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Lactiplantibacillus plantarum FXJKS21M3 alleviates polycystic ovary syndrome by modulating gut microbiota, serum metabolome and facilitating progestational hormone biosynthesis

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ABSTRACT: The abundance of *Lactiplantibacillus plantarum* species was found to be decreased in polycystic ovary syndrome (PCOS) patients, indicating this species may play a superior role in PCOS treatment. Here, the potential of six *Lp. plantarum* strains in reducing PCOS symptoms was assessed in a rat model. *Lp. plantarum* FXJKS21M3 (F21M3) intervention alleviated abnormal ovarian morphology and restored progesterone and luteinizing hormone levels in rats. Butyrate was not involved in this curative effect on PCOS symptoms. Dysbiosis of specific gut microbes, including *Prevotellaceae_Ga6A1_group*, *Candidatus_Saccharimonas*, and *ASF356*, was restored by *Lp. plantarum* F21M3 intervention. Enrichment of *Bifidobacterium* was also observed in *Lp. plantarum* F21M3-treated rats. The impact of *Lp. plantarum* F21M3 on the serum metabolome was more remarkable than that on the fecal metabolome. According to enrichment analysis of differential metabolites, the progestational hormone biosynthesis pathway was enriched after *Lp. plantarum* F21M3 administration. These results demonstrated that *Lp. plantarum* species may alleviate PCOS symptoms by regulating gut microbiota and facilitating progestational hormone biosynthesis.

Keywords: polycystic ovary syndrome, *Lactiplantibacillus plantarum*, gut microbiota, serum metabolome, progestational hormones.

1. Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder with an average incidence of up to 21%, making it the most prevalent endocrine disorder affecting women of reproductive age [1]. Androgen excess, ovulatory dysfunction, and polycystic ovarian morphology are typical clinical presentations of PCOS. Due to the heterogeneous nature of this disease, many therapies have been applied in PCOS treatment. In

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recent years, gut-targeting therapies have been demonstrated to play an emerging role in PCOS symptom alleviation [2].

A bidirectional relationship exists between PCOS and gut microbiota. Dysbiosis of gut microbiota, including decreased microbiota diversity and altered microbial composition, has been observed in PCOS patients [3]. Conversely, the potential role of gut microbiome in PCOS pathogenesis was first proposed by Tremellen and Pearce [4] and subsequently confirmed through gut microbiota manipulation [5]. The related mechanisms may involve gut immunity, gut endocrine function, and gut permeability [3]. Therefore, modification of gut microbiota represents an effective therapeutic strategy for PCOS.

Probiotics are live microorganisms that confer health benefits on the host when consumed in adequate amounts. These microbes can pass through the upper digestive tract and reach the distal gut [6], exerting their roles through direct host interaction or gut microbiota regulation [7,8]. The curative effects of probiotic strains on PCOS symptoms have been demonstrated in several clinical trials [9,10]. Understanding the impact of PCOS on probiotic species composition in patients' gut may provide a reference for screening probiotic strains that can alleviate PCOS. Additionally, several *Lactiplantibacillus plantarum* strains have proven effective in PCOS symptom alleviation in our previous studies [11-12]. A butyrate-gut hormone mechanism was observed in one *Lp. plantarum* strain. However, due to the heterogeneity of biological characteristics within *Lp. plantarum* species, different strains may exert their probiotic effects through alternative mechanisms. Apart from that, how *Lp. plantarum* strains affect sex hormones levels via regulating gut microbiota and their metabolites remains further study.

According to human trial results, the abundance of *Lp. plantarum* species was decreased in PCOS patients, indicating that *Lp. plantarum* species supplementation may play a superior role in PCOS treatment. Therefore, six *Lp. plantarum* strains were screened for their abilities in PCOS alleviation using a rat model. One strain demonstrated significant curative effects on PCOS, in which butyrate may not be involved. To further explore the potential mechanism, gut microbiota, gut metabolites, and serum metabolites were analyzed, and their interactions were investigated. The present study provides a new perspective on probiotic applications for PCOS therapies.

2. Materials and methods

2.1 Design of human trial

To analyze fecal microbiota composition, 54 subjects were recruited by Suzhou Municipal Hospital. Sample collection was approved by the Ethics Committee of Suzhou Municipal Hospital (No. K-2021-004-H01) and registered in the Chinese Clinical Trial Registry (No. ChiCTR2100045475). Written informed consent for research purposes was acquired before sample collection.

Healthy and PCOS subjects were divided into three groups: 16 non-obese healthy subjects in the control group, 18 non-obese PCOS subjects in the PCOS-L group, and 20 obese PCOS subjects in the PCOS-O group. For recruited subjects, body mass index (BMI) <25 was considered non-obese and BMI ≥25 was considered obese. Healthy subject inclusion criteria were as follows: women of reproductive age in good health who had

not been diagnosed with PCOS. PCOS patients were included according to Rotterdam diagnostic criteria. Subject exclusion criteria were as follows: pregnancy; antibiotic use within 30 days; probiotic product use within 30 days; Chinese medicine use within 30 days; suffering from other serious illnesses. Fresh feces from subjects were collected using sterile tubes and stored at -80℃ as soon as possible.

2.2 Bacterial strains and culture conditions

Information on candidate *Lp.* plantarum strains is listed in Table 1. *Lp. plantarum* strains were cultured in DeMan, Rogosa and Sharpe broth and grown anaerobically at 37℃ for 24 hours. Strain cultures were collected by centrifugation at 4℃, 8,000 g for 15 min and washed with sterile saline. A cryoprotectant was added to the washed pellets, which were then freeze-dried into powder and stored at 4℃ before use. The freeze-drying procedure was described in detail previously [13].

2.3 Animal study design

Animal studies strictly followed the Principles of Laboratory Animal Care (NIH publication No. 8023, revised 1978) and were authorized by the Animal Welfare and Ethics Committee of Jiangnan University (JN.No20190930S0901220[241]). Eight-week-old female prepubertal Sprague Dawley rats were purchased from Vital River (Zhejiang). Rats were housed in a specific pathogen-free barrier environment with a 12-h light/dark cycle, stable humidity, and controlled temperature, with free access to standard rodent feed and drinking water.

Rats were randomly allocated to 9 groups: Control, Letrozole, Diane-35, and bacterial groups (n = 6 per group). To induce the PCOS phenotype, rats received letrozole treatment (1 mg kg^{-1} by gavage in 1% carboxymethylcellulose, Jiangsu Hengrui Pharmaceuticals) for 3 weeks. Rats treated with Diane-35 (1 tablet suspended in 50 mL of 1% carboxymethylcellulose administered by gavage at 4.5 mL kg^{-1}, Bayer Pharmaceuticals) served as the clinical PCOS treatment control. Lyophilized bacterial powder was suspended in sterile saline and delivered by gavage (1×10^9 colony-forming units of viable bacteria per day). A detailed schematic of the animal procedures is presented in Figure 1.

Table 1. Details of the *Lp. plantarum* strains used in this study.

.	Strain number	Origin	Regional information
F21M3	FXJKS21M3	Human feces	Xinjiang, China
F35M1	FCQNA35M1	Human feces	Chongqing, China
F14L1	FJSWX14L1	Human feces	Jiangsu, China
F4M3	FJLHD4M3	Human feces	Jilin, China
F7M7	FFJNDD7M7	Human feces	Fujian, China
F4G1	FJSNJPK4G1	Human feces	Jiangsu, China

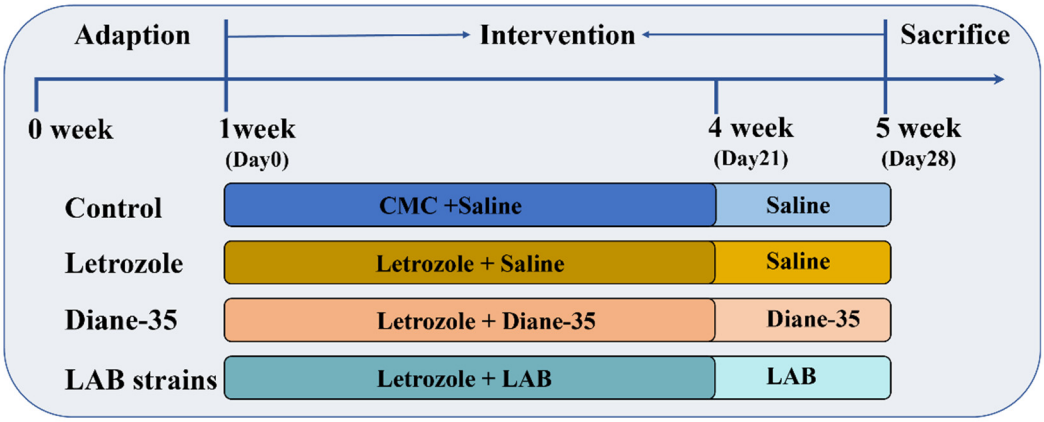


Figure 1. Schematic of the animal treatment procedure. The whole feeding period included 1 week of adaptation, 3 weeks of letrozole molding and 4 weeks of strains/Diane-35 intervention.

