efficacy and microbial profiling, our findings aim to provide evidence-based insights for optimizing endometrial preparation strategies in this unique patient population.

Materials and methods

Study design and sample collection

This retrospective cohort study included women with EMs who underwent either LE + HMG or GnRH-a HRT FET cycles between January 2016 and December 2023. Inclusion criteria were as follows: (i) The first three FET cycles per patient; (ii) Female age under 43 years; (iii) A single blastocyst transfer per cycle. Exclusion criteria included: (i) History of recurrent spontaneous abortion; (ii) Congenital uterine malformations; (iii) Use of preimplantation genetic testing; (iv) Loss to follow-up or incomplete data; (v) Multiple pregnancies.

In addition, a prospective study component involved vaginal sample collection from EMs patients undergoing FET with either the LE + HMG (n = 55) or GnRH-a HRT (n = 50) protocol between January and June 2024. The inclusion criteria for the microbiota cohort included: (i) diagnosis of endometriosis; (ii) female age under 43 years. However, embryo quality was not restricted, as the primary aim of this component was to investigate how two endometrial preparation protocols affect the composition of the vaginal microbiota, without addressing potential associations between microbiota alterations and pregnancy outcomes at this stage. The exclusion criteria were consistent with the retrospective cohort but included additional microbiota-specific considerations: patients were excluded if they had used antibiotics or vaginal probiotics within one month prior to sampling, or had acute reproductive tract infections, diabetes mellitus, or autoimmune diseases.

Vaginal secretions were collected prior to embryo transfer on the day of the procedure, before any surgical manipulation. A sterile speculum was used to expose the cervix, and two sterile cotton swabs were used to obtain secretions from the upper third of the vaginal wall. One swab was used for 16S rRNA gene sequencing and the other for droplet digital PCR (ddPCR) analysis. Importantly, this microbiota cohort was independent of the retrospective cohort. In total, 210 vaginal samples (two per patient) were collected. The study was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University (No. 2023-SR-325).

Diagnosis of EMs and adenomyosis

EMs was diagnosed either through surgical methods (laparoscopy or laparotomy) or based on transvaginal ultrasound findings consistent with ovarian endometriotic cysts. The diagnosis of endometriotic cysts via ultrasound had to be documented in at least two separate menstrual cycles. Adenomyosis was diagnosed based on imaging criteria using transvaginal ultrasound, with assessments conducted by at least two highly skilled radiologists. The diagnosis was established when patients presented with clinical symptoms such as hypermenorrhea or dysmenorrhea and exhibited at least two of the following ultrasound features, as defined by the Morphological Uterus Sonographic Assessment (MUSA) criteria [24].

Endometrial preparation

In the LE + HMG group, LE (Hengrui, Lianyungang, Jiangsu, China) was administered orally at a daily dose of 2.5 mg starting on the 4th day of the menstrual cycle for 5 consecutive days. Additionally, 75 IU of human menopausal Gonadotropin (Lizhu, Zhuhai, Guangdong, China) were given every other day. Follicle monitoring began on the 12th day of the menstrual cycle and continued until the follicle diameter exceeded 18 mm. Subsequently, 5,000-10,000 IU of urinary human chorionic gonadotropin (Lizhu, Zhuhai, Guangdong, China) was injected. Following triggering, oral dydrogesterone (Duphaston; Abbott Laboratories, Chicago, IL, United States) was prescribed at a dose of 10 mg twice daily for luteal phase support. On the 6th day after trigger, blastocyst transfer was performed, and Duphaston was continued until the 10th week of pregnancy (***Fig.1A***).

In the GnRH-a HRT group, long-acting GnRH-a (Diphereline, 3.75 mg; Ipsen Pharma Biotech, Signes, France) was administered by intramuscular injection on menstrual cycle days 1-2. After 30 days, estradiol valerate (Progynova; Bayer, Leverkusen, North Rhine-Westphalia, Germany) was prescribed orally at a daily dose of 4-6 mg to stimulate endometrial proliferation until the endometrial thickness (EM) reached ⩾8 mm. Luteal support consisted of oral dydrogesterone (10 mg twice daily) combined with vaginal progesterone gel (Crinone, 90 mg once daily; Merck Serono, Darmstadt, Hesse, Germany). Blastocyst transfer was scheduled five days after

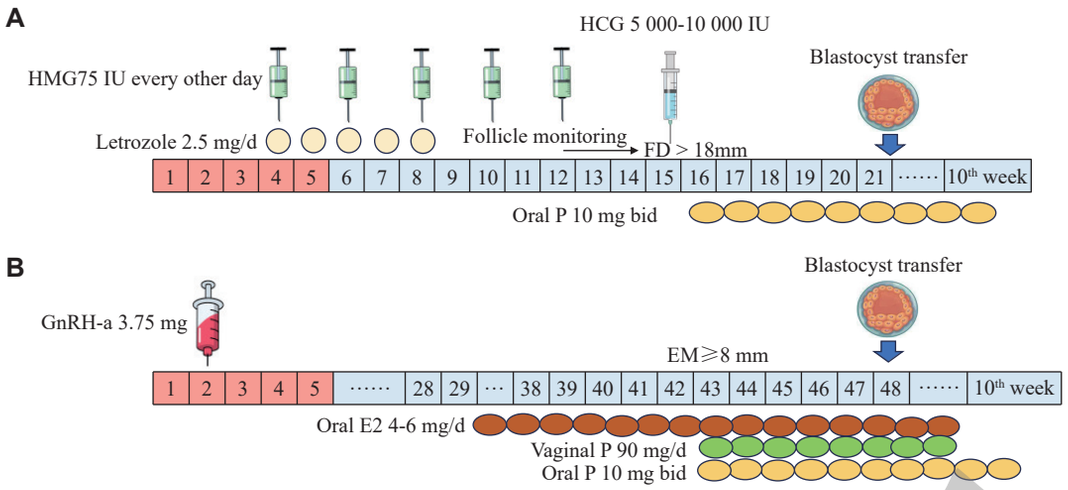

Fig. 1 **Schematic representation of protocols.** A: Letrozole + HMG protocol. B: GnRH-a HRT protocol. Abbreviations: FD, follicle diameter; EM, endometrial thickness; E2, estradiol; P, progesterone.

initiating progesterone support. Upon confirmation of clinical pregnancy, estradiol and vaginal progesterone were tapered off by gestational weeks 7-8, while oral dydrogesterone was maintained until 10 weeks (***Fig.1B***).

