Gut microbiota in women with polycystic ovary syndrome: an individual based analysis of publicly available data

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Summary

Background Polycystic ovary syndrome (PCOS) represents a prevalent endocrine disorder affecting numerous females worldwide. Dysbiosis of gut microbiota has been linked to the occurrence of PCOS; however, research into the characteristics of gut microbiota in PCOS patients, especially those from different regions and with different testosterone level, remains limited. Additionally, it is still unclear whether gut microbiota helps to distinguish different PCOS subtypes.

Methods We searched four electronic databases (PubMed, Web of Science, Cochrane Library, and ClinicalTrials.gov) from Jan 1, 2010 to May 1, 2024. This combined analysis included studies providing the raw data of gut microbiota in PCOS patients. We reanalyzed the characteristics of gut microbiota in PCOS patients from different regions and with different testosterone level.

Findings Fourteen publications satisfying the inclusion criteria were included in the combined analysis. Based on data from 948 individuals, we found alpha-diversity was not significantly different between PCOS and healthy control (HC) groups. However, gut microbiota composition was distinct in PCOS patients compared with healthy individuals. Specifically, *Fusobacterium*, *Ruminococcus_gnavus_group*, and *Escherichia-Shigella* increased, while *Dysosmobacter*, *Schaedlerella*, *Merdimonas*, *Clostridiisalibacter*, *Flintibacter* et al. decreased in PCOS women. Regionally, *Alistipes* was enriched in primarily European patients, while *Blautia* and *Roseburia* were more abundant in Chinese patients. Subtype analysis revealed that the gut microbiota of PCOS patients with higher testosterone level (PCOS-HT) differed significantly from those with lower testosterone level (PCOS-LT). *Prevotella*, *Blautia*, *Dialister*, *Ruminococcus_torques_group* and *UCG-002* were enhanced in PCOS-HT patients, while *Alistipes*, *Dysosmobacter*, *Phocaeicola* and *Faecalibacterium* were diminished. Importantly, a set of eight genera effectively differentiated PCOS-HT patients from PCOS-LT patients with an AUC of 0.95.

Interpretation This systematic anatomization of gut microbiota revealed the microbial characteristics of PCOS patients, particularly those with different testosterone level, thus laying the foundations for further research into pathogenesis of PCOS, and the development of effective diagnostic, treatment, and intervention strategies.

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Keywords: Polycystic ovary syndrome (PCOS); Gut microbiota; Testosterone; Biomarker

Introduction

Polycystic ovary syndrome (PCOS) is a reproductive endocrine disorder affecting women of reproductive age, characterized by increased androgen, polycystic ovarian morphology injury, ovulatory dysfunction, hirsutism and/or acne.[1] According to epidemiological

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Research in context

Evidence before this study
Dysbiosis of gut microbiota has been linked to the occurrence of polycystic ovary syndrome (PCOS), yet characterization of gut microbiota in different subtype PCOS is limited. We searched four electronic databases including PubMed, Web of Science, Cochrane Library, and ClinicalTrials.gov from Jan 1, 2010 to May 1, 2024 using search terms of "((polycystic ovary syndrome OR PCOS) AND (gut microbiota OR gut bacteria with no restrictions on language and region. The search identified 14 publications comprised 513 PCOS patients and 435 healthy controls.

Added value of this study
This combined analysis is the first to characterize gut microbiota in PCOS patients by re-analyzing raw sequencing

data from 14 datasets. This comprehensive analysis of gut microbiota highlights key microbial characteristics in PCOS, particularly in those with higher testosterone, as well as regional differences (China and Europe) and lays the groundwork for future research on pathogenesis, diagnostics, and treatment strategies.

Implications of all the available evidence
Identifying the gut microbiota of PCOS patients in different regions may influence current practices for disease treatment. In addition, gut microbiota may serve as a biomarker to distinguish different subtypes of PCOS, promoting clinical diagnosis and treatment of PCOS.

surveys, 10%–15% of Chinese women suffer from infertility, with PCOS accounting for about 50% of these cases.[2] PCOS is linked to obesity, insulin resistance, metabolic syndrome, cardiovascular disease and cancer,[3] posing a considerable burden on public health systems and the affected women. In spite of being typically diagnosed during early reproductive years, the precise etiology and pathology of PCOS remain unclear.[4] Meanwhile, due to the heterogeneity of clinical phenotypes and individual difference, available therapies are often inadequate for many patients, leading to recurrent pregnancy failure. Therefore, identifying safe and effective strategies for the prevention and treatment of PCOS is a primary focus for reproductive endocrinologists.

For the clinical diagnosis of patients with PCOS, significant progression has been made recently. For example, serum anti-Mullerian hormone (AMH)[5] and small-molecule metabolites in urine can be served as biomarkers for complementary diagnosis of the different phenotypes of PCOS.[6] Additionally, genome-wide association study (GWAS) analysis proposes that hexadecanedioate and dihomo-linolenate can be used to screen for PCOS.[7] Another GWAS analysis revealed that two single nucleotide polymorphisms (SNPs) (rs17186366 and rs11171739) have been regarded as candidate PCOS risk factors.[8] Although the diagnosis of PCOS has made many advances, the screening and diagnostic markers are lacking for different subtypes of PCOS patients, since some women without hyper-androgenism will still be diagnosed as suffering PCOS.

Human gut microbiota aids in food digestion, nutrient absorption, metabolism and immune regulation, influencing the development of various diseases,[9–14] such as PCOS. One prospective, case–control cross-sectional study found that PCOS patients have higher levels of Actinobacteria phylum and Streptococcaceae family, along with the lower levels of

Bacteroidota phylum and Bacteroidaceae, Porphyromonadaceae family.[15] Progressive studies have confirmed that gut microbiota-mediated bile acid metabolism is crucial for PCOS treatment.[16,17] Specifically, *Bacteroides vulgatus* was increased in PCOS women, while glycodeoxycholic acid and tauroursodeoxycholic acid were decreased. Fecal microbiota transplantation (FMT) from PCOS women to mice lead to an increased disruption of ovarian functions and altered bile acid metabolism. Notably, glycodeoxycholic acid can lead to the secretion of intestinal IL-22, thus alleviate PCOS development.[13] Additionally, supplementation with specific gut bacteria, such as *Lactiplantibacillus plantarum* CCFM1019, can alleviate pathological ovarian injury and regulate testosterone and luteinising hormone levels, thus alleviate PCOS symptoms.[18] Although several meta-analysis have reported changes in the gut microbiota of PCOS patients,[19–21] these descriptions are only based on original literature results and cannot find consistent or specific changes in bacteria, since there is significant heterogeneity in gut microbiota research. Additionally, a causal relationship between gut microbiota and PCOS has not been established. Furthermore, it is also unclear if gut microbiota differences exist among PCOS patients from different regions or between those with different testosterone levels, as current findings are preliminary.

To unravel the specific characteristics of gut microbiota in PCOS patients, particularly regional differences, and variations between those with different testosterone levels, we conducted this combined analysis by synthesizing current evidence on the gut microbiota profiles of PCOS patients. By identifying gut microbiota patterns associated with PCOS subtypes, we devoted to identify potential microbial biomarkers for PCOS subtypes, and proposed potential reasons for individual differences in the PCOS treatment. These ongoing endeavors will

promote the understanding of PCOS pathogenesis, and advance personalized treatment strategies for PCOS.

Methods

Ethical statement

This combined analysis was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. The protocol has been registered on the INPLASSY website with the registration number INPLASY202490105.

Search strategy and selection criteria

We performed a comprehensive literature search using multiple databases including PubMed, Web of Science, Cochrane Library and ClinicalTrials.gov online databases from Jan 1, 2010 to May 1, 2024. Our used search terms or text terms were as follows: ("polycystic ovary syndrome" OR "PCOS") AND ("gut microbiota" OR "gut bacteria"). To compensate the omissions in our search strategy, we have also screened the original articles mentioned in the reviews. There were no restrictions on language and region. Unpublished studies were excluded in our analysis. The detailed selection criteria was outlined in the in the Appendix Supplementary 1.