2.4 Serum assay

Rats were sacrificed on day 29 following an overnight fast. Blood samples were collected for serum preparation, held at room temperature for 0.5 h, and centrifuged (3000 r/min, 15 min). Estradiol, testosterone, progesterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were analyzed using enzyme-linked immunoassay kits (Elabscience Biotechnology, Wuhan). A biochemical analyzer (Beckman Coulter, CA) was used to determine concentrations of high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triglyceride (TG), aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

2.5 Ovarian morphology

Following blood sample collection, rats were euthanized and bilateral ovary samples were rapidly removed and weighed. Tissue fixation was performed using 4% paraformaldehyde, and hematoxylin–eosin staining was conducted to analyze ovarian histopathological changes. The detailed experimental procedures and evaluation criteria were described in our previous study [14].

2.6 Short-chain fatty acid assay

Fecal samples were collected on day 28 of the animal study. Fecal concentrations of acetate, propionate, butyrate, isobutyric acid, valeric acid, and isovaleric acid were determined using gas chromatography–mass spectrometry (Shimadzu, Japan). Sample preparation details and detection parameters were described in our previous study [12].

2.7 Fecal microbiota assay

After collection, fecal samples were transferred to sterile tubes and stored at −80°C. Microbial genomic DNA was extracted from feces using a Fast DNA Spin Kit (MP Biomedicals, Irvine). For rat samples, bacterial 16S rRNA genes (V3–V4 hypervariable region) were amplified using universal primers (341F and 806R). For human samples, bacterial *groEL* genes were amplified using *Lactobacillus*-specific primers (Lac-groEL-F: 5-GCYGGTGCWAACCCNGTTGG-3, Lac-groEL-R: 5-AANGTNCCVCGVATCTTGTT-3) and *Bifidobacterium*-specific primers (Bif-groEL-F:

5-TCCGATTACGAYCGYGAGAAGCT-3, Bif-groEL-R: 5-CSGCYTCGGTSGTCAGGAACAG-3) to investigate the composition of *Lactobacillus* and *Bifidobacterium* species [15,16]. The amplification profile of *Lactobacillus*-specific primers was 94℃ 5 min; 94℃ 30 s, 58℃ 30 s, 72℃ 60 s for 35 cycles; 72℃ 7 min. The amplification profile of *Bifidobacterium*-specific primers was 94℃ 8 min; 94℃ 40 s, 60℃ 40 s, 72℃ 40 s for 30 cycles; 72℃ 8 min. The resulting PCR products were purified using a DNA Gel/PCR Purification Miniprep kit (Biomiga, San Diego) and sequenced on an Illumina MiSeq PE300 sequencing platform (Illumina, San Diego).

For bioinformatic analysis, the QIIME2 platform was applied. Valid tags were denoised using dada2, and taxonomy was assigned using the silva-132-99 database. Alpha diversity was represented by Shannon index, observed operational taxonomic units (OTUs), Faith_pd index, and Pielou_e index. Beta diversity was indicated by calculating Bray-Curtis distance and visualized using principal coordinate analysis (PCoA). Linear discriminant analysis effect size (LEfSe) was applied to discover microbial biomarkers between groups.

2.8 Metabolomics assay

Fecal samples (100 mg) were completely homogenized with 500 μL methanol (at 4℃) and centrifuged (4℃, 13,000 r/min, 20 min) to remove proteins. Supernatants (200 μL) were transferred to new tubes, centrifuged (4℃, 13,000 r/min, 20 min), and filtered using 0.22 μm membranes. The filtered supernatants were stored in vials for detection. Serum samples (20 μL) were completely mixed with 80 μL methanol (at 4℃) and centrifuged (4℃, 13,000 r/min, 20 min) to remove proteins. The supernatants were stored in vials for detection.

Fecal and serum metabolites were detected using an UltiMate 3000 ultra-performance liquid chromatography (UPLC) system (T3 chromatographic column) and high-resolution Q-Exactive mass spectrometer (Thermo Scientific, Massachusetts). Compound Discovery 3.2 software was used to extract raw data, filter background, recognize peaks, deconvolute, align peaks, and calculate peak areas. Databases including mzCloud, ChemSpider, and HMDB were used to identify metabolites.

2.9 Quantitative reverse transcriptionime PCR

Ovary tissues were lysed in TRIzol (Invitrogen, Carlsbad) for total RNA extraction. Complementary DNA was prepared using HiFiScript gDNA Removal RT MasterMix (CWBIO, Beijing). Gene expression changes were measured on a BioRad-CFX384 thermocycler using SYBR Green Supermix (Bio-Rad, Hercules). All amplification reactions were performed in triplicate, with no-template controls. Rat *GAPDH* was amplified as an internal control. The amplification profile was 30 s at 95℃ for polymerase activation, followed by 35 cycles of 5 s at 95℃ for DNA denaturation and 30 s at 60℃ for annealing and extension. The $2^{-\Delta\Delta Ct}$ method was used to make relative quantification based on the PCR signal of the target gene in a treated-sample to that of another untreated sample. The size of the expected PCR products of the rat cDNAs is 536 bp for *SCC*, 405 bp for *3β-HSD* and 246 bp for *StAR*. The primer sequences are: *SCC*-F: 5-AGAAGCTGGGCAACATGGAGTCAG-3; *SCC*-R: 5-TCACATCCCAGGCAGCTGCATGGT-3;

3β-HSD-F: 5-ACTGGCAAATTCTCCATAGCC-3; *3β-HSD*-R: 5-TCCTCCCAGCTGACAAGTGG-3; *StAR*-F: 5-CAAACTCACGTGGCTGCTCAGTA-3; *StAR*-R: 5-GCAAGTGGCTGGCGAACTCTA-3.

2.10 Statistics

Statistical analyses and data visualization were performed using GraphPad Prism 9. Data are presented as means ± standard deviations (SDs) or medians ± interquartile ranges. The Kolmogorov-Smirnov test was performed to examine data normality. For normally distributed datasets, one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test was used to evaluate statistical significance of differences between multiple groups. For non-normally distributed data, the nonparametric Kruskal-Wallis test was used to analyze differences. A P value < 0.05 was considered statistically significant. For microbiota or metabolomics assay, p value was adjusted by false discovery rate (Benjamini Hochberg method).

3. Results

3.1 Decreased abundance of Lp. plantarum species was observed in PCOS patients

Fifty-four subjects were recruited in the present study and divided into a control group and two PCOS groups. *Lactobacillus* and *Bifidobacterium* are generally regarded as typical probiotic microbes [17]. Using specific primers, the composition of nine *Lactobacillus* species and seven *Bifidobacterium* species was analyzed based on high-throughput sequencing. Compared with healthy women, both obese and non-obese PCOS subjects exhibited decreased *Lp. plantarum* abundance in their feces (Figure 2). This phenomenon was more pronounced in obese PCOS subjects. Changes in the abundance of other probiotic species were not as obvious as those of *Lp. plantarum*. Therefore, administration of *Lp. plantarum* strains may exert superior beneficial effects on PCOS symptoms.

