










Reproductive endocrinology

Gut bacteriome and mood disorders in women with PCOS

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ABSTRACT

STUDY QUESTION: How does the gut bacteriome differ based on mood disorders (MDs) in women with polycystic ovary syndrome (PCOS), and how can the gut bacteriome contribute to the associations between these two conditions?

SUMMARY ANSWER: Women with PCOS who also have MDs exhibited a distinct gut bacteriome with reduced alpha diversity and a significantly lower abundance of *Butyricicoccus* compared to women with PCOS but without MDs.

WHAT IS KNOWN ALREADY: Women with PCOS have a 4- to 5-fold higher risk of having MDs compared to women without PCOS. The gut bacteriome has been suggested to influence the pathophysiology of both PCOS and MDs.

STUDY DESIGN, SIZE, DURATION: This population-based cohort study was derived from the Northern Finland Birth Cohort 1966 (NFB1966), which includes all women born in Northern Finland in 1966. Women with PCOS who donated a stool sample at age 46 years (n = 102) and two BMI-matched controls for each case (n = 205), who also responded properly to the MD criteria scales, were included.

PARTICIPANTS/MATERIALS, SETTING, METHODS: A total of 102 women with PCOS and 205 age- and BMI-matched women without PCOS were included. Based on the validated MD criteria, the subjects were categorized into MD or no-MD groups, resulting in the following subgroups: PCOS no-MD (n = 84), PCOS MD (n = 18), control no-MD (n = 180), and control MD (n = 25). Clinical characteristics were assessed at age 31 years and age 46 years, and stool samples were collected from the women at age 46 years, followed by the gut bacteriome analysis using 16s rRNA sequencing. Alpha diversity was assessed using observed features and Shannon's index, with a focus on genera, and beta diversity was characterized using principal components analysis (PCA) with Bray–Curtis Dissimilarity at the genus level. Associations between the gut bacteriome and PCOS-related clinical features were explored by Spearman's correlation coefficient. A P-value for multiple testing was adjusted with the Benjamini–Hochberg false discovery rate (FDR) method.

MAIN RESULTS AND THE ROLE OF CHANCE: We observed changes in the gut bacteriome associated with MDs, irrespective of whether the women also had PCOS. Similarly, PCOS MD cases showed a lower alpha diversity (Observed feature, PCOS no-MD, median 272; PCOS MD, median 208, FDR = 0.01; Shannon, PCOS no-MD, median 5.95; PCOS MD, median 5.57, FDR = 0.01) but also a lower abundance of *Butyricicoccus* (log-fold change Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) = −0.90, FDR_{ANCOM-BC} = 0.04) compared to PCOS no-MD cases. In contrast, in the controls, the gut bacteriome did not differ based on MDs. Furthermore, in the

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PCOS group, *Sutterella* showed positive correlations with PCOS-related clinical parameters linked to obesity (BMI, $r^2 = 0.31$, FDR = 0.01; waist circumference, $r^2 = 0.29$, FDR = 0.02), glucose metabolism (fasting glucose, $r^2 = 0.46$, FDR < 0.001; fasting insulin, $r^2 = 0.24$, FDR = 0.05), and gut barrier integrity (zonulin, $r^2 = 0.25$, FDR = 0.03).

LIMITATIONS, REASONS FOR CAUTION: Although this was the first study to assess the link between the gut bacteriome and MDs in PCOS and included the largest PCOS dataset for the gut microbiome analysis, the number of subjects stratified by the presence of MDs was limited when contrasted with previous studies that focused on MDs in a non-selected population.

WIDER IMPLICATIONS OF THE FINDINGS: The main finding is that gut bacteriome is associated with MDs irrespective of the PCOS status, but PCOS may also modulate further the connection between the gut bacteriome and MDs.

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Keywords: gut microbiome / gut bacteriome / mood disorders / anxiety / depression / polycystic ovary syndrome / PCOS

Introduction

Polycystic ovary syndrome (PCOS), which affects one out of eight women, can be diagnosed when at least two of the following symptoms are present according to the Rotterdam consensus criteria: oligo- or anovulation (OA), biochemical or clinical hyperandrogenism (HA), and polycystic ovarian morphology (PCOM), with the exclusion of other androgen excess-related disorders ('Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS)', 2004; Azziz et al., 2006; Teede et al., 2023). The endocrine abnormalities and chronic inflammation observed in PCOS, represented by increased inflammatory markers (e.g. C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α)) (Aboeldaly et al., 2021), are not only associated with metabolic derangements, such as obesity and insulin resistance (IR) (Escobar-Morreale, 2018), but also elevate the risk of mood disorders (MDs), which have been shown to be 4–5 times more common in women with PCOS compared to other women (Cooney et al., 2017; Karjula et al., 2017, 2021; Kolhe et al., 2022). The etiology of MDs in PCOS is complex and multifactorial (Karjula et al., 2017, 2021; Kolhe et al., 2022).

The gut microbiome, particularly the bacterial community known as the bacteriome, communicates with its host by influencing immune maturation and regulation (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Hooper and Macpherson, 2010), sex hormone metabolism (He et al., 2021), metabolic processes (Devaraj et al., 2013), and even brain function (Armeth, 2018; Peirce and Alviña, 2019; Simpson et al., 2021). Changes in the gut bacteriome of women with PCOS, exemplified by lower alpha diversity (Jobira et al., 2020; Liang et al., 2020; García-Bernal et al., 2021; Zhu et al., 2021) and shifts in the abundance of specific bacteria (e.g. *Bacteroides*, *Escherichia/Shigella*) (Liu et al., 2017; Zhang et al., 2019; Chu et al., 2020; Haudum et al., 2020; Chen et al., 2021; Zhu et al., 2021) compared to non-PCOS women, can impact the pathogenesis of PCOS through several intertwined mechanisms. First, the altered gut bacteriome can elevate circulating androgens via beta-glucuronidase enzyme-driven deconjugation (Pellock and Redinbo, 2017; Batra et al., 2022). Second, the altered bacterial composition can trigger systemic low-grade inflammation by compromising gut barrier integrity (Ye et al., 2006; Swidsinski et al., 2007; Tremellen and Pearce, 2012), promoting insulin resistance (Caricilli and Saad, 2013) and HA (He and Li, 2020). Additionally, changes in the gut bacteriome can influence gut hormone secretion, including ghrelin and peptide YY, which

influence pituitary function and further exaggerate the sex hormone imbalance in PCOS (Sola-Leyva et al., 2023). Furthermore, the alterations in the gut bacteriome can influence anxiety and depression, collectively referred to here as MDs, by (i) regulating the synthesis of neurotransmitters and their precursors, such as serotonin, gamma-aminobutyric acid, and tryptophan, (ii) altering neuropeptide and gut hormone release, as well as brain-derived neurotrophic factor, and (iii) promoting neuroinflammation (Peirce and Alviña, 2019; Simpson et al., 2021). Given the involvement of the gut bacteriome in the pathophysiology of both PCOS and MDs, it is important to understand its associations with MDs in women with PCOS.