Data extraction

A standard process was employed to extract data. Specifically, the Microsoft Excel file has been built to capture the key information of literature, including author's name, journal's name, publication year, country where volunteers were recruited, study design, sample size, the general indices of participants, covariates and potential confounding variables, collection and preservation of fecal samples, sequencing techniques, gene sequencing fragments and study outcomes. Data extraction was also completed independently by two authors. If any disagreements existed, two authors discussed by referring back to the original text and reached a consensus.

Quality assessment

The quality of the cohort studies was assessed using Newcastle-Ottawa scale (NOS). The detailed quality assessment criteria is shown in the Appendix Supplementary 2.

Bioinformatics analysis

The raw sequence reads of 14 available datasets were downloaded from the National Center for Biotechnology Information (NCBI) under project, PRJNA326866,[22] PRJEB22972,[23] PRJNA866344,[24] PRJNA934689,[25] PRJNA 779930,[26] PRJNA899143,[27] PRJNA786067,[28] PRJNA 760247,[29] PRJNA737206,[30] PRJNA622999,[31] PRJNA694729,[32] PRJNA634229,[33] PRJNA341567[34] and PRJNA981428[35] using Linux virtual machine (Ubuntu 20.04.6 LTS). By using SRAtoolkits, the downloaded SRA files were converted into

fastq files, and then subjected to quality control using FastQC. After ensuring that the quality meets the standards, QIIME2 (version 2023.05) was employed to process the raw sequence with default parameters (https://docs.qiime2.org/). The further analysis, including noise reduction, correcting marginal sequence errors, removing chimeric sequences, removing singletons, joining paired-end reads, and dereplication, was achieved using DADA2 plugin. The species annotation results were based on the SILVA (version 138.1) (http://www.arb-silva.de/). ASV (amplicon sequence variants) were defined based on 100% sequence similarity between the sequences. The normalized ASV abundance data was subjected to alpha-diversity analysis, beta-diversity analysis, difference analysis and ecological network analysis using R (version 4.1.2). The Vegan package (version 2.6–6.1) was used to calculate the alpha-diversity and beta diversity. The ggplot2 package (version 3.5.0) was used to visualization. The NetMoss analysis was shown in the Appendix Supplementary 3.

Statistical analysis

The statistical difference of beta diversity was assessed by PERMANOVA. The statistical difference of alpha-diversity was assessed by Wilcoxon test or Kruskal–Wallis teest. The differential bacteria were analyzed using Wilcoxon test and P-values were corrected using BH to FDR values. $P < 0.05$ or FDR <0.05 were considered to be statistically significant.

Role of the funding source

The funder had no involvement in data collection, analysis, interpretation, writing of the manuscript and the decision to submit.

Results

Study selection

A total of 563 studies were retrieved after the literature search, including 245 from PubMed, 272 from Web of Science, 21 from Cochrane Library, and 25 from ClinicalTrials.gov. Among these, 188 studies were excluded due to duplication. The remaining 375 studies were subjected to screen via reading their titles and abstracts. Following rigorous screening, we finally collected 52 papers for assessment. According to our exclusion criteria, 38 studies were removed as they did not provide raw sequencing data, did not employ 16S rRNA sequencing, or did not focus on gut microbiota. Ultimately, 14 publications were included in our combined analysis. An outline of the literature screening process was illustrated in Fig. 1.

Overall characteristics of included studies

The included studies consist of 14 cohort studies. A total of 11 studies were conducted in China, and 3 studies were conducted in Austria, Poland and Russia, with one in each country. The total sample comprised 513 PCOS
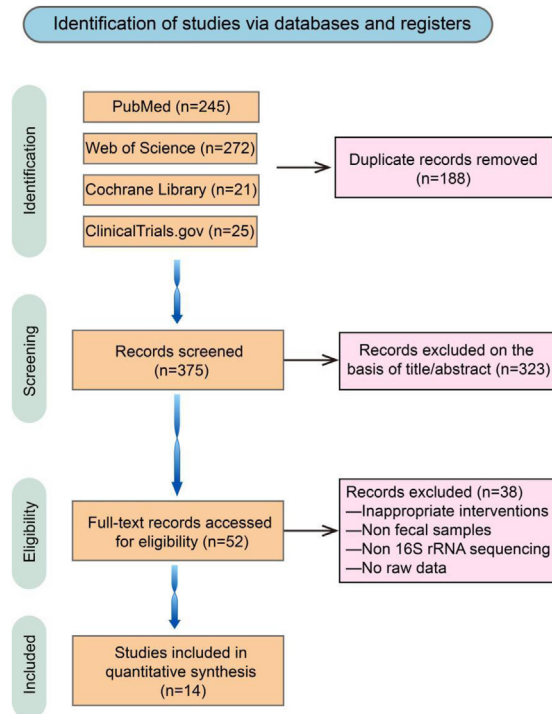
Fig. 1: The PRISMA flow diagram of literature screening. Period from Jan 1, 2010 to May 1, 2024 only.

and 435 healthy controls, with ages ranging from 23 years old to 35 years old. Key PCOS-related indicators that researchers focused on included levels of testosterone, free androgen index, HOMA-IR index, fasting glucose and fasting insulin. Notably, testosterone levels were markedly higher in PCOS patients compared to healthy individuals in 9 studies, with an increasing trend observed in PCOS patients in 2 studies (Table 1). The detailed information of gut microbiota sequencing is shown in Table 2, including sample collection, storage, sequencing technology and primers. The quality assessment, analysis of gut microbiota-related confounding factors, heterogeneity evaluation and publication bias were provided in the Appendix Supplementary 4–6.

Characteristics of gut microbiota in women with PCOS

Overall, analysis of Chao1 and Shannon diversity indices showed no significant differences between the PCOS and HC groups (Fig. 2A and B). However, score plots of principal coordinate analysis (PCoA) indicated the separation of gut microbiota structure among two groups (adjust $P = 0.001$) (Fig. 2C). Analysis at the phylum level revealed slight reductions in Bacillota in PCOS patients, with increases noted in Actinobacteriota (Fig. 2D). At the genus level, there were marginal increase in *Bacteroides, Blautia* and *Bifidobacterium* among

PCOS women, while *Ruminococcus* showed decreased relative abundance (Fig. 2E). Further characterization of gut microbial composition between PCOS and HC using Wilcoxon non-parametric testing identified 12 genera downregulated and 4 upregulated in PCOS (Fig. 3A). Notably, genera such as *Mesomycoplasma, TM7x, Delftia, Ruminococcus_gnavus_group,* were elevated in PCOS patients, whereas *Millionella, Bacteroides_pectinophilus_group, Desulfosarcina,* Lachnospiraceae_UCG-008, were reduced (FDR <0.05) (Fig. 3B). STAMP analysis, a method for intergroup difference comparison, highlighted top 20 genera with significant intergroup differences, including 8 upregulated and 12 downregulated genera in PCOS ($P < 0.05$) (Fig. 3C). Specifically, *Bifidobacterium, Butyricicoccus, Blautia, Mesomycoplasma, Bacteroides, Eggerthella, Bilophila* and *Ruminococcus_gnavus_group* were increased, while *UCG-002, UCG-005, Ruminococcus,* Christensenellaceae_R-7_group, *Clostridia_UCG-014, Holdemanella, Mucispirillum, Desulfosarcina,* Rikenellaceae_RC9_gut_group, *Holdemanella,* Lachnospiraceae_UCG-008, *UCG-010* and *Millionella* were decreased in PCOS women, which is partly consistent with previous reported results (Appendix Supplementary Table S3). To further explore potential biomarkers associated with PCOS, we applied NetMoss analysis,[36] focusing on genera with average abundances exceeding 0.01%. Key taxa *Blautia* was identified in both PCOS and HC individuals, while *Burkholderia* and *Pseudescherichia* were only presented in HC individuals. Additionally, some correlations were altered when comparing PCOS group to HC group. For instance, positive correlation between *Blautia* and Erysipelotrichaceae_UCG-003 was presented in PCOS patients as compared with HC individuals (Fig. 3D). Eighteen bacteria emerged as significant markers based on NetMoss score exceeding 0.8 and a P value less than 0.05 (Appendix Supplementary Table S4), contributing to an overall area under curve (AUC) of 0.77 in distinguishing PCOS patients from healthy controls (Fig. 3E).