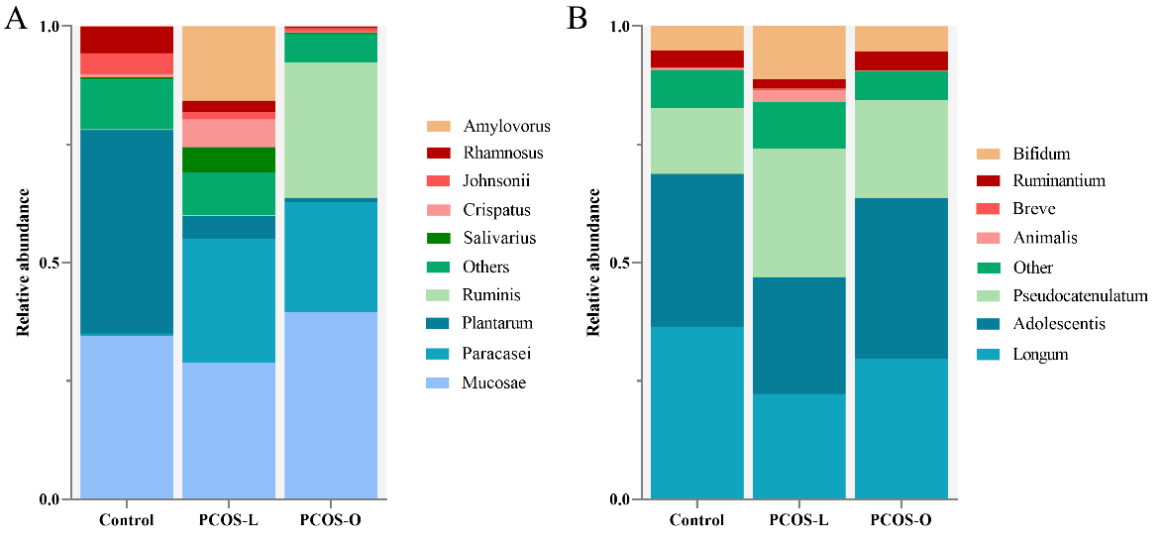


Figure 2. Probiotic species distribution of healthy and PCOS subjects. (A) *Lactobacillus* distribution at the species level. (B) *Bifidobacterium* distribution at the species level.

3.2 Lp. plantarum F21M3 exhibited the best effects on PCOS symptom alleviation compared to other strains

In the subsequent study, six *Lp. plantarum* strains were tested for their abilities to alleviate PCOS symptoms in a rat model. Although these strains were classified as the same species, they were isolated from

subjects in different provinces of China. To mimic PCOS symptoms, Sprague-Dawley rats were treated with letrozole for 3 weeks. As shown in Figure 3, Figure 4, and Figure S1, compared with rats in the control group, letrozole-treated rats exhibited abnormal ovarian morphological features, disturbed sex hormone levels, and metabolic disturbances. These symptoms are similar to the clinical manifestations of PCOS patients. Therefore, the letrozole-induced model is appropriate for assessing the mitigative effects of probiotic strains on PCOS.

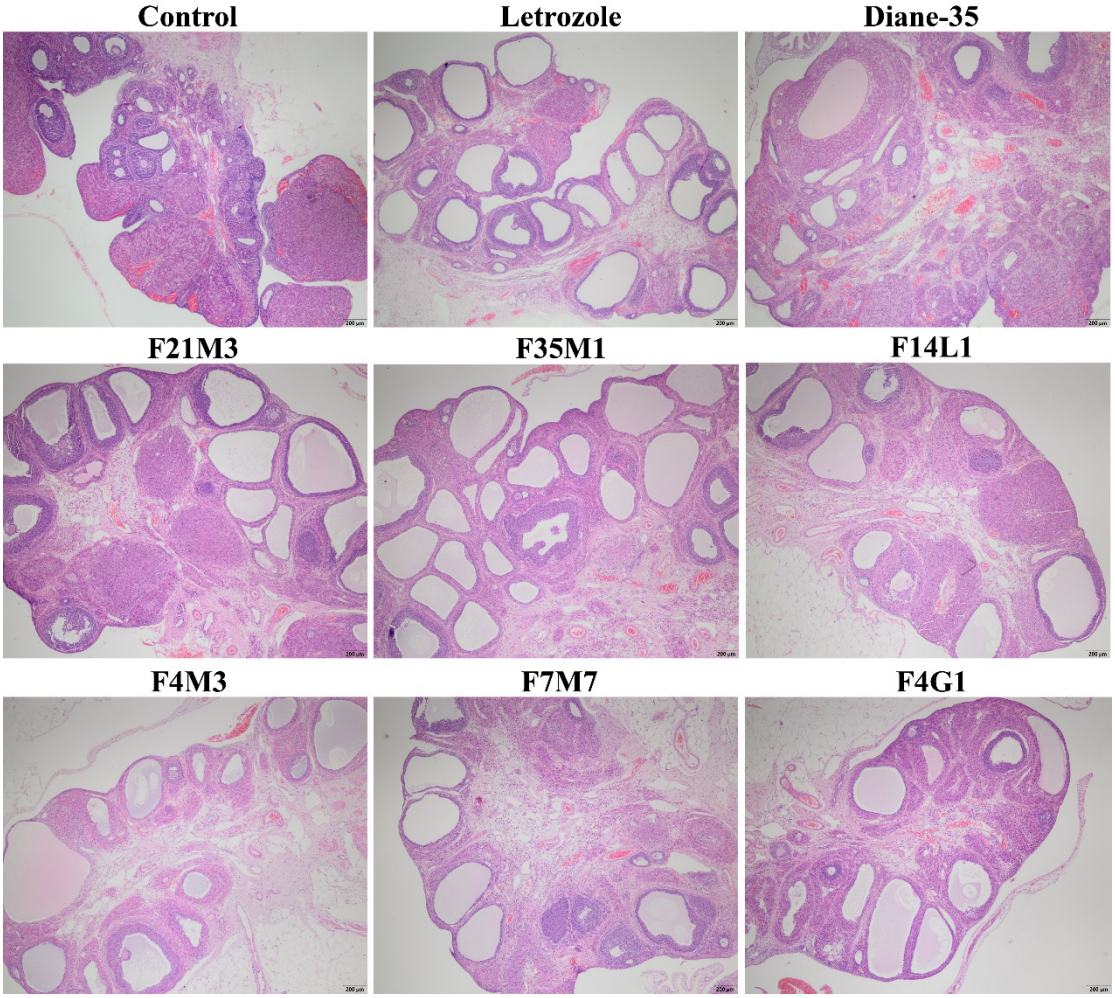


Figure 3. Representative examples of ovarian histopathological sections. Magnification, × 40.

During letrozole treatment, *Lp. plantarum* strains were administered to rats via gavage for 4 weeks. The intervention period for *Lp. plantarum* strains (4 weeks) was slightly longer than letrozole treatment (3 weeks), which may emphasize the alleviative role of probiotic strains. Since letrozole discontinuation may cause PCOS symptoms to vanish, this extended intervention design was implemented. Nevertheless, at the end of the fourth week, PCOS symptoms remained significant in the letrozole group, indicating that the prolonged intervention period of *Lp. plantarum* strains did not compromise the efficacy of the PCOS model. The alleviative effects on PCOS symptoms varied between different strains. As shown in Figure 3 and Table 2, *Lp. plantarum* F21M3 exhibited the best restorative effects on ovarian morphology among the six strains, including decreased ovarian weight index, reduced number of cystic follicles, increased number of corpus lutea, and enhanced granulosa cell layer thickness. In addition to *Lp. plantarum* F21M3, *Lp. plantarum*