Our main hypothesis was that women with PCOS who also had MDs exhibited an altered gut bacteriome compared to women with PCOS but without MDs, as well as non-PCOS women with MDs. By using 102 women with PCOS (84 PCOS no-MD, 18 PCOS MD) and 205 BMI-matched women without PCOS (180 control no-MD, 25 control MD), as a comparator group, our objectives were to (i) characterize the gut bacteriome profile in relation to MDs in women with PCOS and in controls and, (ii) investigate the associations between the gut bacteriome and PCOS-related clinical parameters, with the ultimate goal of elucidating possible facilitating factors between PCOS and MDs.

Materials and methods

Study population

Northern Finland Birth Cohort

The study population was derived from the Northern Finland Birth Cohort 1966 (NFBC1966) dataset, a longitudinal cohort of individuals born in 1966 in Northern Finland. The NFBC1966 subjects have been followed since birth and have been monitored throughout their lives (Rantakallio, 1988). Detailed study protocols, questionnaire forms, data coverage, and related articles can be accessed on the cohort website (www.oulu.fi/nfbc).

PCOS and control samples

The study population included in the current study has been described previously (Lüll et al., 2021). Briefly, the PCOS group was composed of women who either reported PCOS symptoms at 31 years (OA and HA) ($n = 125$) or a history of PCOS at 46 years (reported PCOM/PCOS diagnosis) ($n = 181$). To ascertain the exclusive associations between the gut bacteriome and MDs within the PCOS context, a control group was included, comprising individuals who reported neither OA nor HA at 31 years and no

PCOM/PCOS at 46 years ($n = 1,573$). Participants who were pregnant or using hormonal contraceptives at 31 years were excluded ($n = 1,488$), as well as those who had used antibiotics, antimycotics, or tamoxifen within three months prior to stool sample collection ($n = 849$). All women with PCOS who had donated a stool sample at age 46 years were identified, and two BMI-matched controls were selected for each case. Therefore, the final sample size consisted of 102 women with PCOS and 205 women without PCOS. The study flow chart is shown in [Supplementary Fig. S1](#). The study was approved by the Ethics Committee of the Northern Ostrobothnia Hospital District (EETTMK 94/2011), and informed consent was signed by all cohort participants in this study.

MD assessment

At age 46 years, MDs, including anxiety and depression, were assessed using the following four criteria: Beck Depression Inventory Second Edition (BDI-II), Generalized Anxiety Disorder Assessment (GAD-7), Hopkins Symptom Checklist (HSCL)-25, and a self-reported diagnosis with depression ([Supplementary Table S1](#)) ([Guze, 1995](#); [Beck et al., 1996](#)). Women meeting at least two out of the four criteria were classified as the MD group ([Sinikumpu et al., 2023](#)). Four participants who did not properly report the MD criteria scales were excluded ([Supplementary Fig. S1](#)). To confirm previous findings demonstrating changes in the gut bacteriome related to MDs ([Simpson et al., 2021](#)) using our population, the subjects were grouped based on MDs irrespective of PCOS status (no-MD total $n = 264$, MD total $n = 43$). Following this, the subjects were classified based on both PCOS and MD status. Therefore, the final sample size consisted of 84 PCOS no-MD, 18 PCOS with MD, 180 control no-MD, and 25 control with MD.

Baseline characteristic measurements

Anthropometric measurements

BMI (kg/m^2) was calculated by dividing weight (kg) by height squared (m^2), and waist circumference (cm) was measured at the midpoint between the lowest rib and the iliac crest.

Sex hormone measurements

Serum testosterone (T) and sex hormone-binding globulin (SHBG) were measured using Agilent triple quadrupole 6410 liquid chromatography/mass spectrometry equipment (Agilent Technologies, Wilmington, DE, USA) and chemiluminometric immunoassay (Immulite 2000, Siemens Health Care, Llanberis, UK), respectively. The free androgen index (FAI) was calculated by dividing serum T (nmol/l) by SHBG (nmol/l), then multiplying by 100.

Glucose metabolism

Plasma glucose was analyzed using an enzymatic dehydrogenase method (Advia 1800, Siemens Healthcare Diagnostics, UK), and serum insulin was measured by a chemiluminometric immunoassay (Advia Centaur XP, Siemens Healthcare Diagnostics, UK). IR was assessed using the homeostasis model assessment of insulin resistance (HOMA-IR), calculated as fasting serum insulin [$\mu\text{U}/\text{ml}$] \times fasting plasma glucose [mmol/l]/22.5 ([Matthews et al., 1985](#)).

Inflammatory and gut permeability markers

Serum levels of high-sensitivity CRP (hs-CRP) were measured by nephelometric assay (BN ProSpec, Siemens Healthcare Diagnostics, UK). Zonulin and intestinal fatty acid-binding protein-2 (FABP2) levels in the serum were measured using ELISA (R&D Systems, Minneapolis, MN, USA, and Immundiagnostik AG,

Bensheim, Germany) according to the manufacturer's instructions. The above clinical parameters of the study participants are summarized in [Tables 1](#) and [2](#).

Gut bacteriome analysis

Gut bacterial DNA extraction and 16S rRNA sequencing data analysis

The stool samples were collected in 2012 and stored at -80°C until 2019, when the bacterial DNA was extracted (QIAamp Stool Mini Kit, Qiagen, Venlo, The Netherlands) and sequenced using the V3–V4 regions of the 16S rRNA gene on an Illumina MiSeq sequencing instrument. Further details of the DNA extraction protocol and polymerase chain reaction conditions can be found in our previous study ([Lüll et al., 2021](#)). Quantitative Insights Into Microbial Ecology 2 (QIIME2, version 2021.4) was used to analyze the sequencing data ([Bolyen et al., 2019](#)). The reads were demultiplexed and denoised using DADA2 ([Callahan et al., 2016](#)). The q2-dada2-denoise script was used to truncate the forward reads at position 285 and to trim at position 16 after retrieving the quality scores. Reverse reads were not trimmed; instead, they were truncated at position 240. Chimeras were removed using the q2-dada2-denoise's 'consensus' filter. A phylogenetic tree was drawn using fasttree2 ([Price et al., 2010](#)), and taxonomic classification was assigned using the SILVA database (version 138) ([Quast et al., 2012](#)). Specific reads from cyanobacteria, mitochondria, eukaryota, and archaea were also removed from the final dataset. Ultimately, 4,207 amplified sequence variants (ASVs) were identified as belonging to 253 genera, 86 families, 48 orders, 21 classes, and 11 phyla. Taxa present in $<30\%$ of the samples were filtered out to focus on more common taxa and reduce the burden of multiple testing, resulting in 251 ASVs across 111 genera, 45 families, 28 orders, 13 classes, and 7 phyla.

Statistical analysis

Clinical parameters

The baseline characteristics of the study subjects were analyzed using IBM SPSS Statistics version 27 (IBM Corporation, Armonk, NY, USA). For continuous variables, statistical differences were analyzed using independent samples t-test or the Mann–Whitney U-test for paired comparisons, and the Kruskal–Wallis test was used for multiple comparisons. For categorical variables, Pearson's chi-square test was employed. A P-value for multiple testing was adjusted with the Benjamini–Hochberg false discovery rate (FDR) method, considering a significance level of $P < 0.05$.