Blastocyst morphological evaluation

Embryos were cultured to the blastocyst stage, typically achieved on day 5 or day 6 post-fertilization. Morphological assessment was performed according to the Gardner and Schoolcraft classification system. Embryos with a grade of 3BC or higher were deemed suitable for cryopreservation. Prior to frozen embryo transfer, thawed blastocysts were re-evaluated for structural integrity and developmental quality. High-quality blastocysts were defined as those graded AA, AB, BA, or BB with sufficient expansion, while those classified as AC, CA, BC, CB, or CC were considered of lower quality, despite meeting the minimum expansion criterion of grade 3.

Data collection and outcome measures

Clinical characteristics for this study were obtained from the institution's electronic database. Maternal and fetal outcome data were collected through telephone interviews with parents one to three months after the expected delivery date and recorded in the electronic medical records by trained nurses. The primary outcome was the live birth rate. Secondary outcomes included EM on the transfer day, biochemical pregnancy rate, clinical pregnancy rate, miscarriage rate and perinatal outcomes.

16S rRNA sequencing

Microbial genomic DNA was isolated from vaginal samples using the FastPure Stool DNA Isolation Kit (Vazyme, China). DNA concentration was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and purity was confirmed by an A260/A280 ratio exceeding 1.8. PCR was conducted using primer pair 338F (ACTCCT ACGGGAGGCAGCA) and 806R (GGACTACH VGGGTWTCTAAT). The amplification protocol consisted of an initial denaturation at 95 ℃ for 3 minutes, followed by 27 cycles of denaturation at 95 ℃ for 30 seconds, annealing at 55 ℃ for 30 seconds, and extension at 72 ℃ for 30 seconds, with a final elongation step at 72 ℃ for 10 minutes. PCR reactions were carried out using a T100 Thermal Cycler (Bio-Rad, USA). Sequencing was conducted on the Illumina NextSeq 2000 PE300 platform. Raw data were processed using fastp 0.19.6 software to remove low-quality sequences (length < 50 bp). Sequences were then merged using FLASH 1.2.11 software. Chimeric sequences were filtered out. Alpha and beta diversity, along with LEfSe (linear discriminant analysis effect size), were analyzed using QIIME 2.

Droplet digital PCR quantification

Microbial genomic DNA was extracted using the MagicPure® 32 Microbiome DNA Isolation Kit (Fullgene Biotech, China). Lactobacillus species and *Gardnerella vaginalis* were detected based on 16S rRNA gene sequences. Target sequences for these species were downloaded from the NCBI database, and primers and probes were designed and validated for specificity using the NCBI BLAST tool. The primers and probes used for *Lactobacillus* were: forward primer 338F (AGAGGAGAGTGGAACT CCA), reverse primer 806R (CTCCCAACACTTAGC

ACT), and probe 5'-FAM-CTGAGGCTCGAAAG CATGGGTAG-BHQ1-3'; for *Gardnerella vaginalis*: forward primer F (GGTGAGTAATGCGTGACCAA), reverse primer R (GCCTACAAGCTGATAGGACG), and probe P (5'-HEX-AATAGCTCTTGGAAACG GGTGG-BHQ1-3'). The ddPCR was performed with an initial denaturation at 95 ℃ for 5 minutes, followed by 40 cycles of 95 ℃ for 15 seconds and 58 ℃ for 25 seconds.

Fluorescent signals from each droplet were detected using the AccuONE Pro chip reader (Zhenuo Biotech, China). Fluorescence intensity was categorized by a threshold as "1" (positive) or "0" (negative). The total copy number of the target gene was calculated using a Poisson distribution model.

Statistical analyses

In the retrospective study, propensity score matching (PSM) was used to adjust for imbalanced covariates, including maternal age, infertility type, BMI, type of ART, serum AMH level, blastocyst quality, associated endometrioma, associated adenomyosis, and prior use of GnRH-a within 3 months. The propensity scores were estimated using logistic regression, and LE + HMG cycles were matched with GnRH-a HRT cycles in a 1 ∶ 1 ratio with a 0.05 caliper to ensure comparability, using the nearest neighbor method.

To determine independent predictors of live birth in EMs patients undergoing FET, both univariate and multivariate logistic regression analyses were conducted after PSM. Prior to inclusion in the multivariate model, all variables were screened for multicollinearity. A backward stepwise elimination method was applied to identify significant independent variables.

Additionally, subgroup analyses were undertaken in specific subpopulations. Given that adenomyosis is a significant comorbidity of EMs, the study population was stratified into EMs with or without adenomyosis. Within each subgroup, maternal age, infertility type, BMI, type of ART, serum AMH level, blastocyst quality, associated endometrioma and prior use of GnRH-a within 3 months were matched between the two groups.

The consistency between ddPCR and 16S rRNA sequencing results was assessed using Bland-Altman plots and intra-class correlation coefficients (ICC). Bland-Altman analyses were performed using MedCalc software (version 15.6).

All statistical procedures were conducted using SPSS version 26.0 (IBM Corp., USA) and R software version 4.4.1. For continuous variables, Student's t-test was applied when data followed a normal distribution, while the Mann–Whitney U-test was used for non-normally distributed variables. Categorical data were analyzed using either the chi-square test or Fisher's exact test, as appropriate. A two-sided P-value < 0.05 was considered statistically significant.

Results

Baseline characteristics

A total of 3,235 cycles from patients with EMs who underwent either LE + HMG or GnRH-a HRT cycles were screened. Of these, 1,156 cycles from 948 patients (8 patients had 3 cycles, 192 had 2 cycles, and 748 had 1 cycle) were included. Exclusion reasons were shown in ***Fig.2*** Among the 1,156 cycles, 403 were from the LE + HMG group and 753 from the GnRH-a HRT group. After PSM at a 1 ∶ 1 ratio, 385 matched cycles remained in each group.