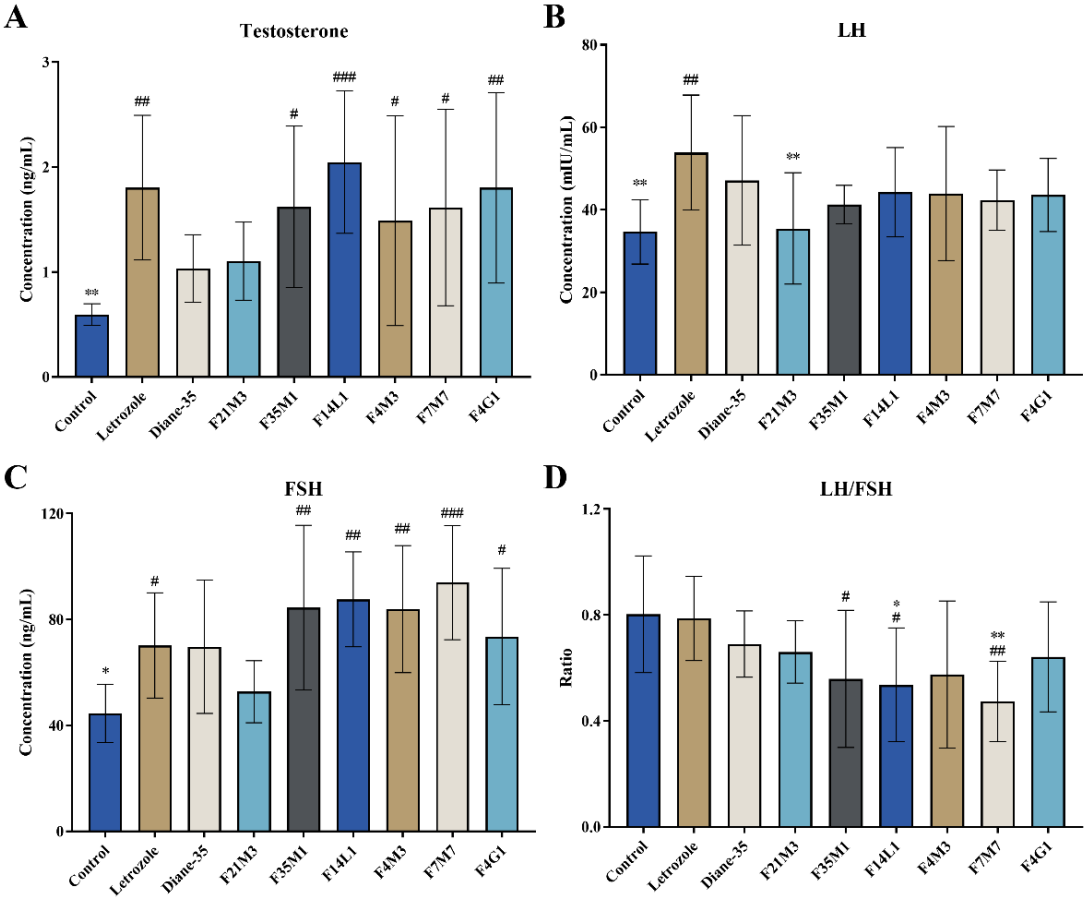
F35M1 and F4M3 also exerted alleviative effects on ovarian morphology (such as decreased ovarian weight index and increased granulosa cells).

Table 2. *Lp. plantarum* strains intervention on ovarian histopathological parameters of rats.

Groups	Ovarian weight index	Number of cystic follicles	Cystic follicle (%)	Number of corpus lutea	Granulosa cell layer thickness (μm)
Control	$0.59 \pm 0.07^{*}$	$0.66 \pm 0.80^{***}$	$6.81 \pm 7.80^{***}$	$7.00 \pm 1.27^{**}$	$46.81 \pm 10.71^{***}$
Letrozole	$0.75 \pm 0.16^{\#}$	$6.16 \pm 0.80^{\#\#\#}$	$43.33 \pm 5.99^{\#\#\#}$	$3.55 \pm 1.05^{\#\#}$	$20.43 \pm 2.78^{\#\#\#}$
Diane-35	$0.73 \pm 0.06^{\#}$	$3.77 \pm 0.80^{**}$	$26.33 \pm 5.29^{*}$	4.67 ± 1.05	$33.66 \pm 7.31^{**}$
F21M3	$0.62 \pm 0.03^{*}$	$3.83 \pm 0.98^{\#*}$	$29.17 \pm 5.67^{*}$	5.00 ± 1.41	$36.85 \pm 4.70^{***}$
F35M1	0.68 ± 0.12	$6.33 \pm 2.34^{\#\#\#}$	$46.33 \pm 12.86^{\#\#\#}$	$3.83 \pm 1.17^{\#\#}$	$29.82 \pm 5.45^{\#\#*}$
F14L1	0.69 ± 0.08	$5.00 \pm 1.79^{\#\#}$	$44.83 \pm 12.17^{\#\#\#}$	$3.67 \pm 0.52^{\#\#}$	$26.94 \pm 2.71^{\#\#}$
F4M3	$0.63 \pm 0.08^{*}$	$4.33 \pm 1.51^{\#\#}$	$42.50 \pm 13.88^{\#\#}$	$3.17 \pm 1.17^{\#\#\#}$	$26.86 \pm 7.59^{\#\#}$
F7M7	$0.76 \pm 0.13^{\#\#}$	$4.61 \pm 1.51^{\#\#}$	$45.00 \pm 10.49^{\#\#\#}$	$4.17 \pm 1.47^{\#}$	$27.44 \pm 4.40^{\#\#}$
F4G1	$0.77 \pm 0.10^{\#\#}$	$5.17 \pm 1.17^{\#\#\#}$	$50.17 \pm 18.73^{\#\#\#}$	$2.50 \pm 0.55^{\#\#\#}$	$27.66 \pm 3.81^{\#\#}$

Ovarian weight index was calculated as ovarian weight/body weight × 1000. Data are means ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the letrozole group using a one-way ANOVA (or Kruskal–Wallis test).

Imbalanced sex hormone levels represent another classical symptom of PCOS. Following letrozole treatment, rats displayed abnormal testosterone, LH, FSH, estradiol, and progesterone levels. *Lp. plantarum* F21M3 exhibited significant regulatory effects on serum LH and progesterone levels, while other strains did not (Figure 4B and F). Although the decreased serum testosterone level mediated by *Lp. plantarum* F21M3 was not significant, *Lp. plantarum* F21M3 still performed better on testosterone regulation than other strains (Figure 4A). Rats in the letrozole group displayed lower serum estradiol levels, which could be restored by different *Lp. plantarum* strains (Figure 4E).



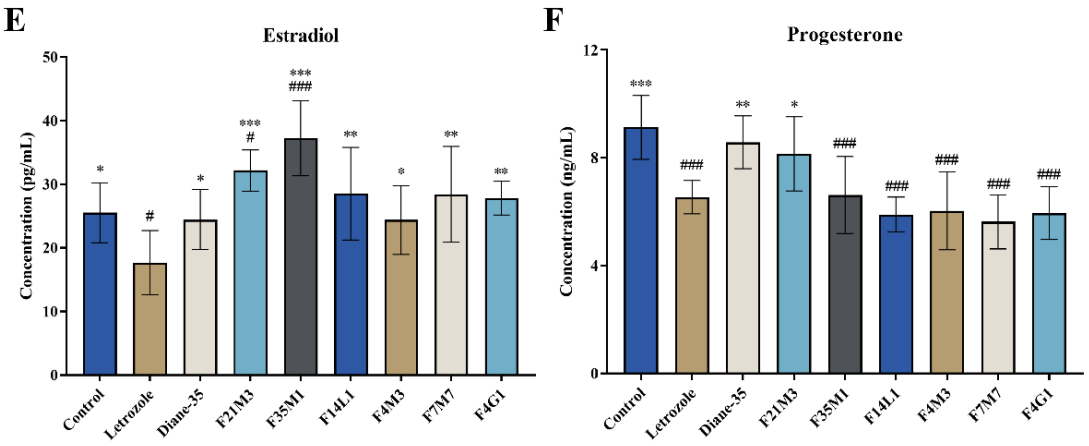


Figure 4 *Lp. plantarum* strains intervention on sex hormone levels of rats. (A) Testosterone levels. (B) LH levels. (C) FSH levels. (D) LH/FSH ratios. (E) Estradiol levels. (F) Progesterone levels. Data are means ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the letrozole group using a one-way ANOVA (or Kruskal–Wallis test).

In our letrozole-induced PCOS model, rats gained more weight and displayed metabolic disturbances. Overall, the four-week intervention with different strains showed minimal effects on metabolic indices of PCOS rats. Serum AST levels slightly increased after letrozole treatment, and this elevation could be restored by *Lp. plantarum* F21M3 intervention (Figure S1H). Metabolic indicators such as body weight, TC, TG, HDL-C, and ALT were not altered by any of these strains. Based on the results regarding ovarian morphology, sex hormone levels, and metabolic indices, we conclude that *Lp. plantarum* F21M3 exhibited the most pronounced effects on PCOS symptom alleviation. Therefore, further investigation into how *Lp. plantarum* F21M3 exerts its probiotic roles in the PCOS model is warranted.