Gut bacteriome analysis

Alpha diversity was assessed using observed features and Shannon's index, with a focus on genera. Differences in alpha diversity between the study groups were analyzed using the Kruskal–Wallis test, and Benjamini–Hochberg method was used to account for multiple testing. Beta diversity was characterized using principal components analysis (PCA) with Bray–Curtis Dissimilarity at the genus level, and differences in beta diversity between the study groups were analyzed using Permutational Analysis of Variance (PERMANOVA) (vegan, v2.6-4) ([Anderson, 2001](#)). Differential abundance (DA) analysis was carried out using the ANOVA-Like Differential Expression tool for compositional data (ALDEx2, v1.30.0), which employs a compositionally consistent approach for DA testing using centered log-ratio transformations for data preprocessing ([Fernandes et al., 2014](#)), and Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC, v2.0.2), which explicitly tests hypotheses about differential absolute taxon abundance, corrects for sampling fraction bias, and considers data compositionality ([Lin and Peddada, 2020](#)).

Table 1. Baseline characteristics of the no-MD and MD cases in the whole population at age 46 years.

	no-MD	MD	FDR
BMI, kg/m ²	27.74 [5.40] (264)	28.82 [5.75] (43)	0.25
Waist circumference, cm	89.16 [12.69] (263)	94.84 [14.75] (42)	0.02
T, nmol/l	0.85 [0.11] (263)	0.94 [0.11] (43)	0.42
SHBG, nmol/l	51.70 [11.80; 224.00] (262)	44.20 [14.00; 157.00] (43)	0.16
FAI	1.59 [0.03; 9.15] (262)	1.89 [0.04; 6.64] (43)	0.33
HOMA-IR index	1.82 [0.50; 11.04] (256)	2.15 [0.37; 58.20] (42)	0.07
Fasting glucose, mmol/l	5.30 [4.20; 8.30] (258)	5.30 [4.40; 6.20] (42)	0.51
Fasting insulin, mU/l	7.65 [5.48; 11.13] (258)	9.20 [6.05; 15.23] (42)	0.08
hs-CRP, mg/l	0.83 [0.17; 17.30] (249)	0.92 [0.20; 19.10] (42)	0.92
Zonulin, ng/ml	128.26 [16.00] (262)	123.23 [16.29] (43)	0.07
FABP2, ng/ml	1.25 [0.34; 6.01] (262)	1.58 [0.39; 6.40] (43)	0.02

Demographic characteristics are presented as mean with [SD] or median with interquartile range [Q1; Q3] based on data distribution and normality. The number of subjects is indicated in parentheses. P-value was determined by the t-test or Mann–Whitney U-test with the Benjamini–Hochberg FDR adjustment, and bold values represent FDR < 0.05.

MD, mood disorder; FDR, false discovery rate; T, testosterone; SHBG, sex hormone-binding globulin; FAI, free androgen index; HOMA-IR, homeostatic model assessment for insulin resistance; hs-CRP, high-sensitive C-reactive protein; FABP2, fatty acid-binding protein 2.

Table 2. Baseline characteristics of the no-MD and MD cases in the PCOS and control groups at age 46 years.

	PCOS		Control		FDR ^a		FDR ^b	
	no-MD	MD	no-MD	MD	PCOS	Control	no-MD	MD
BMI, kg/m ²	27.54 [5.24] (84)	29.22 [6.46] (18)	27.84 [5.48] (180)	28.52 [5.30] (25)	0.24	0.56	0.68	0.70
Waist circumference, cm	88.38 [12.03] (83)	95.91 [16.89] (18)	89.52 [13.00] (180)	94.04 [13.24] (24)	0.09	0.11	0.50	0.69
T, nmol/l	0.94 [0.33] (84)	0.91 [0.32] (18)	0.84 [0.33] (179)	0.90 [0.38] (25)	0.73	0.41	0.03	0.95
SHBG, nmol/l	51.05 [34.73; 72.05] (84)	49.00 [29.23; 63.03] (18)	53.80 [38.58; 73.08] (178)	43.30 [29.85; 70.15] (25)	0.41	0.22	0.74	0.94
FAI	1.75 [1.40; 2.59] (84)	1.72 [1.33; 3.24] (18)	1.49 [1.14; 2.07] (178)	1.98 [1.06; 2.71] (25)	0.97	0.40	0.01	0.77
HOMA-IR index	1.80 [1.27; 2.71] (82)	2.80 [1.50; 4.78] (18)	1.82 [1.22; 2.72] (174)	1.98 [1.14; 3.47] (24)	0.03	0.63	0.94	0.17
Fasting glucose, mmol/l	5.30 [5.10; 5.70] (83)	5.65 [5.20; 5.93] (18)	5.30 [5.10; 5.70] (173)	5.30 [5.00; 5.50] (23)	0.01	0.98	1.00	0.03
Fasting insulin, mU/l	7.45 [5.58; 11.23] (82)	11.45 [6.48; 16.78] (18)	7.80 [5.33; 11.10] (176)	8.25 [5.13; 14.73] (24)	0.04	0.61	0.99	0.18
hs-CRP, mg/l	0.86 [0.17; 8.98] (82)	0.84 [0.22; 19.10] (17)	0.79 [0.16; 17.30] (175)	0.78 [0.20; 6.51] (24)	0.37	0.52	0.52	0.41
Zonulin, ng/ml	129.71 [14.23] (84)	135.30 [12.44] (18)	127.57 [16.77] (178)	130.02 [18.52] (25)	0.13	0.50	0.31	0.30
FABP2, ng/ml	1.31 [0.44; 4.18] (84)	1.74 [0.62; 4.08] (18)	1.29 [0.34; 6.01] (178)	2.17 [0.39; 6.40] (25)	0.13	0.10	0.76	0.96

Demographic characteristics are presented as mean with [SD] or median with interquartile range [Q1; Q3] based on data distribution and normality. The number of subjects is indicated in parentheses. P-value was determined by the t-test or Mann–Whitney U-test with the Benjamini–Hochberg FDR adjustment. ^aFDR is the statistical analysis between no-MD and MD cases in each group (PCOS or control), and ^bFDR is the statistical analysis between PCOS and control cases with the same mood disorder status. Bold values represent FDR < 0.05.

MD, mood disorder; FDR, false discovery rate; T, testosterone; SHBG, sex hormone-binding globulin; FAI, free androgen index; HOMA-IR, homeostatic model assessment for insulin resistance; hs-CRP, high-sensitive C-reactive protein; FABP2, fatty acid-binding protein 2.

Benjamini–Hochberg method was used to account for the multiple testing. Figures were created with RStudio (version 2022.12.0.353 with R version 4.2.2, R Foundation for Statistical Computing, Vienna, Austria) using the ggplot2 tool (v.3.4.0) and GraphPad Prism (version 9.3.0, GraphPad Software, San Diego, CA, USA).