Before PSM, serum AMH level, antral follicle count (AFC), associated endometrioma, adenomyosis, prior use of GnRH-a within 3 months, and blastocyst quality were different between the groups (***Table 1***). After PSM, no significant differences were found.

Pregnancy and obstetric outcomes

As summarized in ***Table 1***, following PSM, live birth rate (54.0% vs. 53.8%, P = 0.942), clinical pregnancy rate (62.6% vs. 67.0%, P = 0.200) and EM on the day of embryo transfer (9.97 mm vs. 9.86 mm, P = 0.356) were similar between the LE + HMG and GnRH-a HRT groups. Notably, the LE + HMG group demonstrated a significantly lower biochemical pregnancy rate (67.0% vs. 75.6%, P = 0.009) and a trend toward a reduced miscarriage rate (13.7% vs. 19.8%, P = 0.070). Moreover, patients in the LE + HMG group experienced significantly fewer cesarean deliveries (64.9% vs. 75.4%, P = 0.020) and a lower incidence of HDP (4.8% vs. 10.1%, P = 0.039) (***Table 2***). ***Table 3*** showed that duration of infertility, serum AMH level, associated adenomyosis, transfer of good-quality blastocysts, and EM on transfer day were independent predictors for live birth in EMs patients undergoing FET.

Subgroup analysis

In the subgroup analysis of women with EMs without adenomyosis, after PSM, 338 matched cycles were included in each group (***Supplemental Table 1***). The GnRH-a HRT group had a higher biochemical pregnancy rate (76.3% vs. 69.2%, P = 0.038), while the LE + HMG group had a lower miscarriage rate (10.9% vs. 17.8%, P = 0.037). In the subgroup of EMs

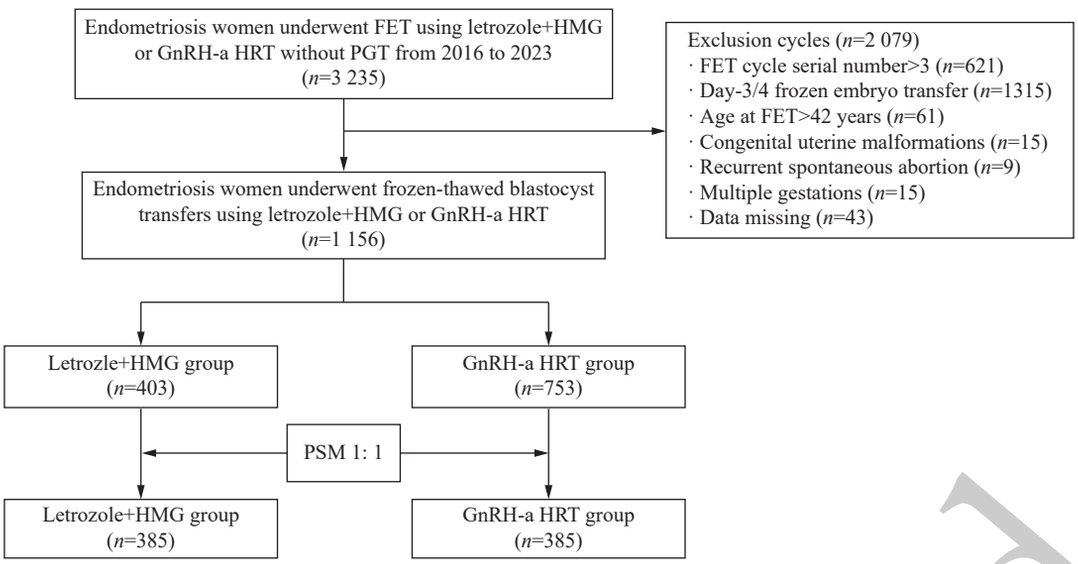

Fig. 2 **Flowchart of patient inclusion and exclusion criteria.** Abbreviations: FET, frozen embryo transfer; HMG, human menopausal gonadotropin; GnRH-a, gonadotropin-releasing hormone agonist; HRT, hormone replacement therapy; PGT, preimplantation genetic testing; PSM, propensity score matching.

patients with adenomyosis, after PSM, 38 matched cycles from each group were included (***Supplemental Table 2***). Conversely, the GnRH-a HRT group had a higher live birth rate (42.1% vs. 21.1%, P = 0.048).

16S rRNA sequencing and OTU analysis

No significant baseline differences were observed between the two groups (***Table 4***). After quality control and merging, 6,097,496 optimized sequences were retained for analysis. A total of 352 operational taxonomic units (OTUs) were identified across all samples, with 207 OTUs shared between the two groups. The LE + HMG group had 58 unique OTUs, while the GnRH-a HRT group had 87 unique OTUs, representing 16.48% and 24.72% of the total OTUs, respectively. Venn diagram analysis (***Fig.3***) shows the overlap between the groups.

Species diversity analysis

Alpha diversity analysis revealed significant differences in the Ace index (P = 0.039) and sequencing depth (Coverage, P = 0.017) between the groups (***Fig.4A***). However, no significant differences were observed in the Shannon and Simpson diversity indices or species evenness (Pielou_e, P = 0.717). Beta diversity analysis, based on unweighted UniFrac distances, indicated no significant differences between the groups (PCoA, P = 0.059; NMDS, P = 0.089) (***Fig.4B***).

Species composition and differential analysis

At the genus level, both groups were dominated by *Lactobacillus* (75.97% vs. 75.54%), *Gardnerella* (7.14% vs. 6.77%), and *Streptococcus* (6.57% vs. 6.98%) (***Fig.5A***). However, in genera with abundance greater than 0.01%, levels of *Escherichia-Shigella* (1.17% vs. 0.06%, P < 0.01), *Limosilactobacillus* (0.63% vs. 0.10%, P < 0.01), and *Staphylococcus* (0.33% vs. 0.22%, P < 0.01) were significantly higher in the GnRH-a HRT group than the LE + HMG group (***Fig.5B***).

LEfSe differential analysis

LEfSe analysis (LDA > 2) revealed that *Pseudomonadota taxa*, including *Gammaproteobacteria*, *Enterobacterales*, *Enterobacteriaceae*, and *Escherichia-Shigella*, were significantly enriched in the GnRH-a HRT group (***Fig. 5C-D***). Additionally, *Staphylococcaceae* and *Staphylococcus* were more abundant in this group. In contrast, the LE + HMG group exhibited significantly higher levels of *Bacillaceae* and *Bacillales*.