3.2 Butyrate was not involved in the therapeutic effects of Lp. plantarum F21M3 on PCOS symptoms

In previous studies, we found that butyrate produced by certain microbes in the distal gut can alleviate PCOS symptoms in rats via a gut-brain mechanism [12,14]. Therefore, short-chain fatty acid (SCFA) levels were analyzed to determine whether the probiotic role of *Lp. plantarum* F21M3 is related to colonic butyrate secretion. Among the six SCFAs, acetate, propionate, and butyrate concentrations were predominant in feces. SCFA levels were not significantly changed in the letrozole group. Notably, *Lp. plantarum* F21M3 intervention led to a significant increase in acetate levels and a slight increase in other SCFA levels (Figure S2A-G). This demonstrates that butyrate and other SCFAs (except acetate) may not be involved in the therapeutic effects of *Lp. plantarum* F21M3 on PCOS symptoms.

3.3 The therapeutic effects of Lp. plantarum F21M3 were accompanied by amelioration of gut microbiota dysbiosis

Since most probiotics can reach and exert their effects in the distal gut, the impact of *Lp. plantarum* F21M3 on the gut microbiota of PCOS rats was further analyzed. Shannon index, observed OTUs, Faith's PD index, and Pielou's evenness index were used to assess alpha diversity of gut microbiota. In letrozole-treated rats, decreased Shannon index and observed OTUs were observed. Administration of *Lp. plantarum* F21M3 significantly restored the reduction in Shannon index and observed OTUs (Figure 5A and B). Additionally,

Pielou's evenness index was found to be decreased by *Lp. plantarum* F21M3 intervention (Figure 5D). As shown in Figure 5E, the overall distribution of gut microbiota in the F21M3 group was significantly different from other groups according to ANOSIM analysis results (F21M3 vs. Control R=0.526 p=0.015, F21M3 vs. Letrozole R=0.665 p=0.017, F21M3 vs. Diane-35 R=0.444 p=0.013). These microbial diversity analysis results indicate that *Lp. plantarum* F21M3 intervention exerted a substantial impact on the gut microbiota of rats.

Abundances of specific microbes were further calculated to explain how *Lp. plantarum* F21M3 alters microbiota composition. As Figure 5F shows, Firmicutes and Bacteroidetes comprised more than 90% of the gut microbiota at the phylum level. The abundance of Actinobacteria was less than 1%, but it was significantly enriched after *Lp. plantarum* F21M3 intervention (Figure 5H). Furthermore, a total of 82 genera were detected. The distribution of the top 15 genera between groups is shown in Figure 5G. *Bacteroides*, *Lachnospiraceae_NK4A136_group*, and *Lactobacillus* were the dominant genera in the distal gut. To further identify biomarkers of different experimental groups, LEfSe analysis was performed. According to these results, five biomarkers were found in both the control group and the F21M3 group, while two biomarkers were observed in the Diane-35 group (Figure 5J). Figure 5K details the abundance of these biomarkers between groups at the genus level. When rats were treated with letrozole, the abundance of *Prevotellaceae_Ga6A1_group*, *Candidatus_Saccharimonas*, and *ASF356* decreased in their gut, and this dysbiosis could be restored by *Lp. plantarum* F21M3 intervention. Notably, *Bifidobacterium* was almost undetectable in the gut of normal rats or letrozole-treated rats but was significantly enriched after *Lp. plantarum* F21M3 administration. Considering the evolutionary relationships between Actinobacteria and *Bifidobacterium*, this result largely explains the enrichment of phylum Actinobacteria. Based on the gut microbiota analysis results, the dysbiosis of gut microbes can be partially restored by *Lp. plantarum* F21M3 administration, and *Bifidobacterium* may play a key role in this process. Amplicon sequencing technique rather than metagenomic sequencing technique was employed for the analysis of the gut microbiota. Due to this technical flaw, functional gene microbiota can not be further investigated. Therefore, some key functional taxa may be overlooked.

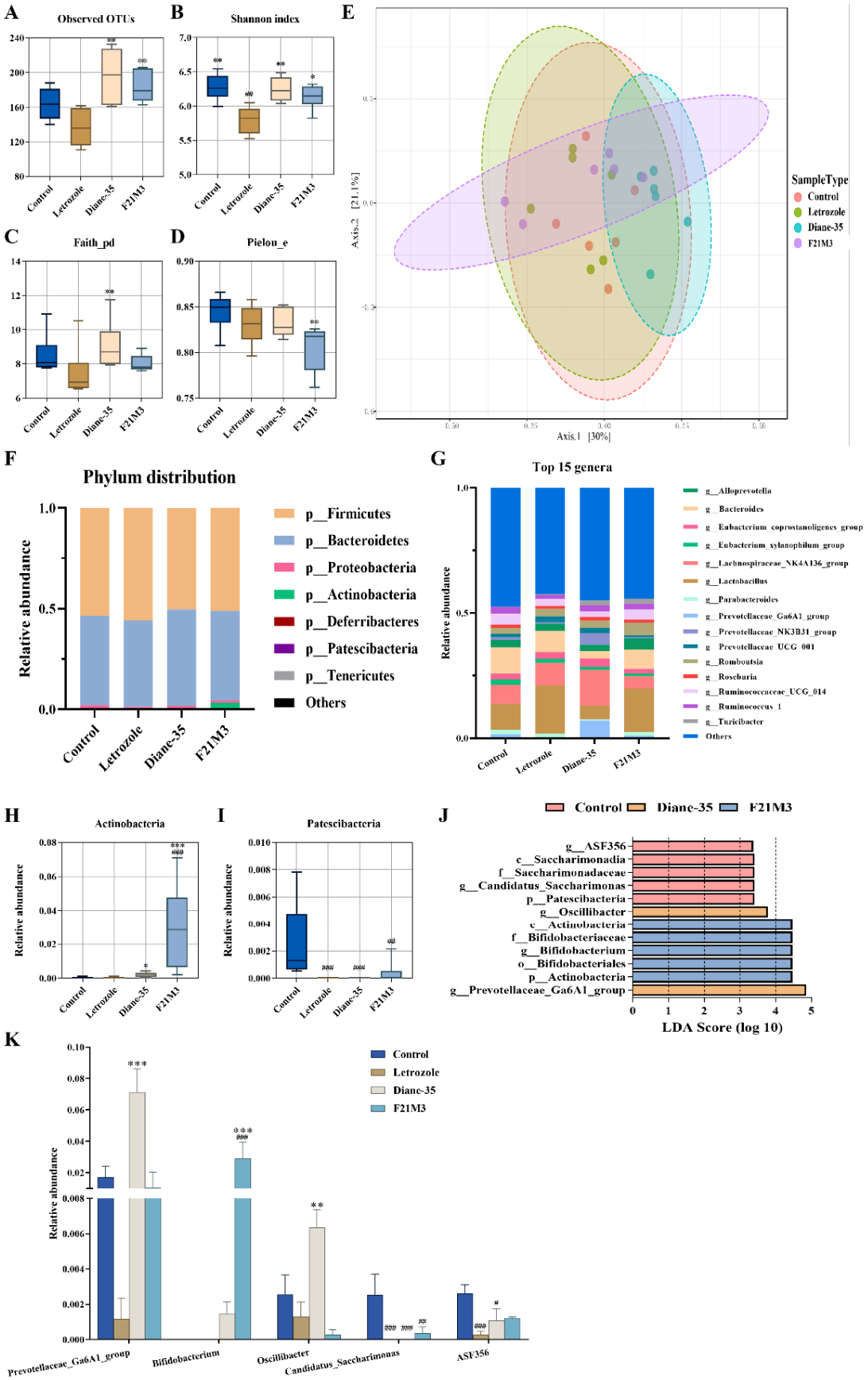


Figure 5. *Lp. plantarum* F21M3 intervention on fecal microbiota parameters of rats. (A) Observed OTUs. (B) Shannon index. (C) Faith_pd index. (D) Pielou_e index. (E) PCoA analysis using Bray-Curtis distance. (F) Microbial distribution at the phylum level. (G) Microbial distribution at the genus level. (H) Actinobacteria abundance. (I) Patescibacteria abundance. (J) LDA scores of LEfSe analysis. (K) Abundance of discrepant genera. Data are means ± SD or medians ± interquartile ranges. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the letrozole group using a one-way ANOVA (or Kruskal–Wallis test).