Machine learning (ML) algorithms facilitate the handling of large-scale microbiome compositional data and the identification of microbial features, which are relevant for classifying the trait of interest. Such approaches have successfully been used to build microbiome-based classification models for various complex diseases (Namkung, 2020; Liu et al., 2022). The ML analyses were implemented using the scikit-learn package (Pedregosa et al., 2011) and the q2-sample-classifier plugin. Given the unique advantages of handling various factors, such as the large number of microbial species, the abundance distribution, and the high-dimensional nature of the data itself, AdaBoost, Extra Trees, and RandomForest (RF) ensemble classifiers were utilized (Freund and Schapire, 1997; Breiman, 2001; Geurts et al., 2006). The receiver operating characteristic (ROC) of the AUC was used to assess the performance of the models. Macro-averaging gives equal weight to each category, while micro-averaging gives equal weight to each sample during classification.

A partial correlation coefficient was calculated to examine the associations between the 10 most abundant taxa and common clinical characteristics related to MD and PCOS, while mitigating the potential influence of confounding factors, using Spearman's correlation coefficient (ppcor, v.1.1) (Kim, 2015). The predominant taxa typically hold a more substantial presence within the gut microbial community, and examining these prevalent taxa can offer valuable insights into the comprehensive structure and composition of the microbiome.

Results

Clinical characteristics of the study subjects

The study population comprised 307 women who responded to both PCOS and MD criteria scales and provided a stool sample at 46 years. To confirm alterations in the gut bacteriome associated with MDs in our study cohort, as aligned with previous findings, we categorized the participants based on MDs: 264 women with no-MD and 43 with MD cases. The whole MD cases (i.e. control MD+PCOS MD) exhibited higher waist circumference (FDR = 0.02) and FABP2 levels (FDR = 0.02) compared to the whole no-MD cases (i.e. control no-MD+PCOS no-MD) (Table 1).

We then compared the clinical characteristics of the participants based on both MDs and PCOS status, resulting in 84 PCOS no-MD, 18 PCOS MD, 180 control no-MD, and 25 control MD cases (Table 2). Notably, we observed higher levels of parameters related to glucose metabolisms, such as HOMA-IR (FDR = 0.03), fasting glucose (FDR = 0.01), and fasting insulin (FDR = 0.04) in the PCOS MD cases compared to the PCOS no-MD cases. There were no significant differences observed in the clinical characteristics among the control cases. Additionally, the prevalence of MDs was 17.65% in the PCOS group and 12.20% in the control group (FDR = 0.41) by Pearson's chi-square test.

Gut bacterial community profile and associations with MDs

We identified 111 genera across all the samples ($n = 307$), with *Bacteroides* being the dominant genus, followed by *Faecalibacterium* and *Alistipes* in each population (Supplementary Table S2). First, we investigated alterations in the gut bacteriome associated with MD status within the whole population. The whole MD cases exhibited a decreased alpha diversity (FDR_{Observed features} = 0.01, FDR_{Shannon} = 0.02) but no distinct differences in beta diversity compared to the whole no-MD cases, as determined by PERMANOVA ($r^2 = 0.01$, $P = 0.05$) (Fig. 1a–c).

We then examined the bacterial composition in individuals with and without MDs considering both shared and distinct features. We explored shared features by calculating median values of the relative abundance of four main bacteria phyla and the top 10 most abundant genera in the study subjects. There were no significant differences in the median relative abundance between the no-MD and MD cases (Fig. 1d, Supplementary Table S3). Next, we assessed 126 taxa to identify distinctive features using ALDEx2 and ANCOM-BC analyses, but none of the taxa reached statistical significance after FDR adjustment (Supplementary Table S4). We then trained ML classifiers using the Extra-Trees model to distinguish cases based on MDs (AUC = 0.65) as both AdaBoost and RF models did not exceed an AUC of 0.6 (Supplementary Fig. S2). We identified 10 important features for the model, with statistically distinct relative frequencies in *Actinomyces* F0332 (FDR = 0.05) and *Aggregatibacter* (FDR = 0.04) between the no-MD and MD cases (Fig. 1e).

The association of gut bacterial community with MDs in the PCOS group

Next, we investigated the gut bacteriome alterations in relation to MDs in the presence of PCOS. Similar to the whole population, the PCOS MD cases showed lower alpha diversity than the PCOS