Characteristics of gut microbiota in PCOS patients across different region

Geographically, we stratified our population (aged from 23 to 35 years old) into Chinese (N = 585) and European (363) subgroups. Alpha-diversity analysis showed a marked decrease in Chinese PCOS patients compared to their healthy counterparts, whereas a increase in European PCOS patients (Appendix Supplementary Fig. S1A-B). Principal coordinate analysis (PCoA) based on bray–curtis distance revealed distinct clustering of gut microbiota between Chinese and European populations, with clear separation observed between PCOS and healthy controls within each region (Appendix Supplementary Fig. S1C-D). The profile of gut microbial community also varied significantly between Chinese and European populations. Specifically, Bacteroidota, Proteobacteriota and Actinobacteriota

Ref.	Country	Sample Size (N)		Age (years)		Testosterone		Free androgen index		HOMA-IR index		Fasting glucose		Fasting insulin	
		HC	PCOS	HC	PCOS	HC	PCOS	HC	PCOS	HC	PCOS	HC	PCOS	HC	PCOS
22	Austria	19	24	32.0	27.0	1.10 nmol/L	1.30 nmol/L	1.30	3.10	0.80	1.70	4.50 nmol/L	4.70 nmol/L	41.40 pmol/L	84.40 pmol/L
23	Poland	48	73	29.4 ± 4.9	27.4 ± 4.9	0.30 ± 0.10 ng/mL	0.56 ± 0.20 ng/mL	N/R	N/R	1.75 ± 0.7	2.27 ± 1.54	4.86 ± 0.34 nmol/L	5.14 ± 1.87 nmol/L	48.5 ± 18.5 pmol/L	61.40 ± 38.20 pmol/L
24	China	12	14	31.5 ± 5.6	26.9 ± 4.2	1.26 ± 0.56	1.72 ± 0.30	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
25	China	N/R	12	N/R	28.0	N/R	74.68 ng/dL	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
26	China	20	20	26.8 ± 5.5	29.0 ± 5.8	0.24 ± 0.12	0.61 ± 0.37	N/R	N/R	N/R	N/R	N/R	N/R	7.80 ± 2.71	12.00 ± 7.35
27	Russia	131	68	35.1 ± 5.7	29.5 ± 5.2	29.20 ± 23.80 ng/dL	46.30 ± 26.10 ng/dL	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
28	China	41	47	29.2	29.0	0.25 ng/mL	0.48 ng/mL	2.07	7.09	3.00	3.86	4.97 nmol/L	5.43 nmol/L	13.35 mIU/L	15.64 mIU/L
29	China	24	24	28.2 ± 3.0	24.9 ± 5.1	N/R	N/R	N/R	N/R	N/R	N/R	4.62 ± 0.47 nmol/L	5.03 ± 2.49 nmol/L	N/R	N/R
30	China	36	98	29.3	29.6	0.28 ng/mL	0.50 ng/mL	1.56	6.77	1.69	3.73	4.81 nmol/L	5.44 nmol/L	7.78 mIU/L	15.19 mIU/L
31	China	20	20	27.6	26.5	1.27 nmol/L	1.63 nmol/L	N/R	N/R	2.36	3.69	4.93 nmol/L	4.92 nmol/L	N/R	N/R
32	China	37	45	31.0	30.0	0.42 ng/mL	0.69 ng/mL	N/R	N/R	1.93	3.94	5.24 nmol/L	5.21 nmol/L	8.00 mU/L	13.9 mU/L
33	China	15	18	24.0 ± 1.0	26.0 ± 4.0	0.46 ± 0.12 µg/L	0.84 ± 0.18 µg/L	N/R	N/R	N/R	N/R	N/R	N/R	N/R	
34	China	15	33	45.4	27.9	0.87 nmol/L	5.07 nmol/L	N/R	N/R	2.42	2.50	5.00 nmol/L	4.89 nmol/L	13.38 uU/mL	11.1 uU/mL
35	China	17	17	23.8 ± 2.8	26.1 ± 4.4	N/R	0.68 ± 0.32	N/R	N/R	N/R	N/R	N/R	5.00 ± 0.64 nmol/L	N/R	17.05 ± 14.36

Note: N/R represents Not reported. Free androgen index and HOMA-IR index have no units. Some studies have not provided the unit of testosterone, fasting glucose and insulin. Additionally, some studies provide mean ± SD and other studies didn't provide.

*Table 1:* Characteristics of studies included in this combined analysis.

Ref.	Sample collection	Sample transport	Sample storage	DNA extraction kit	Sequencing type	Sequencing platform	Sequencing region	Primers
22	Empty stool collection tubes	Stored short-term at −20 °C	−70 °C	MagNA Pure LC DNA Isolation Kit III	16S rRNA	MiSeq desktop sequencer	V1–V2	N/R
23	Cotton swab	In dry ice	−80 °C	PowerSoil DNA Isolation Kit	16S rRNA	Illumina MiSeq platform	V4	515F and 806R
24	N/R	N/R	N/R	N/R	16S rRNA	Illumina MiSeq platform	V3–V4	338F (5′-ACTCCTACGGG AGGCAGCA-3′) and 806R (5′-GGAC TACHVGG GTWTCTAAT-3′)
25	N/R	N/R	N/R	N/R	16S rRNA	N/R	N/R	N/R
26	Sterile plastic spoons and plastic tubes	N/R	−80 °C	Axygen Axy Prep DNA Gel Kit	16S rRNA	Illumina MiSeq platform	V3–V4	F:AYTGGGYDTAAAGNG R:TACNVGGGTATCTAATCC
27	home fecal collection kit	refrigerant	−80 °C	Quick-DNA Fecal/ Soil Microbe Kit	16S rRNA	Illumina MiSeq platform	V1–V3	N/R
28	N/R	N/R	N/R	HiPure Stool DNA Kits B	16S rRNA	Illumina MiSeq platform	N/R	N/R
29	Ethylenediaminetetraacetic acid (EDTA) tube	N/R	−80 °C	Mag-Bind® Pathogen DNA 96 Kit	16S rRNA	Illumina MiSeq platform	V3–V4	N/R
30	Collected on the day of the medical examination	Immediately frozen at −80 °C	−80 °C	HiPure Stool DNA Kits B	16S rRNA	Illumina MiSeq platform	V3–V4	F: 5′-CCTACGGRRBGCASC AGKVRVGAAT-3′ R: 5′-GGACTACNVGGGTW TCTAATCC-3′
31	Sterile plastic bottle	N/R	−80 °C	E.Z.N.A.® Stool DNA Kit	16S rRNA	Illumina MiSeq platform	V4	338F (5′ACTCCTACGGGAGGC AGCAG) and 806R (5′GGACTACHV GGGTWTCTAAT)
32	Fecal DNA storage tubes	Stored and sent to laboratory under room temperature	N/R	Intestinal DNA extraction kit	Full length 16S rRNA	N/R	V1–V9	N/R
33	Sterile plastic spoon and tube	placed in an ice box, transported to the laboratory within 2 h	−80 °C	N/R	16S rRNA	Illumina MiSeq platform	N/R	N/R
34	N/R	Feces was divided into aliquots and was frozen on dry ice immediately upon collection	−80 °C	N/R	16S rRNA	Illumina MiSeq platform	V3–V4	N/R
35	N/R	N/R	N/R	CTAB	16S rRNA	Illumina NovaSeq platform	N/R	N/R

Note: N/R represents Not reported.