3.4 Lp. plantarum F21M3 exerted more pronounced effects on serum metabolites than fecal metabolites

Beyond direct interactions between gut microbiota and host, these microbes also influence the host through metabolite production [18,19]. Therefore, both fecal and serum metabolites of rats were measured using a UPLC-MS-based metabolomics technique. For fecal samples, 10,393 characteristic peaks were detected in positive ion mode and 4,461 characteristic peaks in negative ion mode. Using these characteristic peaks, 459 fecal metabolites were identified by mass spectrometry. For serum samples, the total number of characteristic peaks in positive and negative ion modes was 4,837 and 1,604, respectively. Based on these characteristic peaks, 287 serum metabolites were identified by mass spectrometry. Therefore, fecal metabolites exhibited greater richness than serum metabolites. To further analyze the overall distribution of identified metabolites between groups, principal component analysis (PCA) was performed. As shown in Figure 6A, compared with normal rats, letrozole treatment significantly altered the metabolite pattern of fecal samples. However, the fecal metabolite patterns in the Diane-35 and F21M3 groups were consistent with the letrozole group. For serum samples, the effect of letrozole administration on metabolite patterns was similar to that of fecal samples. Notably, *Lp. plantarum* F21M3 caused a distinct serum metabolite pattern, which also differed from normal rats (Figure 7A). From this result, we can infer that some new metabolites were enriched in the serum of *Lp. plantarum* F21M3-treated rats.

To further identify which metabolites were enriched by *Lp. plantarum* F21M3, samples from the letrozole and F21M3 groups were compared. For fecal samples, 22 metabolites were significantly changed based on Student's t-test results (Figure S3). Due to the overfitting problem of partial least squares-discriminant analysis (PLS-DA) on fecal samples, a random forest model was used for further analysis, identifying 15 differential metabolites (Figure 6B). Finally, 8 metabolites met the requirements of both Student's t-test and PLS-DA and were recognized as fecal differential metabolites (Figure 6C). For serum samples, Student's t-test identified 32 metabolites that differed between the letrozole and F21M3 groups (Figure S3). The overfitting problem was not observed in serum samples (when test number is 1,000, Q^2=0.68, p=0.003); therefore, the PLS-DA model could be used, and 44 metabolites were selected by this method. Combining the results of Student's t-test and PLS-DA, 28 serum differential metabolites were identified (Figure 7B). Based on these results, despite more metabolites being observed in fecal samples, *Lp. plantarum* F21M3 exerted more conspicuous effects on serum samples.

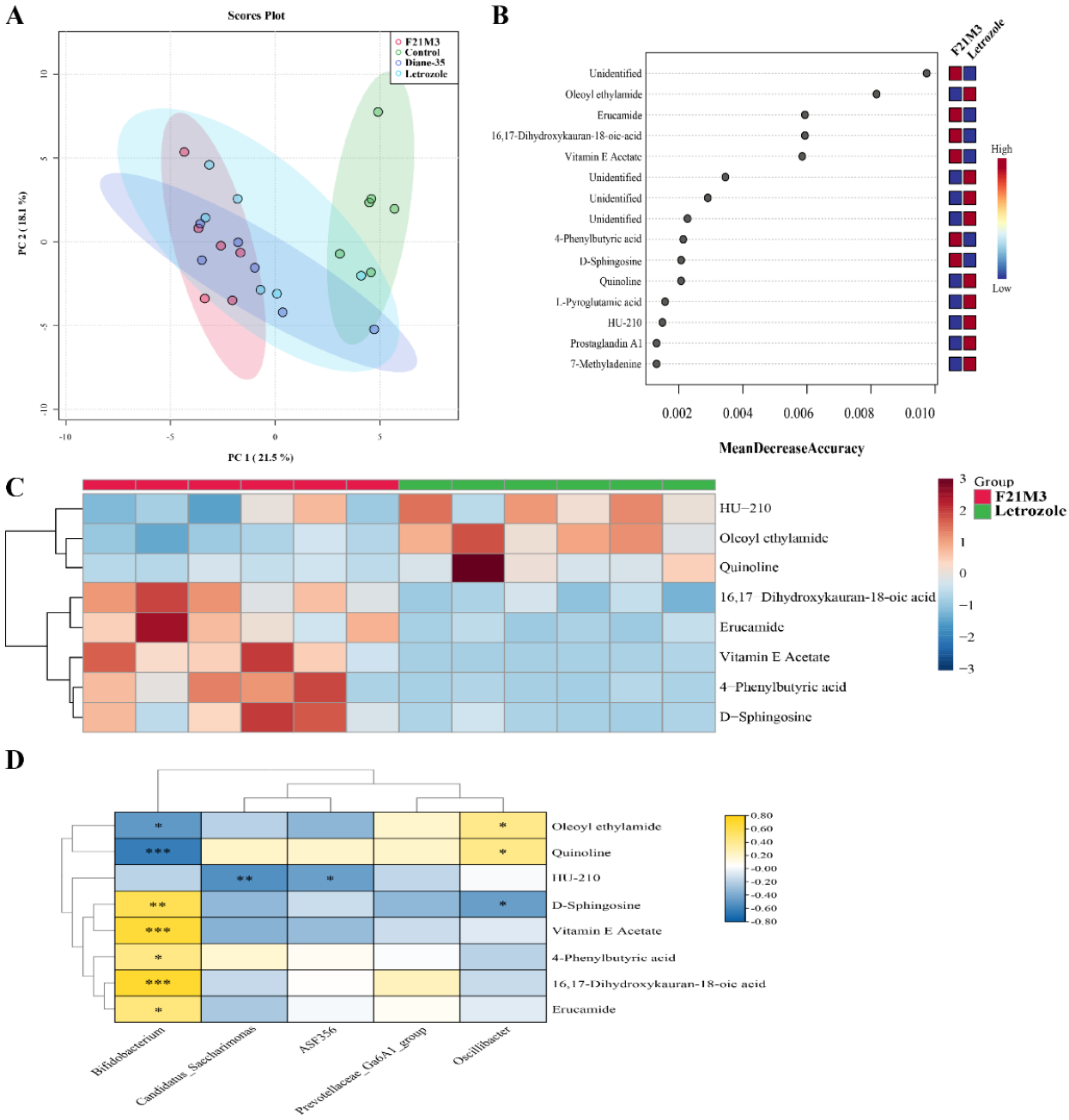


Figure 6. *Lp. plantarum* F21M3 intervention on fecal metabolites of rats. (A) PCA analysis. (B) Mean decrease accuracy scores of random forest analysis. (C) Heat map of differential metabolites. Red indicates higher metabolite abundance and blue indicate lower microbial abundance when all samples are compared together. (D) Correlation between fecal representative taxa and differential metabolites. The nonparametric Spearman's test was performed ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Yellow indicates a positive correlation; blue indicates a negative correlation.

3.5 Progestational hormone biosynthesis was activated after Lp. plantarum F21M3 administration

By searching several databases, these selected differential metabolites were identified, and their variation trends were shown by heatmaps. For fecal samples, vitamin E acetate, 4-phenylbutyric acid, D-sphingosine, 16,17-dihydroxykauran-18-oic acid, and erucamide increased, while oleoyl ethylamide and quinoline decreased after *Lp. plantarum* F21M3 treatment (Figure 6C). Among the 28 differential metabolites in serum samples, 11 metabolites were upregulated and 17 metabolites were reduced (Figure 7B). Notably, higher levels of progesterone, pregnenolone, and 5α-pregnan-3,20-dione were detected in the serum of *Lp. plantarum* F21M3-treated rats, and these compounds are closely associated with progestational hormone biosynthesis.

Based on these differential metabolites, enrichment analysis was performed. Pathways related to steroid hormone biosynthesis and unsaturated fatty acid biosynthesis were significantly enriched (Figure 7C). This also demonstrated that *Lp. plantarum* F21M3 administration enhanced progestational hormone biosynthesis activity. Additionally, the activated progestational hormone biosynthesis was not observed in the distal gut.