Detection of *Lactobacillus* and *Gardnerella* by ddPCR

Lactobacillus and *Gardnerella* are among the most prevalent bacterial genera in the vaginal microbiota. In this study, we explored the potential application of ddPCR in reproductive medicine by targeting these two genera. To evaluate the consistency between ddPCR and 16S rRNA sequencing, we log-transformed the copy numbers obtained from ddPCR and the relative abundances from 16S rRNA data for both *Lactobacillus* and *Gardnerella*. The ICC was 0.875 (95% CI: 0.815 - 0.916), indicating excellent

Table 1 **Baseline characteristics and assisted reproductive pregnancy outcomes between the two groups before and after PSM**

Variables	Before PSM			After PSM		
	LE + HMG (n=403)	GnRH-a HRT (n=753)	*P*-value	LE + HMG (n=385)	GnRH-a HRT (n=385)	*P*-value
Maternal age at FET (y)	30.4±3.6	30.8±3.3	0.053	30.5±3.6	30.7±3.3	0.426
Paternal age at FET (y)	31.3±3.8	31.7±4.0	0.150	32.0±3.9	31.7±4.1	0.389
Duration of infertility (y)	3.6±2.7	3.3±2.5	0.067	3.6±2.6	3.5±2.6	0.530
BMI (kg/m²)	21.7±2.7	21.5±2.8	0.257	21.6±2.7	21.7±2.9	0.871
AMH (ng/mL)	6.0±4.2	5.2±3.9	0.001	5.8±4.0	5.7±3.9	0.825
Basal FSH level (IU/L)	6.9 (5.8-8.1)	7.1 (5.8-8.5)	0.104	6.9 (5.9-8.2)	6.9 (5.7-8.3)	0.848
AFC	16.0±6.1	14.6±6.2	<0.001	15.9±6.2	15.7±6.3	0.686
Type of infertility, %(n)			0.551			0.530
Primary	70.7 (285)	72.4 (545)		70.9 (273)	68.8 (265)	
Secondary	29.3 (118)	27.6 (208)		29.1 (112)	31.2 (120)	
Associated endometrioma, %(n)	52.1 (210)	70.0 (527)	<0.001	54.3 (209)	53.0 (204)	0.718
Associated adenomyosis, %(n)	10.2 (41)	17.9 (135)	<0.001	10.7 (41)	11.4 (44)	0.730
OS protocol, %(n)			0.047			0.099
Agonist protocol	65.3 (263)	67.9 (511)		66.5 (256)	64.7 (249)	
Antagonist protocol	28.3 (114)	28.8 (217)		27.0 (104)	31.7 (122)	
Other protocols	6.5 (26)	3.3 (25)		6.5 (25)	3.7 (14)	
Type of ART, %(n)			0.478			0.531
IVF	80.7 (325)	82.3 (620)		80.5 (310)	78.7 (303)	
ICSI	19.4 (78)	17.7 (133)		19.5 (75)	21.3 (82)	
No. of oocytes retrieved	11.9±4.7	11.1±4.4	0.003	11.7±4.6	11.6±4.5	0.807
No. of 2PN	9.8±4.2	9.2±4.0	0.024	9.6±4.1	9.8±4.1	0.604
Fertilization rate	0.8±0.2	0.8±0.2	0.335	0.8±0.2	0.8±0.2	0.189
Viable embryos	8.9±3.9	8.3 (3.6)	0.011	8.8±3.8	8.8±3.8	0.901
Blastocyst formation rate	0.6±0.3	0.6±0.2	0.081	0.6±0.3	0.6±0.2	0.279
Prior GnRH-a within 3 months, %(n)	17.9 (72)	28.8 (217)	<0.001	18.7 (72)	20.0 (77)	0.648
Good-quality blastocyst transfer, %(n)	65.0 (262)	57.9 (436)	0.018	63.4 (244)	62.6 (241)	0.823
EM on the transfer day (mm)	10.0±1.7	9.9±1.6	0.822	10.0±1.7	9.9±1.6	0.356
Biochemical pregnancy rate, %(n/N)	67.7 (273/403)	73.3 (552/753)	0.046	67.0 (258/385)	75.6 (291/385)	0.009
Clinical pregnancy rate, %(n/N)	63.3 (255/403)	73.3 (552/753)	0.403	62.6 (241/385)	67.0 (258/385)	0.200
Live birth rate, %(n/N)	55.1 (222/403)	53.5 (403/753)	0.610	54.0 (208/385)	53.8 (207/385)	0.942
Miscarriage rate, %(n/N)	12.9 (33/255)	18.6 (92/495)	0.049	13.7 (33/241)	19.8 (52/258)	0.070

Abbreviations: LE, letrozole; HMG, human menopausal gonadotropin; FET, frozen embryo transfer; GnRH-a, gonadotropin-releasing hormone agonist; HRT, hormone replacement therapy; BMI, body mass index; AMH, anti-Mullerian hormone; FSH, follicle-stimulating hormone; AFC, antral follicle count; OS, ovarian stimulation; ART, assisted reproductive technique; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; 2PN, two pronuclei; EM, endometrial thickness. PSM, propensity score matching.

agreement (ICC ⩾ 0.75), which was further supported by Bland-Altman analysis (***Fig. 6A***).

For *Lactobacillus*, the median copy number detected by ddPCR was 1.03×10^8 (IQR: 6.28×10^6 - 3.97×10^8) in the GnRH-a HRT group and 1.90×10^8 (IQR: 1.78×10^7 - 7.35×10^8) in the LE + HMG group. The difference between the two groups was not statistically significant (P = 0.280) (***Fig. 6B***), a result consistent with 16S rRNA sequencing data (P = 0.601) (***Fig. 6C***).