Table 2: **Information of gut microbiota collection and processing methods across studies.**

were more enriched in Chinese. Within the Chinese cohort, the PCOS group showed a notably higher relative abundance of Actinobacteriota phylum and *Bifidobacterium* genus compared to healthy controls. In the European cohort, *Faecalibacterium* was decreased in PCOS patients compared with healthy controls (Appendix Supplementary Fig. S1E and F). Intriguingly, the specific bacteria characteristic of PCOS patients varied distinctly across regions. In Chinese populations, Wilcoxon non-parametric testing revealed one significantly increased bacterium and thirty-two decreased bacteria in PCOS compared to HC (Appendix Supplementary Fig. S2A and B). On the contrary, among European, twenty-nine bacteria were upregulated and thirteen were downregulated in the PCOS group compared to the HC group (Appendix Supplementary Fig. S2D and E). Statistical Analysis of Metagenomics Profiles (STAMP) analysis further highlighted distinct patterns in the top twenty genera altered in PCOS patients between Chinese and European populations, with two genera (*Lachnoclostridium* and *Ruminococcus_gnavus_group*) enriched and eighteen reduced in Chinese (Appendix Supplementary Fig. S2C), while eighteen genera increased and two decreased in
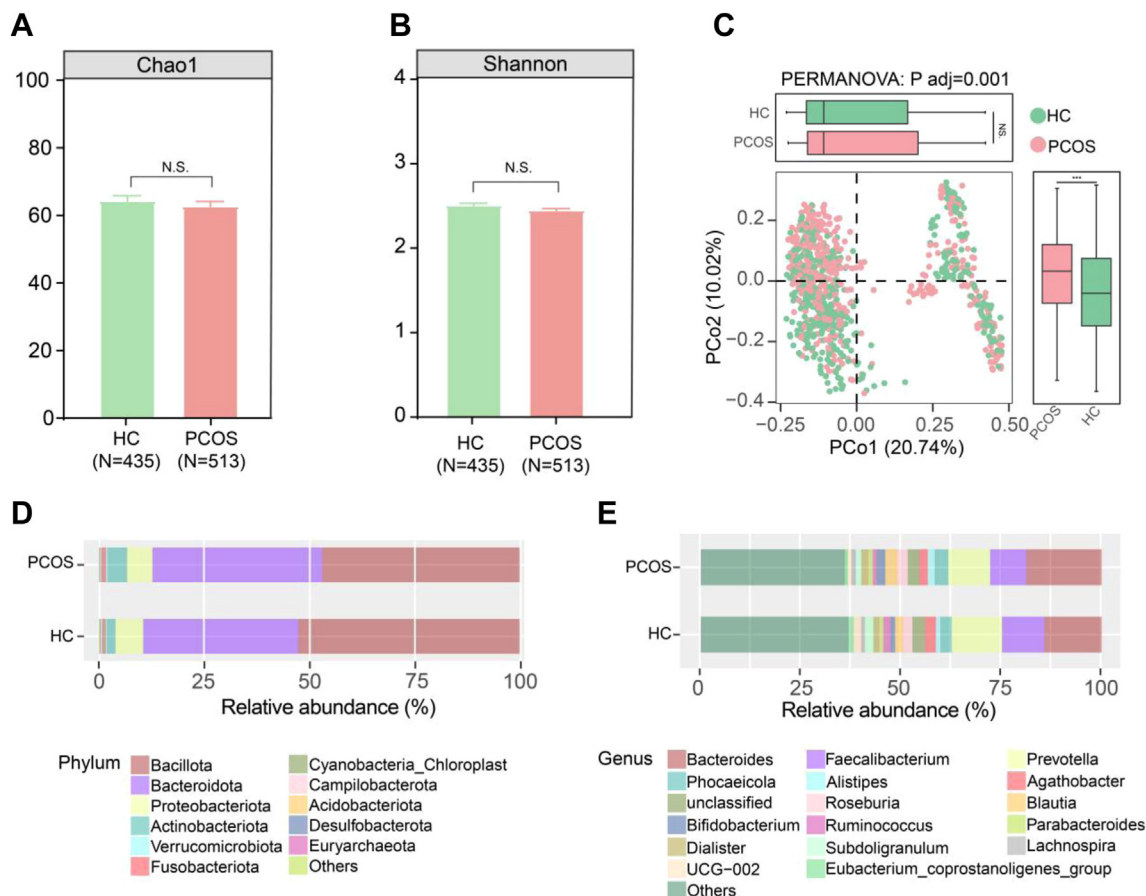
Fig. 2: The diversity and composition of gut microbiota among PCOS and HC groups. (A-B) Difference of alpha-diversity in the two groups is assessed by the Chao1 and Shannon indices. (C) Beta-diversity of the gut microbial community is indicated by principal coordinate analysis (PCoA) (Bray–Curtis distances) at the sample level. (D) The composition of gut microbiota at the phylum level. (E) The composition of gut microbiota at the genus level. The Wilcoxon test was used for alpha diversity analysis, and permutational multivariate analysis of variance (PERMANOVA) was used for PCoA analysis. Statistical data are presented as mean values ± standard deviations. ***$P < 0.001$. PCOS, polycystic ovary syndrome; HC, healthy control.

European (Appendix Supplementary Fig. S2F). To delve deeper into the ecological interactions of gut microbiota in PCOS across regions, NetMoss analysis was performed. The network structures differed notably between Chinese and European. In Chinese, the key taxa *Faecalibacterium*, *Gemmiger*, *Burkholderia*, and *Duncaniella* in HC individuals were changed in PCOS patients, which *Burkholderia* and *Duncaniella* were disappeared, while *Aeromicrobium* existed (Appendix Supplementary Fig. S3A). In contrast, the key taxa in European networks showed minimal changes (Appendix Supplementary Fig. S3B). Using a NetMoss score threshold of 0.8, fourteen genera were recognized as markers in Chinese populations with an AUC of 0.72 (Appendix Supplementary Fig. S3C, Table S5), while eleven genera served as markers in Europeans with an AUC of 0.72 (Appendix Supplementary Fig. S3D; Table S5). Overall, these collective data underscore substantial regional variations in gut microbiota

composition among PCOS patients, suggesting these differences may contribute to individualized treatment approaches based on geographic considerations.

Characteristics of gut microbiota in PCOS patients with different testosterone levels