As gut microbiota dysbiosis was recovered by *Lp. plantarum* F21M3, certain gut microbes may also contribute to progestational hormone biosynthesis activation. Therefore, Spearman correlation analysis was performed between representative taxa and serum differential metabolites. As shown in Figure 7D, *Bifidobacterium* was positively correlated with 5α-pregnan-3,20-dione levels, while *Candidatus_Saccharimonas* and *ASF356* were positively correlated with progesterone and pregnenolone levels. Therefore, higher levels of progestational hormone biosynthesis may be the direct cause of PCOS symptom alleviation. Behind this, the recuperative abundance of *Candidatus_Saccharimonas* and *ASF356*, and the enrichment of *Bifidobacterium* may play key roles from the perspective of gut microecology. To further confirm the progestational hormone biosynthesis activation, qPCR analysis of genes controlling the rate of progesterone synthesis were performed. When rats were treated with letrozole, decreased expression of *StAR*, *SCC* and *3β-HSD* were observed in the ovary. Intervention of *Lp. plantarum* F21M3 recovered the expression of *StAR* and *SCC* (Figure 7E, F and G). The *Lp. plantarum* strain F21M3 has been preserved at the Food Biotechnology Centre of Jiangnan University under the serial number CCFM1202.

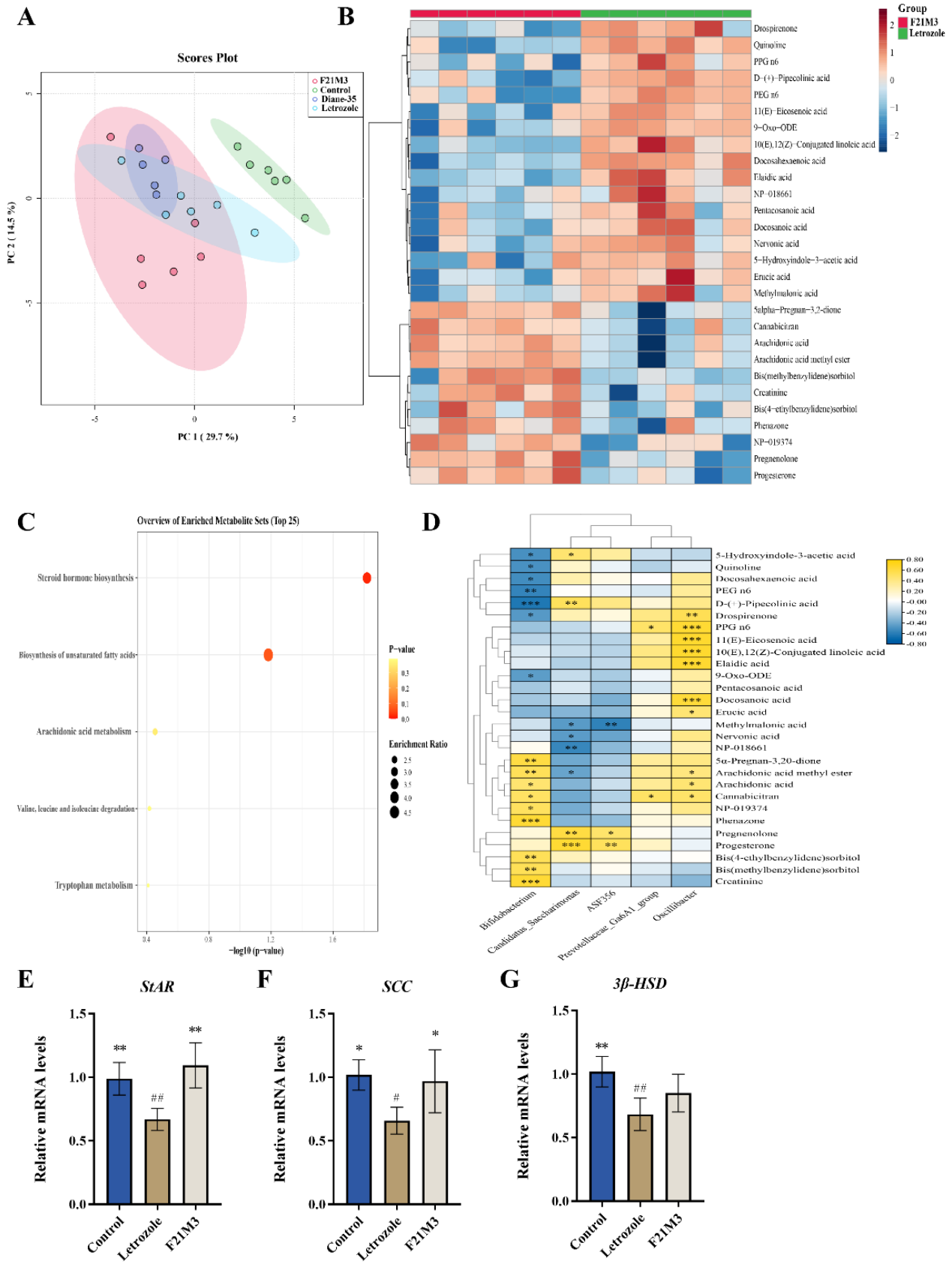


Figure 7. *Lp. plantarum* F21M3 intervention on serum metabolites of rats. (A) PCA analysis. (B) Heat map of differential metabolites. Red indicates higher metabolite abundance and blue indicate lower microbial abundance when all samples are compared together. (C) Enrichment analysis of differential metabolites. (D) Correlation between fecal representative taxa and differential metabolites. The nonparametric Spearman's test was performed ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Yellow indicates a positive correlation; blue indicates a negative correlation. (E) *StAR* expression (n=4). (F) *SCC* expression (n=4). (G) *3β-HSD* expression (n=4). Data are means ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the letrozole group using a one-way ANOVA (or Kruskal–Wallis test).

4. Discussion

As the pathogenic mechanism of PCOS has not been clearly elucidated, PCOS therapies always aim at reducing clinical symptoms [20]. In this case, lifestyle modification should be the preferred treatment strategy. Among different lifestyle modifications, improving dietary patterns is the easiest to implement, and the effectiveness of this strategy has been demonstrated by several studies [21,22]. Probiotics are widely accepted by consumers as dietary supplements. Screening probiotic strains that can alleviate PCOS symptoms will contribute to the value of dietary modification. Most *Lactobacillus* species are considered probiotics. However, among these species, it is unclear which ones are more suitable for PCOS treatment. In addition to the biological characteristic differences of the species themselves, microecological results may also provide valuable answers to this question. Due to the decreased gut abundance of *Lp. plantarum* in PCOS patients, administration of *Lp. plantarum* strains may be a promising treatment strategy. Because decrease of *Lp. plantarum* abundance was more pronounced in obese PCOS subjects. Thus, *Lp. plantarum* may exert superior role in obese PCOS patients. The curative role of *Lp. plantarum* strains on PCOS symptoms has been demonstrated by our previous studies [11,12]. Considering the heterogeneous nature of PCOS, *Lp. plantarum* strains may alleviate PCOS symptoms via different mechanisms. In the present study, six *Lp. plantarum* strains were screened for their ability to reduce PCOS symptoms. *Lp. plantarum* F21M3 was selected, and the PCOS-mitigating mechanism of this strain was further investigated from the perspective of gut microecology.

As a highly selective aromatase inhibitor, letrozole inhibits the conversion of testosterone and androstenedione to estradiol and estrone, resulting in decreased estrogen levels. Letrozole treatment induces elevated androgen concentrations, which can be utilized to simulate PCOS symptoms in animal models. *Lp. plantarum* strains are generally recognized as safe and widely employed in food production. However, regarding probiotic functions, different strains may exhibit substantial variations, even when classified within the same species [23,24]. In this study, the therapeutic efficacy of six *Lp. plantarum* strains on PCOS symptoms was evaluated. Overall, only *Lp. plantarum* F21M3 demonstrated significant effects on PCOS symptom alleviation. According to the animal study, continuous consumption of *Lp. plantarum* F21M3 (viable count more than 10^9 CFU/d) for no less than one month may yield satisfactory results for PCOS patients. In our previous investigation, five *Lp. plantarum* strains were screened, with only one strain proving effective [12]. Both studies demonstrated that intraspecific differences should be thoroughly considered when utilizing lactic acid bacteria for disease symptom relief.