For *Gardnerella*, ddPCR detected positive samples in 73.3% of cases, compared to 58.1% detected by 16S rRNA sequencing, suggesting that ddPCR may offer greater sensitivity. The median *Gardnerella*

Table 2 **Perinatal outcomes between the two groups before and after PSM**

Outcomes	Before PSM			After PSM		
	LE + HMG (n=222)	GnRH-a HRT (n=403)	*P*-value	LE + HMG (n=208)	GnRH-a HRT (n=207)	*P*-value
Gestational age(weeks)	38 (38-39)	39 (38-39)	0.459	38 (38-39)	39 (38-39)	0.185
Birth weight (g)	3 410.4±511.3	3 412.3±503.2	0.966	3 404.9±518.7	3 435.7±523.6	0.547
Delivery mode, %(n)			<0.001			0.020
Vaginal birth	35.6 (79)	21.6 (87)		35.1 (73)	24.6 (51)	
Caesarean section	64.4 (143)	78.4 (316)		64.1 (135)	75.4 (156)	
Newborn sex, %(n)			0.849			0.404
Female	41.9 (93)	42.7 (172)		41.4 (86)	45.4 (94)	
Male	58.1 (129)	57.3 (231)		58.7 (122)	54.6 (113)	
LBW, %(n)	3.2 (7)	4.2 (17)	0.507	3.4 (7)	5.3 (11)	0.330
Macrosomia, %(n)	9.9 (22)	9.9 (40)	0.995	10.1 (21)	11.6 (24)	0.624
LGA, %(n)	2.7 (6)	3.7 (15)	0.499	2.9 (6)	5.8 (12)	0.145
SGA, %(n)	17.6 (39)	17.9 (72)	0.926	16.4 (34)	18.8 (39)	0.505
PTB, %(n)	9.0 (20)	7.7 (31)	0.565	9.1 (19)	7.3 (15)	0.483
GDM, %(n)	10.8 (24)	10.4 (42)	0.880	11.1 (23)	13.0 (27)	0.534
HDP, %(n)	4.5 (10)	9.2 (37)	0.034	4.8 (10)	10.1 (21)	0.039
Placenta previa, %(n)	3.2 (7)	5.5 (22)	0.190	3.4 (7)	3.4 (7)	0.993

Abbreviations: LE, letrozole; HMG, human menopausal gonadotropin; GnRH-a, gonadotropin-releasing hormone agonist; HRT, hormone replacement therapy; LBW, low birthweight; LGA, large for gestational age; SGA, small for gestational age; PTB, preterm birth; GDM, gestational diabetes mellitus; HDP, hypertensive disorders of pregnancy; PSM, propensity score matching.

Table 3 **Univariate and multivariate logistic regression analysis of the live birth after PSM**

Variables	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Maternal age at FET (years)	0.94 (0.90, 0.98)	0.002		
Paternal age at FET (years)	0.98 (0.95, 1.02)	0.359		
Duration of infertility (years)	0.95 (0.90, 1.01)	0.087	0.94 (0.88, 0.99)	0.019
BMI (kg/m^2)	1.01 (0.96, 1.06)	0.680		
AMH (ng/mL)	1.07 (1.03, 1.11)	<0.001	1.06 (1.02, 1.10)	0.003
Basal FSH level (IU/L)	1.00 (0.99, 1.02)	0.564		
AFC	1.02 (1.00, 1.05)	0.055		
Type of infertility (Secondary vs. Primary)	0.88 (0.65, 1.20)	0.427		
Associated endometrioma (Yes vs. No)	0.87 (0.66, 1.16)	0.339		
Associated adenomyosis (Yes vs. No)	0.38 (0.24, 0.61)	<0.001	0.44 (0.27, 0.72)	0.001
Prior use of GnRH-a within 3 months (Yes vs. No)	0.84 (0.59, 1.20)	0.332		
Endometrial preparation protocol (GnRH-a vs. LE + HMG)	0.99 (0.75, 1.31)	0.942		
Good-quality blastocyst transfer (Yes vs. No)	1.95 (1.45, 2.62)	<0.001	1.71 (1.26, 2.33)	0.001
EM on the transfer day (mm)	1.12 (1.02, 1.22)	0.015	1.11 (1.03, 1.12)	0.013

Abbreviations: LE, letrozole; HMG, human menopausal gonadotropin; FET, frozen embryo transfer; GnRH-a, gonadotropin-releasing hormone agonist; BMI, body mass index; AMH, anti-Mullerian hormone; FSH, follicle-stimulating hormone; AFC, antral follicle count; EM, endometrial thickness; PSM, propensity score matching.

Table 4 **Baseline characteristics of endometriosis patients for investigation of the vaginal microbiota**

Variables	LE+ HMG (n=55)	GnRH-a HRT (n=50)	*P*-value
Maternal age at FET (y)	33.1±3.8	32.8±3.9	0.705
Duration of infertility (y)	3.0±2.7	3.3±2.7	0.544
BMI (kg/m²)	21.8±2.1	22.3±3.0	0.312
AMH (ng/mL)	4.7±4.1	3.8±2.5	0.213
Basal FSH level (IU/L)	7.0±2.2	7.7±2.1	0.172
AFC	13.7±5.7	14.2±6.8	0.679
Primary infertility, %(n)	65.5 (36)	52.0 (26)	0.161
Associated endometrioma, %(n)	47.3 (26)	58.0 (29)	0.329
Associated adenomyosis, %(n)	14.6 (8)	20.0 (10)	0.605

Abbreviations: LE, letrozole; HMG, human menopausal gonadotropin; FET, frozen embryo transfer; GnRH-a, gonadotropin-releasing hormone agonist; HRT, hormone replacement therapy; BMI, body mass index; AMH, anti-Mullerian hormone; FSH, follicle-stimulating hormone; AFC, antral follicle count.