Elevated testosterone levels are a clinical hallmark of PCOS. Based on average testosterone levels reported in the literature, we categorized PCOS patients into high testosterone (PCOS-HT) and low testosterone (PCOS-LT) groups, using a 30% increase as the threshold. Nine studies were grouped into high testosterone group, and two studies into low testosterone group. There was no significant difference in alpha diversity (Fig. 4A and B), but the structure of gut microbiota of each group was significantly separated (Fig. 4C and D). The profile of gut microbiota in PCOS-HT group was similar with HC group but was different from PCOS-LT group, which may be due to the small samples of the PCOS-LT group
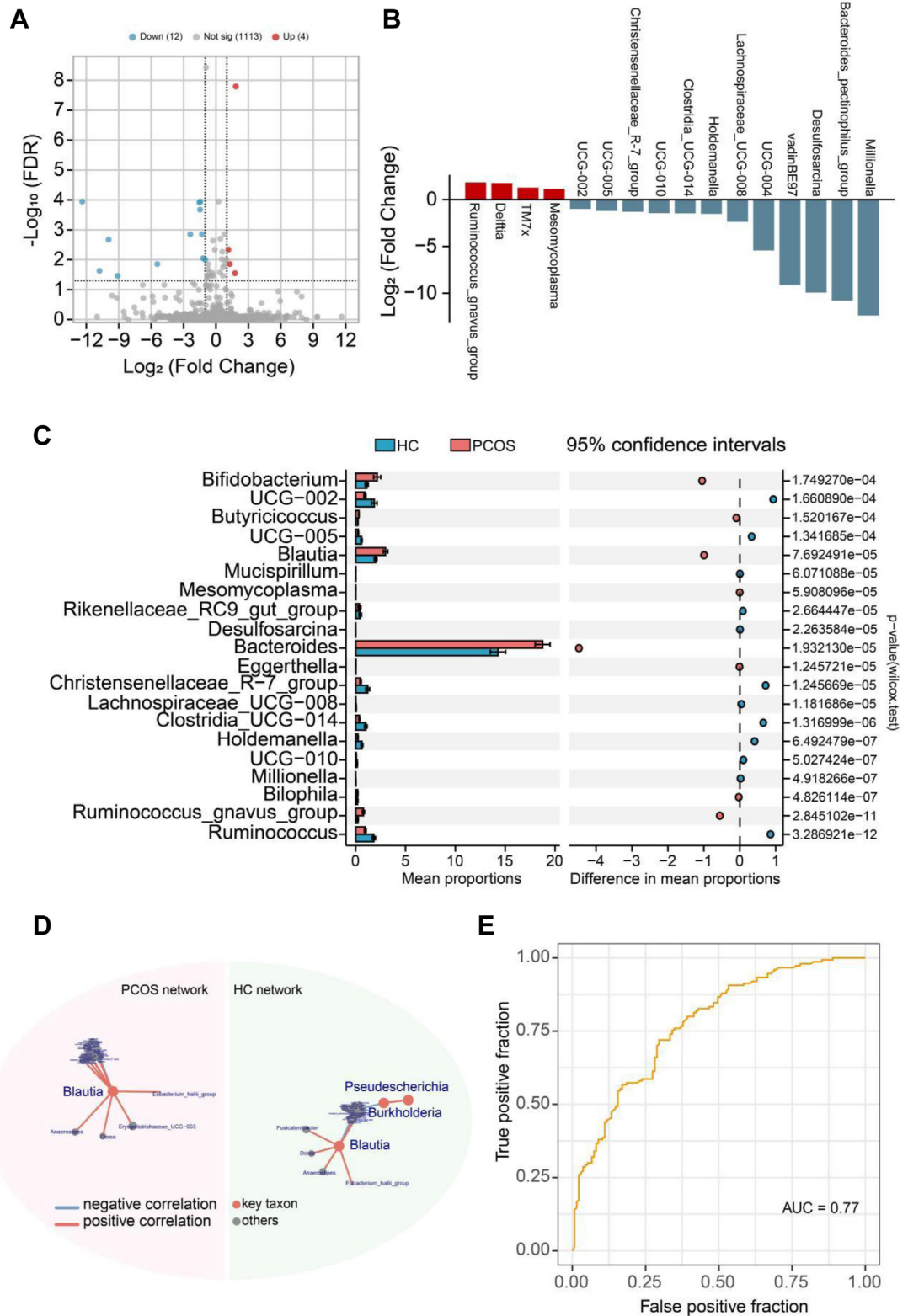
Fig. 3: **The characteristics of the gut microbiota in PCOS patients**. (A) The volcano plot shows distinct genera between the two groups. (B) Two-way bar chart displays specific upregulated and downregulated genera in PCOS patients. (C) STAMP analysis is used to analyze the different

(Fig. 4E). The gut microbiota composition of PCOS-HT patients was obviously different from PCOS-LT patients. Specifically, *Prevotella* and *Phocaeicola* were more abundant on the PCOS-HT patients but less so in PCOS-LT patients. Meanwhile, *Faecalibacterium*, *Alistipes*, *Agathobacter*, *Ruminococcus* and *Barnesiella* were reduced in PCOS patients with high level of testosterone (Fig. 4F). Further comparisons using the Wilcoxon non-parametric test revealed 42 genera upregulated and 49 downregulated in the PCOS-LT group versus HC (Appendix Supplementary Fig. S4, Table S6), whereas 9 genera were upregulated and 61 downregulated in the PCOS-HT group versus HC (Appendix Supplementary Fig. S4, Table S7). STAMP analysis identified top 20 genera with striking changes in PCOS-LT patients, with three bacteria reduced and seventeen enhanced (Appendix Supplementary Fig. S4). In contrast, fourteen bacteria reduced and six enhanced in the PCOS-HT patients (Appendix Supplementary Fig. S4).

Subsequently, the NetMoss network reaffirmed that PCOS-LT patients possessed relatively complex ecological microbial interactions compared to PCOS-HT patients. In PCOS patients with lower testosterone, the key taxon in network were *Prevotella*, and *Faecalibacterium*, *Bacteroides*, *Dialister* and *Incertae_Sedis* (Fig. 5A). 10 bacterial genera in this group had a NetMoss score greater than 0.8, with an AUC of 0.97 as markers for distinguishing PCOS-LT and HC (Fig. 5B, Supplementary Table S8). In contrast, the key taxon in PCOS-HT patients consisted of *Prevotella*, *Blautia*, *Alistipes* and *UCG-002* (Supplementary Fig. S5C). 18 genera in the PCOS-HT group, namely *Prevotella*, *Blautia*, *Dialister*, *UCG-002*, *Alistipes*, *Ruminococcus_torques_group*, *Lactobacillus*, unidentified, *Megasphaera*, *Bilophila*, *Clostridium_sensu_stricto*, *Kineothrix*, *Varibaculum*, *Enterobacter*, *Anaerobium*, *Fournierella*, *Oribacterium* and *Flintibacter*, had a NetMoss score greater than 0.8, with an AUC of 0.72 for distinguishing PCOS-HT from HC (Fig. 5D, Supplementary Table S8). These data implied that gut microbiota can discriminate PCOS patients from healthy controls to some extent and serve as markers for PCOS. Intriguingly, further comparison between PCOS-LT and PCOS-HT revealed a stunning phenomena, that is, although the key taxon in both groups were identical, their elaborate correlations were distinct (Fig. 5E). Compared to PCOS-LT patients, the intestinal environment of PCOS-HT patients expanded the presence of genera like *Prevotella*, *Phocaeicola*, *Dialister*, and *Ruminococcus_torques_group*, whereas diminished *Alistipes*, *Eubacterium_eligens_group*, *Bifidobacterium* and *Faecalibacterium* (Fig. 5F). Notably, six genera, including *Bacteroides*, *Prevotella*, *Faecalibacterium*, *Blautia*, unclassified and *Dialister*, were selected as marker with a NetMoss score greater than 0.8 and *P* value less than 0.05 (Appendix Supplementary Table S9). The ROC curve analysis revealed that this marker had an AUC of 0.93, effectively distinguishing PCOS-HT from PCOS-LT patients (Fig. 5G).

Comparative genomic analysis of PCOS-related gut microbiota

To unveil the potential mechanism of gut microbiota causing PCOS occurrence, we chose several common strains of gut microbial biomarkers (*Bacteroides*, *Prevotella*, *Faecalibacterium*, *Blautia*, and *Dialister*) to performer comparative genomic analysis. The pan-genome analysis revealed that PCOS-HT patients enriched *Prevotella* and *Dialister* possessed unique combinations of genes than *Faecalibacterium*, *Bacteroides* and *Blautia* (Fig. 6A). Then, we compared the complete genome encoded proteins of each strain and annotate gene functions based on eggNOG database (Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups). We found KEGG pathways annotated by PCOS-related gut microbiota consisted of lipopolysaccharide biosynthesis, lipid metabolism (primary/secondary bile acid biosynthesis). Notably, *Dialister* annotated more lipopolysaccharide biosynthesis than other strains (Fig. 6B), which may be a potential reason why PCOS-HT patients differing from PCOS-LT patients.