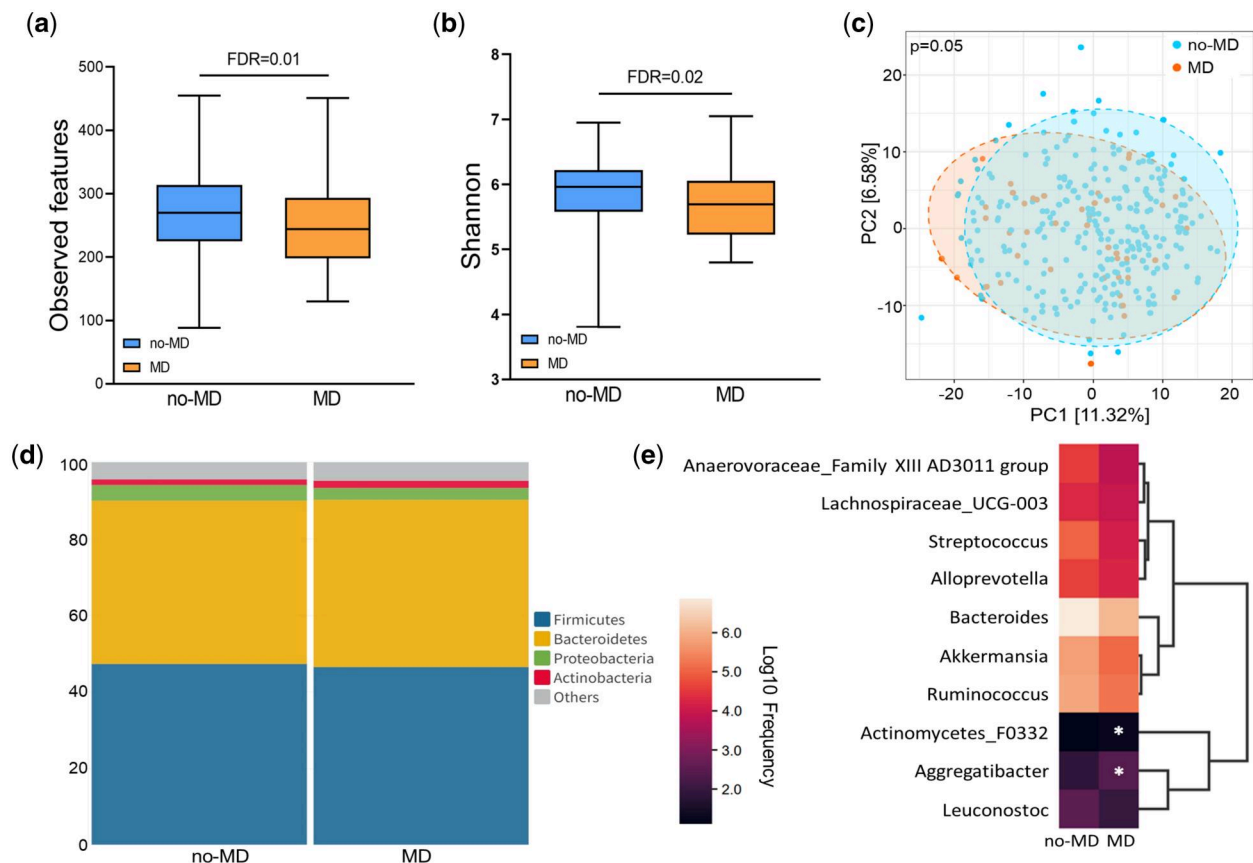


Figure 1. Comparisons of the gut bacteriome in relation to MDs in the whole population. The gut bacteriome profile of the subjects in the whole population, including both women with PCOS and control women, was analyzed. Alpha richness was analyzed using observed features and the Shannon index, and beta diversity was assessed using Bray–Curtis dissimilarity. In (a) observed features and (b) Shannon index. The box plots show the IQR, and the middle line represents the median values. Whiskers in the box plots denote minimum to maximum values. Blue represents no-MD and orange represents MD. A P -value was defined using the Kruskal–Wallis test adjusted with the Benjamini–Hochberg method. In the Bray–Curtis distances of (c) no-MD and MD cases, each dot represents a single individual, and the variation is shown by the percentages at the two axes. Blue represents no-MD and orange represents MD. A P -value was defined by PERMANOVA analysis. (d) The relative abundances of the four major phyla are represented as median values. The phyla with a relative abundance of <1% were grouped as others. (e) The bacterial features classifying the cases based on MDs were analyzed using the Extra-Trees model. The abundance of each feature (log₁₀ frequency) is indicated by the color scale of the heatmap. A P -value was calculated by the Mann–Whitney U -test adjusted with the Benjamini–Hochberg method. FDR, false discovery rate; IQR, interquartile range; MD, mood disorder; PC, principal component; PERMANOVA, permutational analysis of variance.

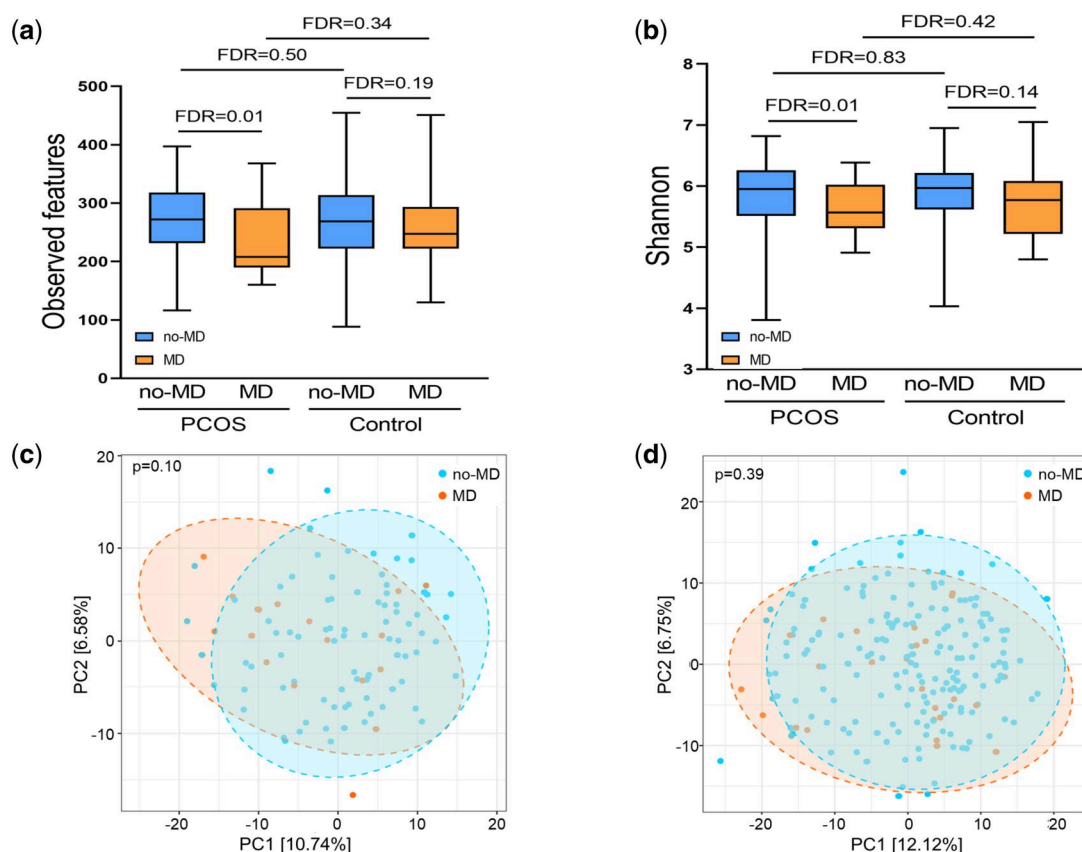


Figure 2. Comparisons of the gut bacteriome in relation to MDs in the PCOS and control groups. Alpha richness was analyzed using observed features and the Shannon index, and beta diversity was assessed using Bray–Curtis dissimilarity. In (a) observed features and (b) Shannon index, The box plots show the IQR, and the middle line represents the median values. Whiskers in the box plots denote minimum to maximum values. Blue represents no-MD and orange represents MD. A P-value was defined using the Kruskal–Wallis test adjusted with the Benjamini–Hochberg method. In the Bray–Curtis of (c) PCOS and (d) control groups, each dot represents a single individual, and the variation is shown by the percentages at the two axes. Blue represents no-MD and orange represents MD. A P-value was defined by PERMANOVA analysis. FDR, false discovery rate; IQR, interquartile range; MD, mood disorder; PC, principal component; PERMANOVA, permutational analysis of variance.

no-MD cases ($FDR_{\text{Observed features}} = 0.01$, $FDR_{\text{Shannon}} = 0.01$) (Fig. 2a and b), and no difference in beta-diversity was observed (PERMANOVA; $r^2 = 0.01$, $P = 0.10$) (Fig. 2c). Differences in diversity related to MDs could not be detected within the control group ($FDR_{\text{Observed features}} = 0.19$, $FDR_{\text{Shannon}} = 0.14$, as shown in Fig. 2a and b, PERMANOVA; $r^2 = 0.01$, $P = 0.39$, as shown in Fig. 2d). Additionally, PCOS status did not influence community diversity when comparing cases with MDs (i.e. control MD vs PCOS MD), including both alpha diversity ($FDR_{\text{Observed features}} = 0.34$, $FDR_{\text{Shannon}} = 0.42$, as shown in Fig. 2a and b) and beta diversity (PERMANOVA; $r^2 = 0.02$, $P = 0.96$, data not shown).