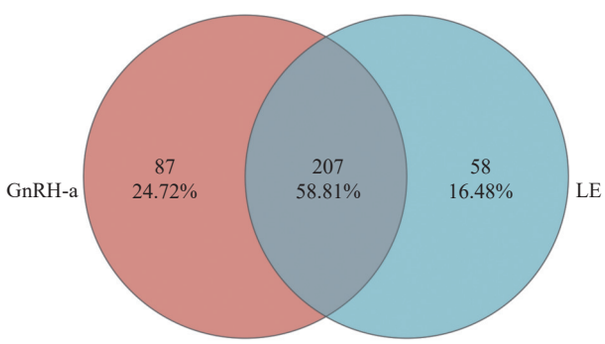

Fig. 3 **Venn diagram** showing shared and unique operational taxonomic units (OTUs) between the GnRH-a HRT group (GnRHa) and the letrozole + HMG group (LE).

copy number in the GnRH-a HRT group was 6.63×10^3 (IQR: 0 - 5.75×10^5), while in the LE + HMG group it was 2.81×10^4 (IQR: 0 - 3.94×10^5), with no significant intergroup difference (P = 0.393) (***Fig. 6D***). This finding was also consistent with 16S rRNA sequencing results, which showed similar relative abundances of *Gardnerella* between the two groups (P = 0.429) (***Fig. 6E***).

Discussion

To our knowledge, this is the first large-scale study comparing pregnancy and perinatal outcomes between the LE + HMG and GnRH-a HRT groups in women with EMs. To date, only two studies have attempted to evaluate different endometrial preparation protocols in this population. One study compared GnRH-a HRT, HRT, and NC protocols but did not include any LE-based regimens[7]. The other study incorporated LE + HMG in its comparison alongside GnRH-a HRT, HRT, and NC protocols[8]; however, it involved only 42 LE + HMG cycles, substantially limiting its statistical power and the generalizability of its findings.

The LE + HMG group showed lower rates of miscarriage, cesarean delivery, and HDP. These findings may be partly explained by the presence or absence of the CL in different endometrial preparation protocols. In conventional HRT cycles, with or without GnRH-a pretreatment, the hypothalamic–pituitary–ovarian axis is suppressed, resulting in the absence of a functional CL. The CL plays a pivotal role in early pregnancy by producing not only estradiol and progesterone but also key vasoactive and angiogenic factors such as relaxin and vascular endothelial growth factor (VEGF). These hormones are essential for embryo implantation, endometrial decidualization, and proper placental development[25]. A deficiency in these factors may impair vascular remodeling and placental formation, potentially contributing to abnormal implantation and an increased risk of miscarriage. Moreover, relaxin is involved in cardiovascular adaptations during pregnancy; its absence has been implicated in the pathophysiology of preeclampsia and other hypertensive complications[15]. The LE + HMG protocol, by preserving ovulation and CL function through mild ovarian stimulation, ensures endogenous production of these crucial hormones. This may underlie the observed reduction in HDP incidence and cesarean section rates in the LE group. Nonetheless, further investigation is warranted to elucidate the association between HRT protocols and obstetric complications, such as miscarriage and HDP.

Blastocyst quality emerged as an independent predictor of live birth in our analysis. Accumulating evidence suggests that impaired endometrial receptivity may not be the principal factor underlying implantation failure in ART, even in patients with EMs[26]. Rather, embryo quality appears to play a more critical role in determining successful pregnancy outcomes[27]. Additionally, the presence of adenomyosis was independently associated with reduced live birth rates among women with EMs, emphasizing the importance of considering adenomyosis as a significant confounding factor. Its detrimental effect on reproductive outcomes may surpass that of EMs alone[28]. In our subgroup analysis involving patients diagnosed with both EMs and adenomyosis, those undergoing GnRH-a HRT demonstrated a significantly higher live birth rate compared to those treated with the LE + HMG protocol. This finding suggests that GnRH-a