Discussion

PCOS is a major cause of anovulatory subfertility and can adversely affect the endocrine system of offspring.[37] Multiple factors contribute to PCOS pathogenesis, with gut microbiota potentially playing a crucial role. Due to limited research on gut microbiota in different PCOS subgroups, current treatments remain a formidable challenge, particularly due to individual variations. As a major strength, this combined analysis is the first to characterize the gut microbiota among PCOS patients across different cohorts and detect regional microbial differences. More importantly, as hyperandrogenism is a typical clinical feature of PCOS patients, we therefore refine the characteristics of gut microbiota in PCOS patients with higher or lower testosterone levels. As a result, our reanalyses revealed the obvious difference in gut microbiota between Chinese and European PCOS populations. Simultaneously, PCOS patients with higher testosterone levels (PCOS-HT) showed markedly disordered gut microbiota, distinct from both lower testosterone patients (PCOS-LT) and non-PCOS

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genera between PCOS and HC. (**D**) Sparcc network diagram between PCOS and HC is constructed by NetMoss2. (**E**) NetMoss2-constructed ROC plots of PCOS and HC. The Wilcoxon nonparametric test was used for statistical tests and *P* value was adjusted by BH to FDR value. Statistical data are presented as mean values ± standard deviations. PCOS, polycystic ovary syndrome; HC, healthy control. STAMP, Statistical Analysis of Metagenomics Profiles.
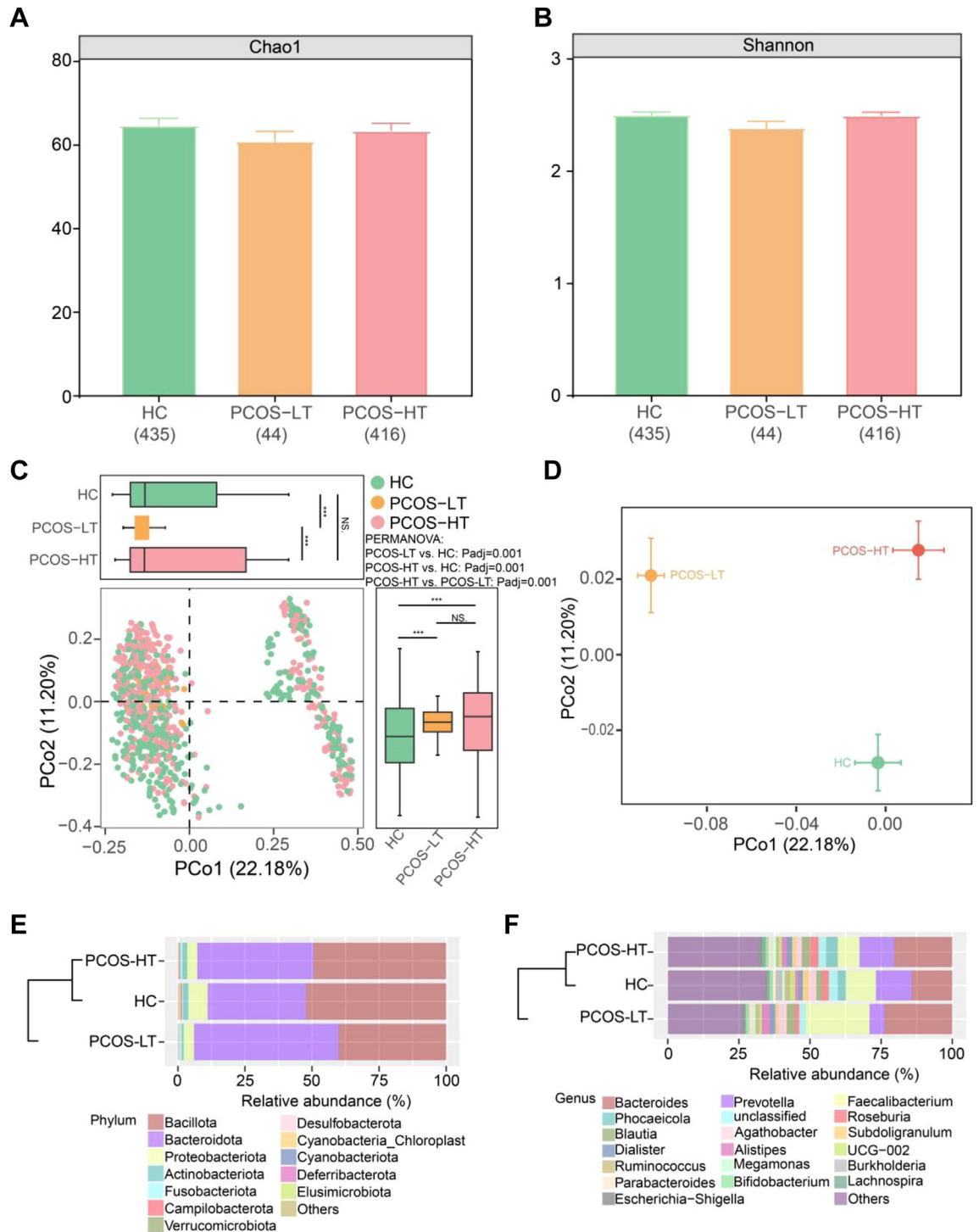