The most abundant phyla in the PCOS population are shown in Supplementary Fig. S3. In the comparisons of the median-based relative abundance of the 10 most abundant genera in the PCOS group, *Sutterella* showed a higher abundance in the PCOS MD cases compared to the PCOS no-MD cases (PCOS no-MD, median 0.00%; PCOS MD, median 0.14%, $FDR < 0.001$) (Table 3). In the DA analyses, while the control group showed no significant variation in relation to MDs (Supplementary Table S5), *Butyrivibrio* displayed a DA associated with MDs in the PCOS group, even after multiple testing corrections (ALDEx2, log-fold change (lfc) = -0.52 , $FDR = 0.06$; ANCOM-BC, lfc = -0.90 , $FDR = 0.04$) (Table 4). *Peptococcus* and *Agathobacter* showed DA between the control MD and PCOS MD cases in the ANCOM-BC analysis, but the significances were no longer present after multiple testing corrections (*Peptococcus*, $FDR_{\text{ANCOM-BC}} = 0.99$; *Agathobacter*, $FDR_{\text{ANCOM-BC}} = 0.99$).

Associations of the gut bacteriome in relation to common clinical features of PCOS and MDs

To explore potential connections between PCOS and MDs mediated by the gut bacteriome, we conducted partial correlation analyses between the most abundant genera and common clinical features of PCOS and MDs using two different study groups: (i) entire PCOS group (i.e. PCOS no-MD+PCOS MD) and (ii) entire MD group (i.e. control MD+PCOS MD) in order to mitigate their potential influence. In the PCOS group, *Sutterella* exhibited positive correlations with BMI ($r^2 = 0.31$, $FDR = 0.01$), waist circumference ($r^2 = 0.29$, $FDR = 0.02$), fasting glucose level ($r^2 = 0.46$, $FDR < 0.001$), fasting insulin level ($r^2 = 0.24$, $FDR = 0.045$), and zonulin ($r^2 = 0.25$, $FDR = 0.03$) (Fig. 3a). Additionally, we observed positive correlations between *Parabacteroides* and BMI ($r^2 = 0.44$, $FDR = 0.02$) and *Bifidobacterium* and zonulin level ($r^2 = 0.51$, $FDR = 0.01$) in the MD group (Fig. 3b).

Discussion

Previous studies have provided evidence that women diagnosed with PCOS are at higher risk for MDs compared to women without PCOS. However, currently, there is a lack of studies exploring the associations of the gut bacteriome with MDs in women with PCOS, even though the gut bacteriome can contribute to the underlying mechanisms of both disorders. Here we report, for the first time, the link between the gut bacteriome and MDs in PCOS.

Table 3. Relative abundance of the 10 most abundant genera in the PCOS group.

	PCOS		Control		FDR ^a		FDR ^b	
	no-MD (n = 84)	MD (n = 18)	no-MD (n = 180)	MD (n = 25)	PCOS	Control	no-MD	MD
<i>Bacteroides</i>	21.10 [14.62; 29.46]	27.06 [15.03; 36.73]	27.56 [16.19; 40.83]	24.65 [18.47; 37.92]	0.30	0.75	0.00	0.51
<i>Faecalibacterium</i>	11.35 [6.41; 18.61]	9.21 [5.09; 18.17]	13.33 [7.55; 19.42]	11.68 [8.17; 15.32]	0.55	0.24	0.18	0.56
<i>Alistipes</i>	8.25 [3.58; 13.45]	8.68 [4.13; 12.76]	10.62 [6.22; 16.59]	8.67 [4.60; 14.72]	0.82	0.35	0.02	0.77
<i>Parabacteroides</i>	1.30 [0.04; 3.76]	3.26 [0.41; 6.04]	1.07 [0.08; 4.49]	1.43 [0.30; 4.11]	0.22	0.85	0.60	0.44
<i>Ruminococcus</i>	1.59 [0.88; 4.05]	1.23 [0.59; 4.99]	1.67 [0.66; 3.43]	1.37 [0.54; 2.46]	0.72	0.31	0.46	0.51
<i>Subdoligranulum</i>	2.83 [1.10; 4.91]	1.55 [0.60; 4.56]	3.24 [1.37; 6.10]	2.47 [1.27; 3.80]	0.26	0.14	0.27	0.58
<i>Oscillospiraceae_UCG_002</i>	1.68 [0.40; 4.63]	1.78 [0.01; 2.98]	2.41 [0.60; 5.54]	1.65 [0.04; 7.39]	0.12	0.56	0.27	0.30
<i>Barnesiella</i>	1.57 [0.00; 4.62]	0.37 [0.00; 4.70]	0.57 [0.00; 3.01]	0.01 [0.00; 1.47]	0.99	0.12	0.09	0.14
<i>Sutterella</i>	0.00 [0.00; 0.00]	0.14 [0.13; 1.41]	0.00 [0.00; 0.45]	0.00 [0.00; 0.63]	<0.001	0.95	0.00	0.26
<i>Prevotella</i>	0.00 [0.00; 0.23]	0.00 [0.00; 0.01]	0.01 [0.00; 0.23]	0.00 [0.00; 3.60]	0.32	0.89	0.26	0.35

The 10 most abundant genera within the PCOS group are shown. The relative abundance of genera is presented as a median with an interquartile range [Q1; Q3]. P-value was determined by the Mann–Whitney U-test with the Benjamini–Hochberg FDR adjustment. FDR^a is the statistical analysis between no-MD and MD cases in each group (PCOS or control), and FDR^b is the statistical analysis between PCOS and control cases with the same mood disorder status. Bold values represent FDR < 0.05.

MD, mood disorder; FDR, false discovery rate.

Table 4. Differential abundant taxa between no-MD and MD cases in the PCOS group.

ALDEx2			ANCOM-BC		
Taxa	Effect size	FDR	Taxa	Effect size	FDR
<i>Butyricicoccus</i>	−0.52	0.06	<i>Butyricicoccus</i>	−0.90	0.04
<i>Oscillospiraceae_uncultured</i>	−0.55	0.09	<i>Oscillospiraceae_uncultured</i>	−0.72	0.47
<i>Holdemania</i>	−0.36	0.16	<i>Izomoplasmatales</i>	1.43	0.47
<i>Ruminococcaceae_DTU089</i>	−0.37	0.20	<i>Holdemania</i>	−1.23	0.47
<i>Oscillibacter</i>	−0.45	0.26	<i>Ruminococcaceae_DTU089</i>	−1.02	0.60
<i>Ruminococcus_torques_group</i>	−0.39	0.53	<i>Oscillibacter</i>	−0.52	0.60
<i>Bacteroides</i>	−0.37	0.54	<i>Ruminococcus_torques_group</i>	−0.54	0.60

The seven differentially abundant genera between no-MD and MD cases within the PCOS group are shown. A positive effect size value indicates a higher abundance of a taxon in the MD cases, while a negative value indicates a higher abundance in the no-MD cases. P-value was adjusted using the Benjamini–Hochberg method. Bold values represent FDR < 0.05.

MD, mood disorder; FDR, false discovery rate.