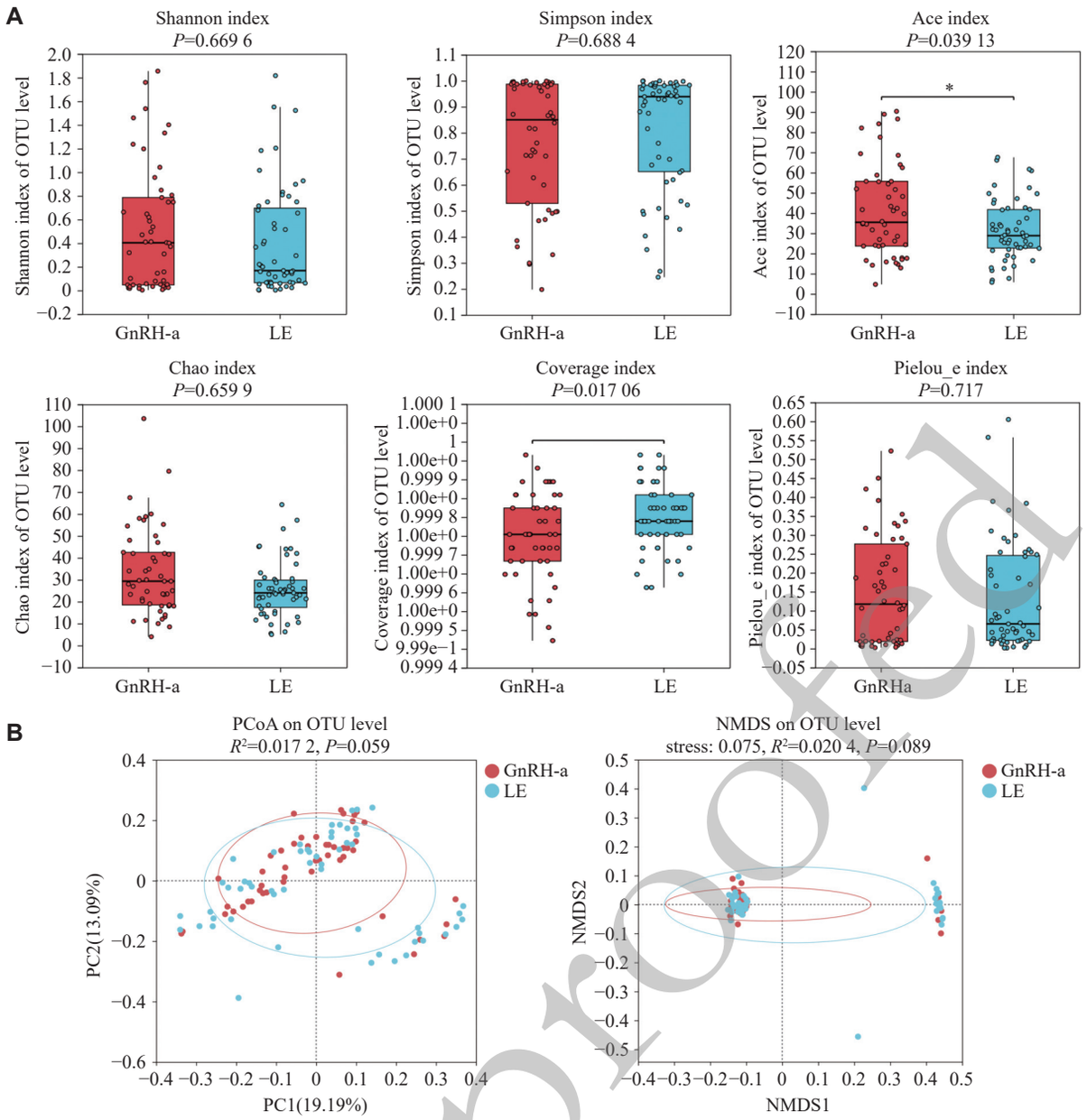

Fig. 4 **Comparison of vaginal microbiota diversity between GnRH-a HRT (GnRHa) and letrozole + HMG (LE) groups.** A: Alpha diversity indices (Shannon, Simpson, ACE, Chao, Coverage, and Pielou_e). B: Beta diversity analysis by Principal Coordinates Analysis (PCoA) and Non-metric Multidimensional Scaling (NMDS). P-values < 0.05 were considered statistically significant.

pretreatment may be particularly beneficial for women with adenomyosis, aligning with previous studies that support the use of GnRH-a to improve reproductive outcomes in this subgroup[29].

This study also systematically evaluated the impact of the two endometrial preparation protocols on the structure of the vaginal microbiota and the abundance of key bacterial taxa. Notably, the GnRH-a HRT protocol was associated with an increased prevalence of potentially pathogenic bacteria, such as *Escherichia-Shigella* and *Staphylococcus*. This may be attributable to the hypoestrogenic state induced by GnRH-a treatment. Estrogen is known to play a crucial role in maintaining mucosal immunity by modulating the expression of antimicrobial peptides (AMPs), such as defensins and secretory leukocyte protease inhibitors, within the reproductive tract [30-31]. A reduction in estrogen levels may lead to decreased AMP expression, thereby compromising local defense mechanisms and facilitating colonization by opportunistic pathogens in the vaginal environment.

While 16S rRNA sequencing is widely used for qualitative analysis and to determine microbial diversity and relative abundance, it has limitations such as lower resolution, reduced detection efficiency for certain genera, and longer testing periods

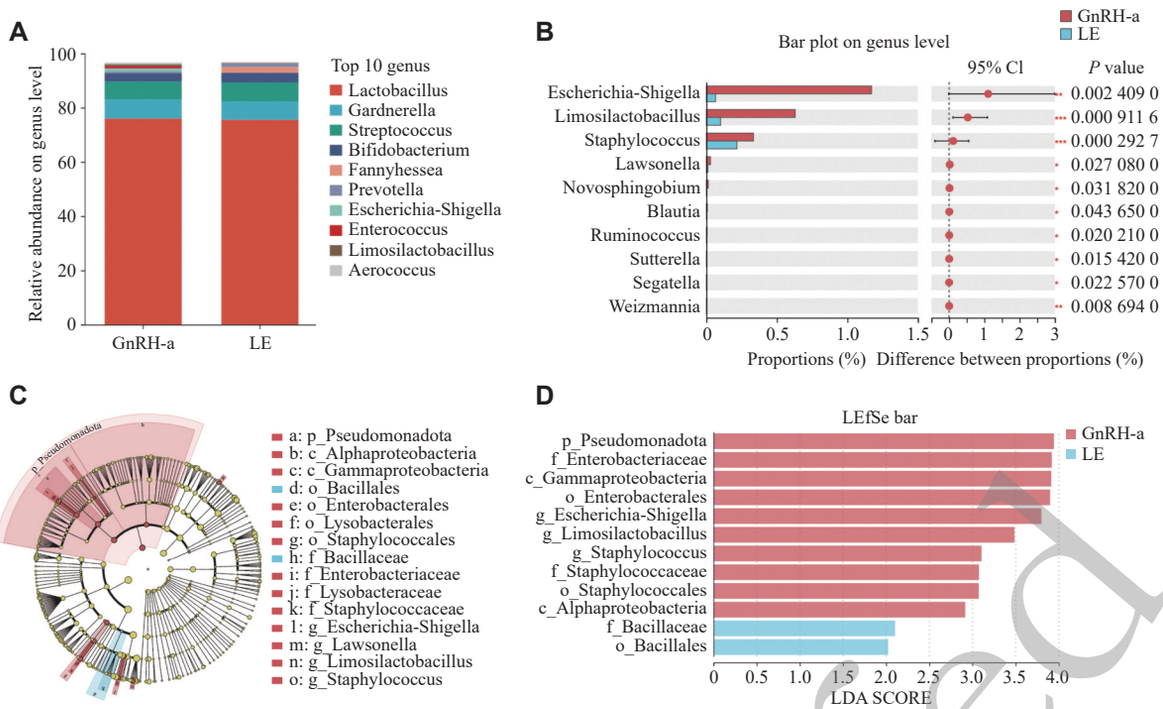

Fig. 5 **Comparison of vaginal microbiota composition between GnRH-a HRT (GnRHa) and letrozole + HMG (LE) groups.** A: Bar plots depicting the relative abundance of the top 10 genera at the genus level. B: Genus-level microbial taxa showing statistically significant differences in abundance between the two groups. C: Cladogram generated by LEfSe analysis. Each concentric circle represents a taxonomic level, from phylum (center) to class, order, family, and genus (outer layers). D: Linear Discriminant Analysis (LDA) score plot showing the effect size of taxa with significant intergroup differences.