SCFAs are crucial bacterial metabolites derived from dietary sources. Diseases including inflammatory bowel diseases, diabetes, and atherosclerosis have been associated with decreased intestinal SCFA levels [25,26]. Additionally, butyrate can function as an endocrine regulator by stimulating gut peptide secretion and reducing PCOS symptoms [14, 27]. Therefore, it is essential to determine whether the therapeutic effect of *Lp. plantarum* F21M3 on PCOS symptoms relates to enhanced butyrate production. However, based on our findings, fecal butyrate levels remained unchanged following *Lp. plantarum* F21M3 treatment. This suggests that the butyrate-gut endocrine mechanism is not the sole pathway through which *Lp. plantarum* strains reduce

PCOS symptoms. Although a slight increase in fecal acetate levels was observed in the F21M3 group, acetate may not be the compound responsible for PCOS symptom reduction. Studies have demonstrated the role of oral acetate intake on PCOS-related epigenetic regulators, with the hypothalamus potentially serving as the target organ [28,29]. However, no evidence suggests that PCOS symptoms can be ameliorated by gut-derived acetate. Apart from SCFA mechanisms, immune-related mechanisms were not investigated in the current study. Abnormal glucose and lipid metabolism are usually accompanied by chronic inflammation, but the effects of *Lp. plantarum* F21M3 intervention on metabolic indicators were not significant. This suggested *Lp. plantarum* F21M3 may not alleviate PCOS symptoms via anti-inflammatory mechanisms.

Hundreds of billions of microbes inhabit the human gut, creating a complex ecosystem. Gut microecological dysbiosis, characterized by decreased levels of beneficial microbes and metabolites, has been linked to numerous diseases [30]. The relationship between PCOS and gut microbiome has been established as bidirectional [3]. Gut dysbiosis is observed in PCOS patients, while gut microbiome modification can alleviate PCOS symptoms [31]. Therefore, the therapeutic effect of probiotic strains on PCOS may operate through gut microbiome-related mechanisms. The observed OTUs index reflects microbial community richness, while the Pielou_e index reflects biocoenosis evenness. The Shannon index is determined by both microbial community richness and evenness [32]. Alpha diversity analysis revealed that decreased gut microbial richness and evenness in PCOS rats was insignificant, indicating that excess androgen levels exert negligible effects on microbial alpha diversity. Similar results have been observed in both animal studies and clinical trials [33,34]. Although microbial species number was restored by *Lp. plantarum* F21M3 administration, gut microbial evenness was significantly decreased by this strain, suggesting that certain microbes were markedly enriched in the gut of *Lp. plantarum* F21M3-treated rats. Among these microbes, *Bifidobacterium* represents the most notable taxa. As an important member of the innate human gut microbial community, *Bifidobacterium* is generally considered a biomarker of healthy gut microbiota [35]. Decreased *Bifidobacterium* abundance is observed in PCOS subjects [33]. Therefore, *Bifidobacterium* enrichment may indicate gut dysbiosis recovery. Although more than 10^9 CFU *Lp. plantarum* cells were ingested daily in the F21M3 group, *Lactobacillus* abundance in gut microbiota remained unchanged. The total cell number of genus *Lactobacillus* can reach 10^{12} CFU in the rat gut, which is 1000-fold greater than the ingested *Lp. plantarum* cells. Therefore, *Lp. plantarum* F21M3 intervention was insufficient to directly impact total *Lactobacillus* bacterial cells. Indirect mechanisms, such as regulation of other microbial species and metabolites, may play key roles in PCOS alleviation by *Lp. plantarum* F21M3.

Metabolites are considered important mediators between the host and gut microbiota. Microbially-derived bile acids, amino acids, and fatty acids have demonstrated beneficial impacts on numerous diseases [36,37]. These metabolites can directly interact with enterocytes or enter peripheral circulation via enterohepatic circulation, extending their impact beyond the gut. Using high-resolution mass spectrometry, the distribution and composition of small molecule metabolites can be systematically analyzed. Metabolite profiles of PCOS patients have been investigated in recent studies, with metabolites in serum,

feces, follicular fluid, and urine reported to be altered in PCOS patients [38-40]. When fecal bacterial taxa, fungal taxa, and serum metabolites were used to build PCOS predictive models, the serum metabolite-based model exhibited the highest accuracy [41]. Therefore, metabolites may be considered more efficient predictive targets for PCOS than microbes. It was worth noting that quinoline was both identified as serum and fecal differential metabolites. The ratio of quinoline was reduced after *Lp. plantarum* F21M3 treatment. Quinoline and its derivatives are toxic to animals and humans. Therefore, the decrease of quinoline ratio both in serum and fecal samples can be considered as a positive signal. However, there are no research indicating an association between quinoline and PCOS yet. Thus, the decrease of quinoline content may not participate in PCOS alleviation.

Previous studies have consistently established connections between microbiota and metabolites [42,43]. Beyond these findings, the present study compared differences between serum and fecal metabolites in PCOS models. Although fecal metabolites exhibited higher richness than serum metabolites, *Lp. plantarum* F21M3 exerted more significant effects on serum metabolites. Based on our results, serum progesterone levels increased and progestational hormone biosynthesis was activated by *Lp. plantarum* F21M3 administration. As antagonists of androgens, progestational hormones have been proven to decrease androgen levels and reduce PCOS symptoms [44,45]. This mechanism may explain how *Lp. plantarum* F21M3 exerts anti-PCOS effects. Significant correlations were identified between differential metabolites and gut microbes, particularly between progestational hormones and *Bifidobacterium*. Progesterone has been demonstrated to promote *Bifidobacterium* growth during late pregnancy [46]. Additionally, enrichment of *Bifidobacterium* has been observed in the salivary microbiota of pregnant women [47]. This evidence indicates that increased *Bifidobacterium* abundance may result from activated progestational hormone biosynthesis. Conversely, increased progestational hormone levels were not observed in fecal samples, indicating that progestational hormones were not directly synthesized by gut microbiota. Cholesterol side-chain cleavage enzyme (SCC) is an inner mitochondria membrane protein that catalyzes the conversion of cholesterol to pregnenolone. This process can also be facilitated by steroidogenic acute regulatory protein (StAR). Once produced, pregnenolone diffuses from the mitochondria to the cytoplasm, where 3β-hydroxysteroid dehydrogenase (3β-HSD) converts it to the first biologically active hormone, progesterone [48]. Therefore, SCC, 3β-HSD and 3β-HSD are the rate-limiting enzymes that regulates the synthesis of progestational hormones in the ovary. The qPCR results further confirmed progestational hormones biosynthesis was activated in the ovary. Compared with feces, the changes in metabolites in serum should have a more direct impact on the biosynthesis of progestational hormones.

Notably, vitamin E acetate (a derivative of vitamin E with similar biological activities) was enriched in feces following *Lp. plantarum* F21M3 treatment. Studies have indicated that plasma progesterone concentrations increase with vitamin E acetate administration [49,50]. Therefore, activation of progestational hormone biosynthesis may be mediated by vitamin E. However, the detailed interactions between vitamin E and progestational hormones biosynthesis production require further investigation. PCOS related cells can be

regarded as ideal models used to verify this conjecture. Gut microbiota shaped by *Lp. plantarum* F21M3 may explain vitamin E enrichment. This hypothesis is supported by another study revealing relationships between vitamin E degradation and gut microbiota [51].

5. Conclusions

In this work, *Lp. plantarum* F21M3 was screened from six *Lp. plantarum* strains and demonstrated significant curative effects on abnormal ovarian morphological features and disturbed sex hormone levels. Results from gut microbiota and metabolome analyses suggest that *Lp. plantarum* F21M3 may alleviate PCOS symptoms by regulating gut microbiota and facilitating progestational hormone biosynthesis. Vitamin E may act as a key mediator promoting progestational hormone levels, which requires confirmation in future studies. In addition, the number of candidates used for strain screening is relatively small, which may not enough to prove the superior efficacy of *Lp. plantarum* F21M3. These findings indicate the potential benefits of *Lp. plantarum* strains as PCOS treatments and highlight the possibility of targeting gut microbiota to regulate progestational hormones.

Conflicts of Interest:

The authors declare no conflicts of interest.

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