Here, we observed decreased alpha diversity in the entire MD cases (i.e. control MD+PCOS MD) compared to the entire no-MD cases (i.e. control no-MD+PCOS no-MD), consistent with the previous findings (Simpson *et al.*, 2021), and identified *Actinomycetes* F0332 and *Aggregatibacter* as features classifying subjects based on the presence or absence of MDs. Taking into account their associations with inflammation (Kamma *et al.*, 2004; Looh *et al.*, 2022; Miao *et al.*, 2022), in addition to our results of elevated FABP2 levels and a positive correlation between abundance of *Bifidobacterium* and zonulin across all of the MD cases, these findings are relevant given the literature on systemic inflammation resulting from gut barrier compromise and its role in MD development (Arneith, 2018; Peirce and Alviña, 2019; Simpson *et al.*, 2021).

Previous studies have reported (i) altered gut bacterial profiles and a decline in diversity among women with PCOS and their associations with clinical characteristics of PCOS (Lindheim *et al.*, 2017; Torres *et al.*, 2018; Jobira *et al.*, 2020; Lüll *et al.*, 2021), and (ii) compositional changes in the gut bacterial community and reduced diversity along with a decrease in short-chain fatty acids (SCFA) synthesis in MD patients (Huang *et al.*, 2018; Jiang *et al.*, 2018). Interestingly, here, we revealed a shift in gut bacterial diversity within the PCOS group in relation to MDs. These findings align with the general understanding that greater diversity corresponds to higher community stability and functionality, indicative of the beneficial ecological value of the gut bacterial system (Shade, 2017). Nevertheless, the precise causal relationship between PCOS and changes in the gut bacteriome remains unknown. It is also important to acknowledge that gut bacterial

diversity associated with PCOS (Rizk and Thackray, 2021; Sola-Leyva *et al.*, 2023) and MDs (Järbrink-Sehgal and Andreasson, 2020) varies between studies. Indeed, we did not observe any differences in the gut bacteriome profiles between women with and without PCOS. This discrepancy could stem from variations in the criteria we utilize to select study subjects in the present study. Unlike previous studies, we consider criteria for both PCOS and MDs. Furthermore, similar gut bacteriome profiles between PCOS cases and controls could be explained by meticulously matching of age and BMI. Since these confounding factors can affect clinical features of PCOS (e.g. HA, IR) (Hsu, 2013; Moran *et al.*, 2015), MDs (Scott *et al.*, 2008; Faravelli *et al.*, 2013; Sharafi *et al.*, 2020; Gallagher *et al.*, 2023), and the microbiome (Maruvada *et al.*, 2017; Badal *et al.*, 2020; Walrath *et al.*, 2021; Sola-Leyva *et al.*, 2023), they should be carefully considered when comparing case and control groups. Additionally, it is likely that metrics, such as diversity indices, only offer a simplified view of the gut bacteriome and may not be sufficient to explain the complex crosstalk with the host (Simpson *et al.*, 2021).

The relative abundance of *Butyricicoccus* in the MD cases significantly differed from the no-MD cases within the PCOS group. *Butyricicoccus*, as a butyrate producer, can suppress inflammatory responses by regulating immune activation in the colon (Li *et al.*, 2018) and contribute to brain function by elevating the expression of brain-derived neurotrophic factor, known for its antidepressant-like effects and support of neuroplasticity (Zhou *et al.*, 2020b). Consistent with our findings, previous studies have reported a reduced abundance of *Butyricicoccus* in a PCOS-like rat model (Chang *et al.*, 2021), in GAD patients (Jiang *et al.*, 2018), and

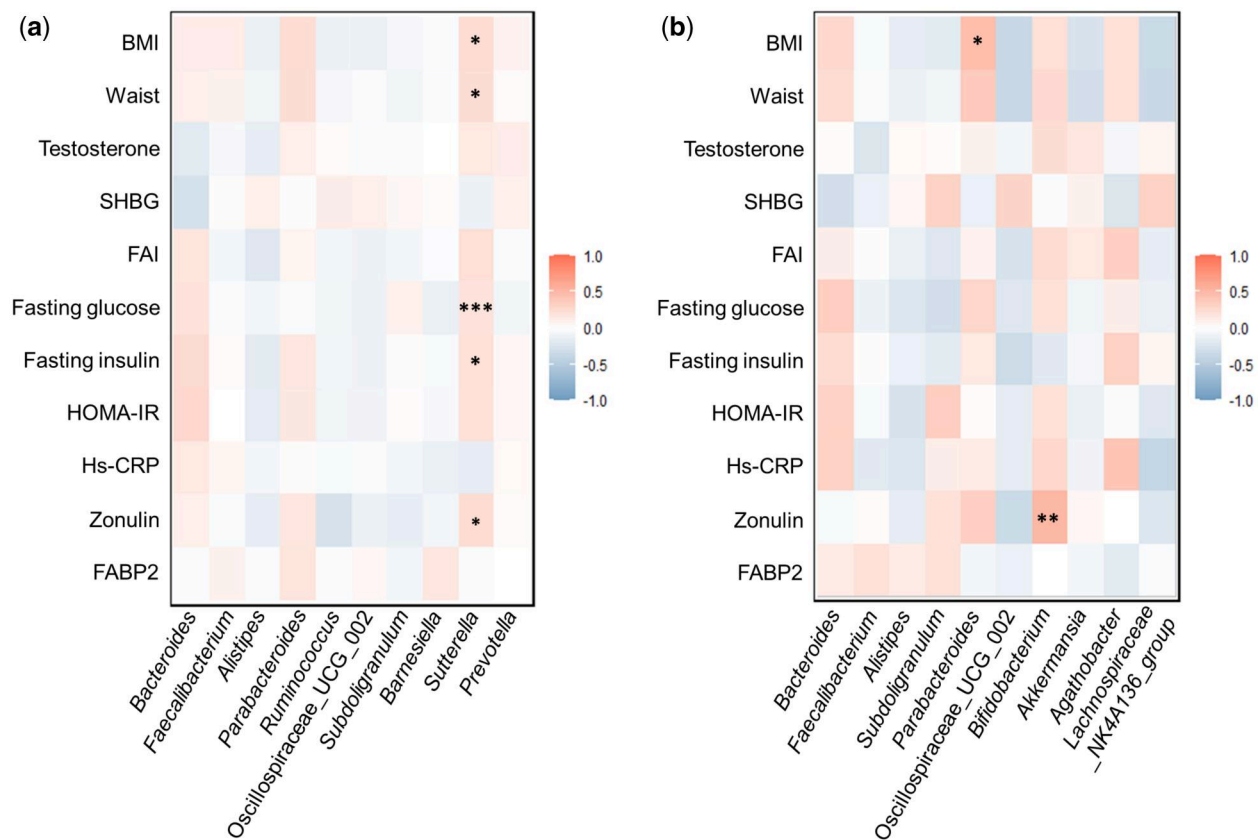


Figure 3. The partial correlation of the top 10 most abundant genera with common clinical characteristics of PCOS and MDs. Correlations between the 10 most abundant genera in (a) the PCOS group (i.e. PCOS no-MD+PCOS MD cases, $n = 102$), controlled by the presence of MDs, and (b) the MD group (i.e. control MD+PCOS MD, $n = 43$), controlled by the presence of PCOS. A negative correlation is indicated as blue and a positive correlation is indicated as red in the color key. A P-value was determined by the Kruskal–Wallis test adjusted with the Benjamini–Hochberg method. *FDR < 0.05, **FDR < 0.01, and ***FDR < 0.001. FDR, false discovery rate; MD, mood disorder; SHBG, sex hormone-binding globulin; FAI, free androgen index; HOMA-IR, homeostatic model assessment for insulin resistance; hs-CRP, high-sensitive C-reactive protein; FABP2, fatty acid-binding protein 2.

in women with depressive symptoms (Zhou et al., 2020b; Takeda et al., 2022).