(typically 5-7 days). In contrast, ddPCR offers high sensitivity, specificity, and rapid diagnostic capabilities (within 3 hours). In our study, we successfully established a ddPCR assay for the detection of *Lactobacillus* and *Gardnerella* by designing specific primers and optimizing reaction conditions. The ddPCR results showed excellent concordance with 16S rRNA sequencing, further validating its clinical applicability. Notably, ddPCR exhibited superior sensitivity in detecting *Gardnerella*, a low-abundance but potentially pathogenic bacterium that may be underrepresented in sequencing-based analyses. This highlights the advantage of ddPCR in precisely identifying clinically relevant microorganisms within the reproductive tract. Taken together, these findings support a two-step microbial detection strategy: initial screening using 16S rRNA sequencing to identify microbiota shifts associated with reproductive outcomes, followed by targeted ddPCR analysis for rapid and accurate pathogen detection.

This study has several limitations that should be acknowledged. First, it was a single-center, retrospective analysis, which may be subject to inherent selection biases. Prospective, multicenter randomized controlled trials (RCTs) are needed to validate our findings. Second, NC and pure HRT protocols were not included due to their limited application at our center, which may restrict the generalizability of the results. Third, some patients underwent multiple FET cycles, which could introduce intra-patient variability and potential confounding. Additionally, perinatal outcomes were collected through telephone interviews, which may be less accurate than those obtained from standardized medical record reviews.

For the microbiota analysis, we intended to compare the vaginal microbiota between live birth and non-live birth groups. However, after matching for key confounders (e.g., age, blastocyst quality, adenomyosis), only 11 samples remained in each group. Preliminary results showed no significant differences, and the small sample size limited interpretability; thus, detailed data were not presented. Further studies with larger sample sizes are needed to clarify the potential relationship between vaginal microbiota and reproductive outcomes.

Finally, the ddPCR analysis in this study was limited to *Lactobacillus* and *Gardnerella*, and did not encompass other potentially pathogenic taxa such as *Escherichia*, *Shigella*, *Staphylococcus*, *Streptococcus*, and *Enterococcus*.

In conclusion, our findings suggest that live birth rates were comparable between the LE + HMG and

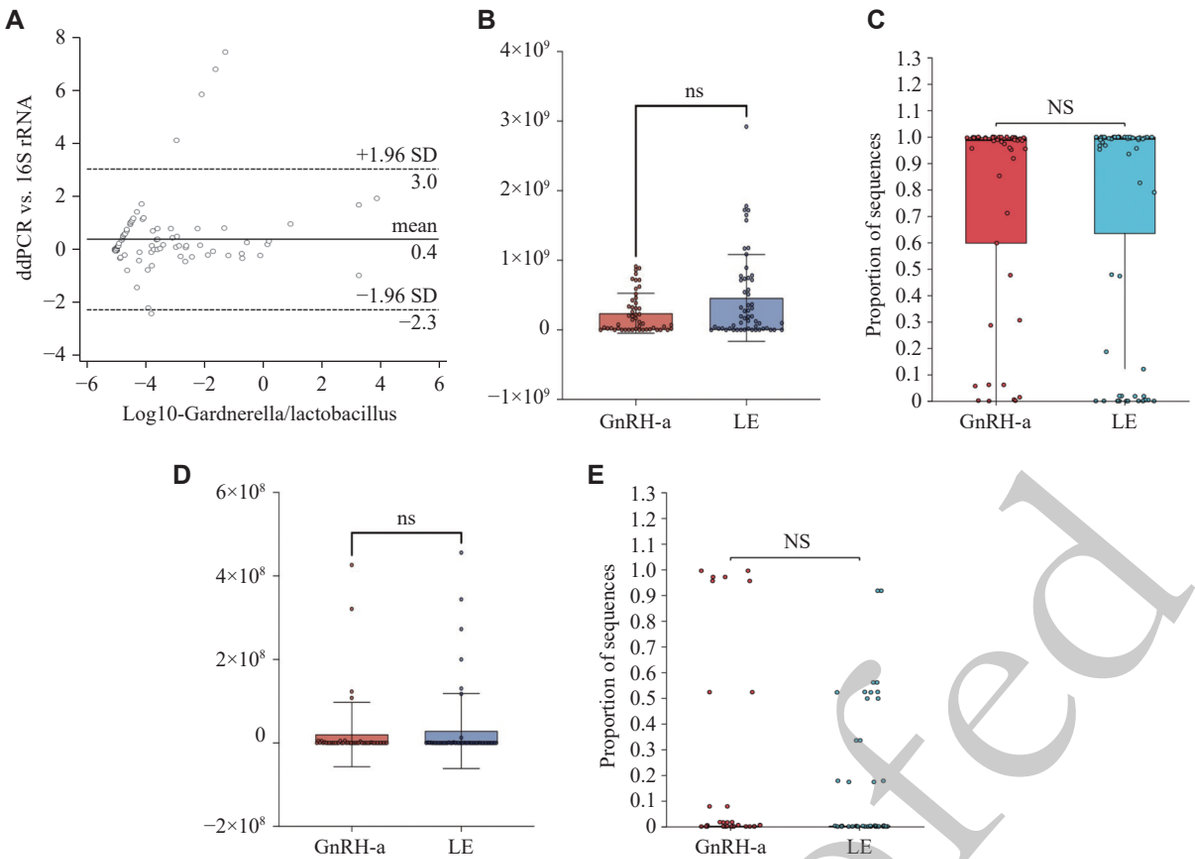

Fig. 6 **ddPCR and 16S rRNA sequencing in quantifying core vaginal microbiota.** A: Bland–Altman plot demonstrating that most data points fall within the 95% confidence interval, indicating strong concordance between the two methods. B: Detection of *Lactobacillus* by ddPCR. C: Detection of *Lactobacillus* by 16S rRNA sequencing. D: Detection of *Gardnerella* by ddPCR. E: Detection of *Gardnerella* by 16S rRNA sequencing. P-values < 0.05 were considered statistically significant.

GnRH-a HRT protocols in women with EMs undergoing FET. However, the LE + HMG group was associated with a lower incidence of pregnancy complications. The GnRH-a HRT protocol may be linked to an increased risk of colonization by pathogenic vaginal bacteria, underscoring the importance of monitoring microbial changes and considering appropriate interventions in patients receiving this protocol. Moreover, ddPCR demonstrated promising clinical utility as a rapid and precise tool for the detection of key vaginal microorganisms, supporting its potential role in clinical practice.

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