Fig. 4: **The diversity and composition of gut microbiota among PCOS–H, PCOS–L and HC groups**. (A-B) Difference of alpha-diversity in the three groups is assessed by the Chao1 and Shannon indices. (C-D) Beta-diversity of the gut microbial community is indicated by PCoA analysis (Bray–Curtis distances) at the sample and group levels, respectively. (E) The composition of gut microbiota at the phylum level. (F) The composition of gut microbiota at the genus level. The Wilcoxon test was used for alpha diversity analysis, and permutational multivariate analysis of variance (PERMANOVA) was used for PCoA analysis. Statistical data are presented as mean values ± standard deviations. **$P < 0.01$, ***$P < 0.001$. PCOS-LT, polycystic ovary syndrome patients with low level of testosterone; PCOS-HT, polycystic ovary syndrome patients with high level of testosterone; HC, healthy control.
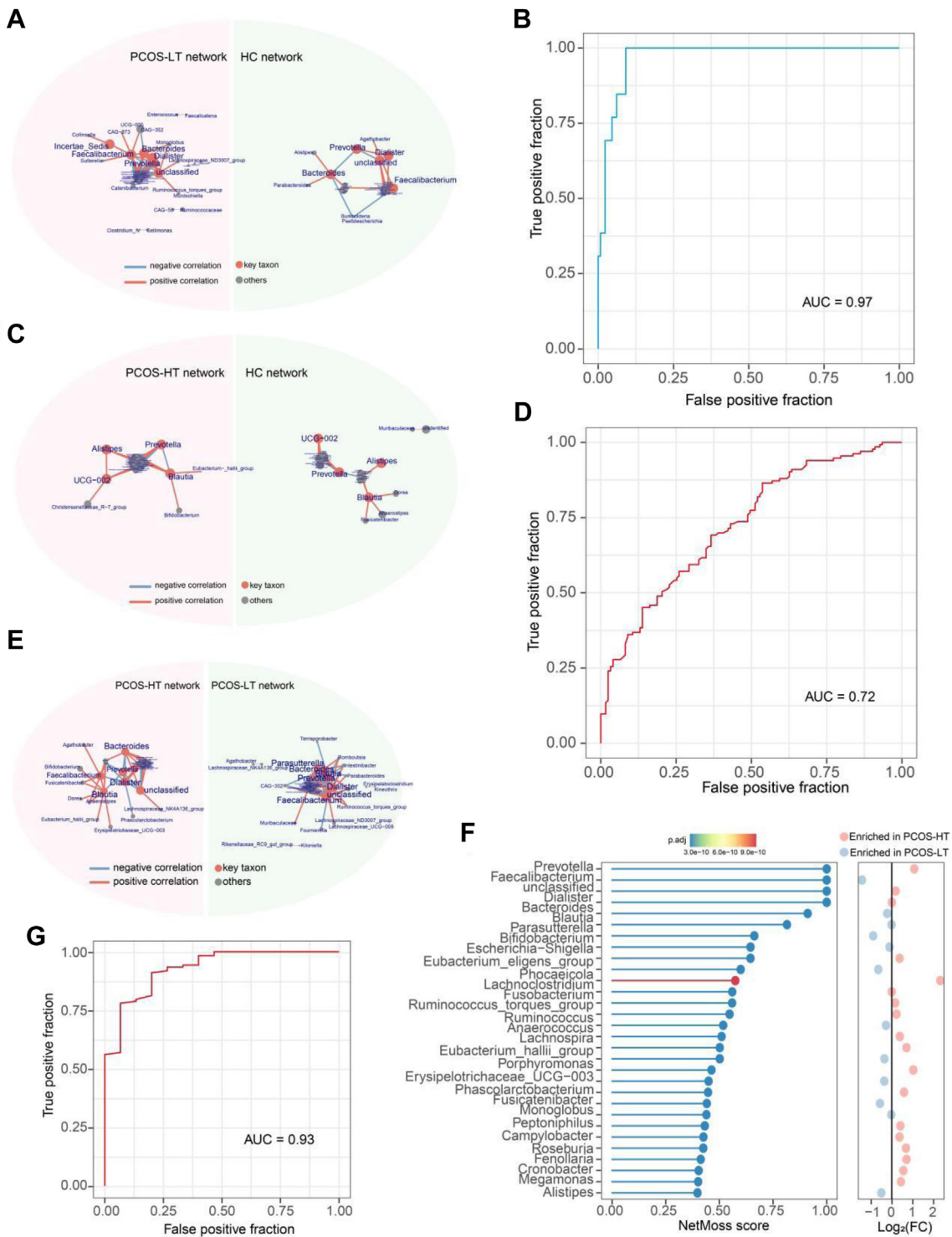
Fig. 5: **NetMoss2 identified biomarkers for PCOS patients with different testosterone level**. (**A**) Sparcc network diagram between PCOS-LT and HC is constructed by NetMoss2. (**B**) NetMoss2-constructed ROC plots of PCOS-LT and HC individuals. (**C**) Sparcc network diagram between PCOS-HT and HC is constructed by NetMoss2. (**D**) NetMoss2-constructed ROC plots of PCOS-HT and HC individuals. (**E**) Sparcc network diagram between PCOS-HT and PCOS-LT is constructed by NetMoss2. (**F**) NetMoss2 identifies top 30 specific bacterial taxa in PCOS-HT and PCOS-LT patients. (**G**) NetMoss2-constructed ROC plots of PCOS-LT and PCOS-HT patients. PCOS-LT, polycystic ovary syndrome patients with low level of testosterone; PCOS-HT, polycystic ovary syndrome patients with high level of testosterone.
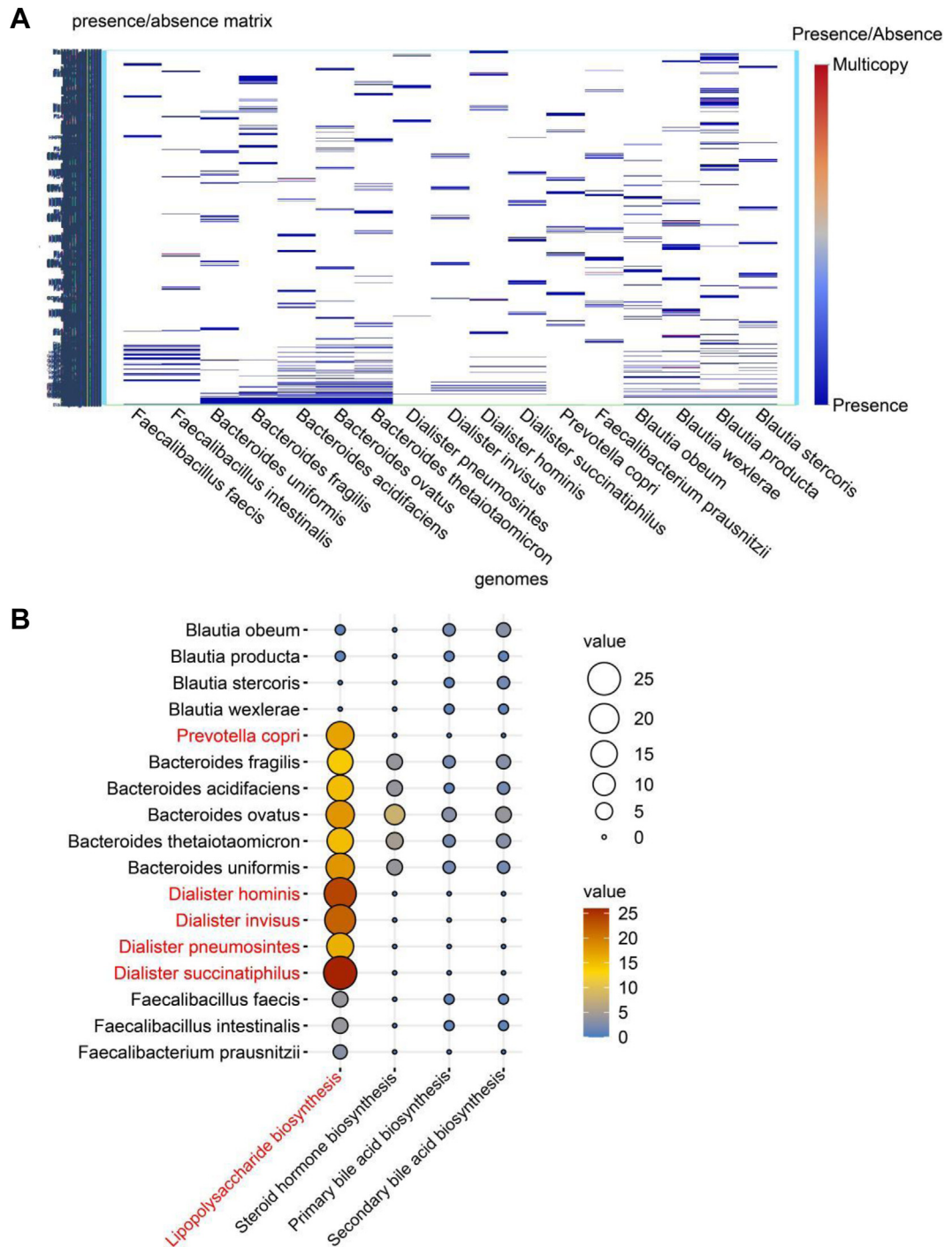
Fig. 6: Comparative genomic analysis of PCOS-related gut microbiota. (A) The pan-genome analysis of seventeen gut microbial strains. (B) KEGG pathway for whole genome encoded protein annotation based on eggNOG database. KEGG, Kyoto Encyclopedia of Genes and Genomes.