These changes in the gut bacteriome may be linked to altered clinical features observed in the PCOS group. The PCOS MD cases showed altered glucose metabolism-related markers compared to the PCOS no-MD cases, thus supporting previous studies demonstrating associations between IR and MDs (Cooney et al., 2017; Leonard and Wegener, 2020). Given that inflammation plays a role not only in insulin signaling (Caricilli and Saad, 2013; Leonard and Wegener, 2020) but also in the pathophysiology of PCOS (Shorakae et al., 2018; Oróstica et al., 2020) and MDs (Peirce and Alviña, 2019), it can be hypothesized that changes in the gut bacteriome and its metabolites in PCOS may trigger peripheral and local inflammation while simultaneously diminishing the synthesis of neurotransmitters like SCFAs, thereby contributing to neuroinflammation via vagal and spinal afferent neurons, ultimately impacting mood (Armeth, 2018; Simpson et al., 2021).

Our findings demonstrated correlations between the relative abundance of *Sutterella* and PCOS-related clinical traits in the PCOS group. *Sutterella* adheres to host intestinal mucosal epithelium and appears to have pro-inflammatory functions (Hiippala et al., 2016). Studies have shown associations between the relative abundance of *Sutterella* and (i) metabolic disorders (Lim et al., 2017; Ferrocino et al., 2018) and (ii) MDs (Williams et al., 2012; Dong et al., 2021) and a negative correlation has been reported between *Sutterella* and SCFAs, such as propionate and butyrate (Zhou et al., 2023). Most importantly, women with PCOS and PCOS-like rodent models showed a higher abundance of *Sutterella*

(Arroyo et al., 2019; Zhou et al., 2020a; Li et al., 2022), and a reduction of SCFA-producing bacteria (Torres et al., 2018; Zhang et al., 2019; Chu et al., 2020), which influence both inflammation (Peirce and Alviña, 2019; Silva et al., 2020; He et al., 2021) and the gut-brain axis (Lal et al., 2001; Silva et al., 2020). Negative associations between neurotransmitters (i.e. serotonin, ghrelin, and peptide YY) and serum T have also been detected (Liu et al., 2017). However, there is conflicting evidence regarding *Sutterella* in relation to MDs (Dong et al., 2021; Simpson et al., 2021), as the virulence (Hiippala et al., 2016) and function (Wang et al., 2020; Zhou et al., 2023) of *Sutterella* can vary among different species, limiting the interpretation of data obtained from animal studies.

The low ethnic and genetic variation of the study subjects and careful BMI-matching helped to minimize confounding factors. This study employed ML analysis as part of the methodologies, providing a data-driven approach to identify relevant patterns and relationships. Despite the strength of this study, discrepancies with previous research may arise from variations in study designs, including the sample size (Yurtdaş and Akdevelioğlu, 2020), sequencing methods (Lüll et al., 2021), and criteria for defining MDs (Deeks et al., 2011; Karjula et al., 2017; Dokras et al., 2018). For example, there are various psychiatric outcome measures, such as the Hospital Anxiety and Depression Scale, the State-Trait Anxiety Inventory, and the Center Hamilton depression rating scale, to assess MDs, apart from the criteria we used in this study (Dokras et al., 2018). Thus, these discrepancies may lead to distinct categorizations of MDs, potentially impacting different landscapes of the gut bacteriome. Additionally, extended

duration of sample storage after sample collection may influence sequencing outcomes, including changes in bacterial community diversity and composition (Shaw *et al.*, 2016; Ezzy *et al.*, 2019; Kim *et al.*, 2023), as well as bacterial functional stability (Kim *et al.*, 2023). While the integrity of DNA may degrade over time, even when stored at ultra-low temperatures like -80°C (Shaw *et al.*, 2016; Ezzy *et al.*, 2019), we mitigated the potential impact of prolonged sample storage through meticulous experimental protocols. For example, we collected samples within a short timeframe and stored them in the same freezer without any thawing (Cardona *et al.*, 2012; Gorzelak *et al.*, 2015). Furthermore, we employed advanced DNA extraction methodologies (Costea *et al.*, 2017), cutting-edge sequencing techniques (Caporaso *et al.*, 2012; Allali *et al.*, 2017), and enhanced error correction strategies using DADA2 (Callahan *et al.*, 2016). Focusing on a small segment of the 16S rRNA gene (V3–V4 region) instead of the entire genome or the full length of the 16S rRNA gene also helped mitigate potential DNA degradation, while still providing sufficient data for microbiome analysis (Cruz-Flores *et al.*, 2022). Even though the present study was the largest of its type to date, the number of women with PCOS stratified by the presence of MDs remains limited, thereby affecting the attainability of statistical power. Despite these limitations, our study was the first to elucidate alterations in the gut bacteriome in women with PCOS and MDs, spanning from the community level to the specific taxa level.

Conclusion

Based on the study results, it appears that the women with co-occurring PCOS and MDs have a distinct gut bacteriome with reduced alpha diversity and a significantly lower abundance of *Butyrivibrio* compared to women with PCOS but without MDs. Notably, the relative abundance of *Sutterella* showed associations with common clinical features of both PCOS and MDs, particularly those related to obesity and gut barrier integrity. This study opens a new avenue for investigating the pathophysiology of MDs in the context of PCOS.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The 16S RNA sequencing data were submitted in the Sequence Read Archive (SRA) (Reference No. PRJNA669650) (<https://trace.ncbi.nlm.nih.gov/Traces/sra/?study=SRP287519> <https://www.ncbi.nlm.nih.gov/bioproject/669650>). NFBC data are available from the University of Oulu, Infrastructure for Population Studies. Permission to use the data can be requested for research purposes via electronic material request portal. In the use of data, we follow the EU general data protection regulation (679/2016) and Finnish Data Protection Act. The use of personal data is based on cohort participant's written informed consent at his/her latest follow-up study, which may cause limitations to its use. Please, contact NFBC project center (NFBCprojectcenter@oulu.fi) and visit the cohort website (www.oulu.fi/nfbc) for more information.

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Authors' roles

This study was designed by S.L., R.K.A., and T.T.P. and supervised by T.T.P., R.K.A., A.S., O.A., and E.O. T.T.P., S.L., M.V.T., and R.K.A. chose the study population. J.P.-D. and S.A. conducted microbiome sequencing. S.L., M.V.T., and E.H. performed the data analysis and statistical analysis. T.S.R. supervised microbiome analysis, machine learning, and interpretation of the results. All authors revised and approved the final version.

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Conflict of interest

We have no conflicts of interest to declare.

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