individuals. Specifically, in PCOS-HT patients, *Alistipes, Eubacterium_eligens_group, Bifidobacterium* and *Faecalibacterium* were decreased, while *Prevotella, Phocaeicola, Dialister,* and *Ruminococcus_torques_group* were increased compared to PCOS-LT patients. The gut microbiota, as a biomarker, can not only distinguish between PCOS and HC, but also effectively distinguish PCOS-HT from PCOS-LT with an AUC of 0.93.

Recently, several studies have uncovered the fundamental importance of gut microbiota in the occurrence

and development of PCOS based on both animal studies and human trials. Jiang et al. found that transplanting the microbiota of PCOS patients to female mice can lead to PCOS-like symptoms.[17] Notably, transplantation of gut microbiota from PCOS women mainly enriched *Bacteroides vulgatus*, then modulated bile acid profiles and reduced ILC3-produced IL-22 levels, thus leading to insulin resistance and ovarian dysfunction.[17] Additionally, it is reported that DHEA-shaped gut microbiome can affect hepatic glucolipid metabolism and immune response, thus result in the metabolic and endocrinal malfunction.[38] These studies suggest that imbalance of gut microbiota represents an important cause of PCOS. It does not mean, of course, that gut microbiota is the only contributing factor. Several studies have demonstrated that insulin resistance, oxidative stress, lipid metabolism and chronic low-grade inflammation are all supposed to involve in pathogenesis of PCOS.[39,40] Importantly, all of these have been found to be related to gut microbiota. For example, PCOS-enriched *Ruminococcus_gnavus_group* is positively correlated with TC, TG and LDL-c, but negatively associated with HDL-c levels,[41] indicating this genus might lead to the lipid metabolism perturbations of PCOS patients. Therefore, identifying alterations of gut microbiota of PCOS patients can contribute to the diagnosis and treatment of PCOS to a certain extent.

It is reported that PCOS patients display heightened levels of *Fusobacterium*,[25] *Escherichia-Shigella*,[26] *Ruminococcus torques*,[28,32] but showed lessened levels of *Faecalibacterium*,[30] *Alistipes*,[28] *Subdoligranulum*,[33] *Clostridium IV*.[34] Our reanalysis yielded similar trends. Additionally, we also saw some varying outcomes across our cross-cohort study. For example, *Bifidobacterium*, *Butyricicoccus*, *Blautia*, *Mesomycoplasma*, *Bacteroides*, *Eggerthella*, *Bilophila* and *Ruminococcus_gnavus_group* were increased, while *UCG-002*, *UCG-005*, *Ruminococcus*, Christensenellaceae_*R-7_group*, *Clostridia_UCG-014*, *Holdemanella*, *Mucispirillum*, *Desulfosarcina*, Rikenellaceae_*RC9_gut_group*, *Holdemanella*, Lachnospiraceae_*UCG-008*, *UCG-010* and *Millionella* were reduced in PCOS patients. Thus, more studies must be conducted among a broader range of PCOS populations to portray their specific gut microbial profile. This will further enhance the understanding of the pathogenesis of PCOS and provide potential treatments for women of reproductive age. Despite our cross-cohort analysis integrated the results from multiple studies, there are still shortcomings due to heterogeneity among the studies. More investigations are still needed to define the characteristics of gut microbiota among PCOS patients more precisely.

Our included populations come from China and Europe, each with unique dietary and lifestyle habits. Since diet and lifestyle habit are critical factors influencing gut microbiota, comparing these two regional populations is essential. Intriguingly, we found that the abundance of *Alistipes* is obviously higher in the Europeans, likely due to their carnivorous, high-fat diet, which supports the growth of *Alistipes*.[42] Conversely, *Blautia* and *Roseburia* are more abundant in Chinese, who consume plant-based diets, as these bacteria can digest dietary fiber and produce short chain fatty acids.[43] When compared to their corresponding healthy controls, Chinese PCOS patients exhibited a decline in many genera, while European PCOS patients showed an increase. Altogether, there are notable differences between Chinese and European PCOS patients in terms of gut microbiota, which may explain the discrepancies observed in treatment.

Hyperandrogenism is defined as excessive serum levels of androgens in woman and serves as one of the hallmark of PCOS. A high testosterone level is often an indicator of hyperandrogenism among women with PCOS. Generally, the enhancement of free testosterone promotes insulin secretion and exacerbates insulin resistance. The interactions between hyperandrogenism and insulin resistance lead to the progression of PCOS in a form of vicious circle.[44] Gut microbiota can encode specific enzymes, such as 17-Hydroxysteroid dehydrogenases[45] and 3β-Hydroxysteroid dehydrogenase,[46] to regulate testosterone levels, thus become a key mediator for PCOS intervention. Simultaneously, compelling evidence has proven that genera such as *Alistipes*, *Agathobacter*, *Phascolarctobacterium*, *Butyricimonas* et al. are depleted in the hyperandrogenic subtype of PCOS,[47] indicating that gut microbiota has potential in distinguishing different subtypes of diseases. Our reanalysis found that the relative abundance of *Prevotella*, *Phocaeicola*, *Dialister*, and *Ruminococcus_torques_group* were enhanced in PCOS-HT patients, while *Alistipes*, *Eubacterium_eligens_group*, *Bifidobacterium* and *Faecalibacterium* were reduced compared with PCOS-LT patients, suggesting that these genera may emerge as a signature gut bacteria in PCOS patients with high testosterone levels. More importantly, we identified a bacteria cluster, including *Bacteroides*, *Prevotella*, *Faecalibacterium*, *Blautia*, *Dialister*, as a marker to differentiate PCOS-HT and PCOS-LT patients with the AUC of 0.93.

Notably, through comparative genomic analysis of PCOS related gut microbiota strains, we found that the functions of these strains were annotated in the primary/secondary bile acid biosynthesis.. Several studies found PCOS patients possessed higher levels of conjugated primary bile acids, which showed positive association with high levels of serum total testosterone and androstenedione.[48] However, gavage with glycodeoxycholic acid can significantly reverse hormone abnormalities, estrous cycle disorders, ovarian polycystic change, fertility decline and insulin resistance in PCOS-like mice.[17] It follows that primary/secondary bile acid biosynthesis play a crucial role in PCOS, but the specific effect of each bile acid still needs further exploration. As

a pro-inflammatory cytokine, lipopolysaccharide can induce the release of proinflammatory cytokines interleukin (IL)-6, resulting the inflammatory response. Intriguingly, existing studies have revealed that the inflammation usually increased in PCOS patients with elevated androgen levels, and testosterone could increase the IL-6 in 3T3-L1 adipocyte responses to LPS.[49] Therefore, inflammatory responses induced by lipopolysaccharide may explain the difference between PCOS-HT and PCOS-LT patients, as PCOS-HT patients possess higher levels of *Dialister* and *Prevotella*.

Despite its contributions, this study has limitations that need to be addressed in future research. Firstly, the study population is not evenly distributed. The Asian population is all Chinese, and there is a lack of cohort from other countries besides Chinese and European. These limitations impeded the comprehensive understanding of the gut microbiota characteristics of PCOS patients. More research from different regions should be conducted to promote the global treatment of PCOS. Secondly, additional clinical studies are necessary to investigate gut microbiota characteristics in PCOS patients at the species or strains levels, which could aid in developing anti-PCOS microbial treatments. Thirdly, comprehensive analyses of gut microbiota in various PCOS subtypes are required to understand individual treatment differences and to develop personalized therapies in the precision medicine era. Fourthly, while our study suggests that gut microbiota could serve as a biomarker for diagnosing and differentiating PCOS subtypes, large-scale clinical trials are needed to validate this potential. Finally, once the gut microbiota associated with PCOS are identified at the species or strain level, it is necessary to employ high-quality reference genomes of the strain from open-source databases for functional gene analysis to address the current inability to enrich metagenomic functional pathways, as well as explore the potential mechanisms of gut microbiota mediated the development of PCOS.

In summary, this combined analysis is the first to characterize gut microbiota in PCOS patients by reanalyzing raw sequencing data from 14 datasets. We unraveled the difference in gut microbiota between Chinese and European PCOS patients, which may be due to variations in Eastern and Western dietary habits. Importantly, gut microbiota can potentially distinguish PCOS patients with different testosterone levels. This comprehensive analysis of gut microbiota highlights key microbial characteristics in PCOS, particularly in those with different testosterone levels, and lays the groundwork for future research on pathogenesis, diagnostics, and treatment strategies.

Contributors

CMW and WYL conceived the study idea and designed the study. YNY conducted the database searches. YNY and JLC evaluated the risk of bias of the included studies, evaluated the quality of evidence. JLC performed the raw data analysis. CYL download the sequence data of the gut bacteria strain. YNY and JLC verified the underlying data. YNY contributed to the interpretation of the results and wrote the first draft of the manuscript. CMW, XPZ, NM and ZZ made substantial contributions to the critical revision of the manuscript. All authors read and approved the final version of the manuscript.

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