

The correlation between gut metabolites and microbiota in patients with rheumatoid arthritis

Haifeng Yun

Suzhou TCM Hospital, Nanjing University of Chinese Medicine

Xinxin Wang

Graduate Institute of China Academy of Chinese Medical Sciences

Na Li

Suzhou TCM Hospital, Nanjing University of Chinese Medicine

Ming Chen

Suzhou TCM Hospital, Nanjing University of Chinese Medicine

Guoxing Zhang

Medical College of Soochow University

Dawei Cui

daweicui@zju.edu.cn

Zhejiang University School of Medicine

Rui Liu

Suzhou TCM Hospital, Nanjing University of Chinese Medicine

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Abstract

Background: Rheumatoid arthritis (RA) is a chronic autoimmune disease that can cause cartilage and bone damage and lead to disability. The lack of early interventional therapy is closely associated with the increased disability rate of RA patients worldwide. This study aimed to investigate the role of fecal metabolisms and microbiomes in the pathogenesis of RA.

Methods: A total of 46 RA patients and 9 healthy volunteers were enrolled in the study. Fecal samples were analyzed using microbiome (16S rRNA gene sequencing) and metabolomics (liquid chromatography-mass spectrometry, LC-MS) techniques. Cytokines are detected by the chemiluminescence immunoassay. These results were analyzed using bioinformatics, metabolomics, correlation analysis, and various other methods.

Results: Significant differences were observed in the gut microbes and metabolites of RA patients compared to healthy controls (HC). The dominant intestinal flora in RA patients included *Blautia*, *Oscillospira*, and *Coprococcus*. Furthermore, multiple bacteria are associated with clinical indicators of RA. In particular, bile-philic bacteria were found to be significantly negatively correlated with IL-6, IL-10, and TNF- α . The predominant metabolites in the intestines of RA patients include Lysine-Proline peptides (Lys-Pro), Tauro lithocholic Acid Sulfate, L-Leucine, etc, which are highly significantly correlated with clinical indicators and cytokines of RA, especially leucine, which may act through the mechanistic target of rapamycin (mTOR) signaling pathway.

Conclusions: These findings provided substantial evidence indicating a novel interaction between the gut microbiome and metabolites, which contributed to clinical indicators and cytokines in RA patients, and L-leucine was identified as a potential diagnostic biomarker for RA progression.

1. Introduction

Rheumatoid arthritis (RA) is a multifactorial autoimmune disease of unknown etiology, primarily affecting the joints and often involving extra-articular manifestations (1). Historically referred to as the “cancer that never dies,” RA results from immune dysfunction caused by interactions between genetic and environmental factors, leading to the production of numerous inflammatory cytokines and autoantibodies (2, 3). Cardiovascular disease is the most common cause of death in RA patients, followed by respiratory disease (4). Without formal treatment, about 75% of patients become disabled within three years.

According to CREDIT data (Chinese Registry of Rheumatoid Arthritis), the average time from symptom onset to definite diagnosis for RA patients in China is more than two years, while the optimal treatment window is 6 months to 1 year after onset. Consequently, most patients fail to receive early diagnosis and treatment, resulting in more cases of moderate and severe disease and complications (5). Early diagnosis and intervention could significantly control disease progression and improve patients’ quality of life.

Microbiome and metabolomics are rapidly developing omics techniques. Previous studies have shown that glucose metabolism plays a key role in the fibroblast-like synoviocytes of RA, and inhibition of glycolysis may directly regulate synovium-mediated inflammatory function (6). It has also been reported that RA T cells are unable to repair mitochondrial DNA, leading to metabolic dysfunction (7). In a mouse model of adjuvant arthritis, the probiotic *Lactobacillus casei* significantly inhibited the induction of adjuvant-induced arthritis (AIA) and protected rats from bone damage by restoring intestinal microbiome balance (8). Furthermore, the pathobiology of RA appears to share several pathways with atherosclerosis, including endothelial dysfunction associated with underlying chronic inflammation (9). Moreover, *Prevotella* species (including *P. histicola* and *P. oulorum*) show an increased presence in the mouths of RA patients compared to healthy individuals (10).

Our present study focused on fecal metabolisms and microbiomes in RA patients, investigating the correlation between gut metabolites and microbiota in the context of RA. Our findings may offer novel insights into the pathogenesis of RA and provide evidence for the application of clinical biomarkers in RA patients.

2. Materials and Methods

2.1 Study Design

In this study, 46 RA patients and 9 healthy volunteers were recruited from the physical examination center of Suzhou Hospital of Traditional Chinese Medicine between September 2020 and September 2021. All participants were Chinese Han from Suzhou, sharing similar geographical regions and dietary habits. The inclusion criteria for patients were: (i) a diagnosis of RA according to the European League Against Rheumatism (EULAR) criteria (11), and (ii) signed informed consent approved by the ethics committee. Patients were excluded if they: (i) had received any antibiotic or probiotic treatment in the three months prior to recruitment; (ii) suffered from infectious diseases or malignant tumors; (iii) had diabetes, hyperthyroidism, or other metabolic diseases; (iv) had severe liver or kidney function impairment; (v) had inflammatory bowel disease (IBD) or other autoimmune diseases; or (vi) were on an extreme diet. After overnight fasting, fecal samples from both groups were collected in the morning (≥ 8 h) using sterile fecal containers and stored at -80°C for subsequent processing.

2.2 Detection of Serum Inflammatory Cytokines

Blood samples were obtained from each patient after an overnight fast upon admission. The blood samples were allowed to clot at room temperature for 30 minutes and centrifuged for 10 minutes at 3000 rpm. The serum samples were separated as soon as possible from the clot of red blood cells after centrifugation. Separated sera were measured immediately.

The levels of tumor necrosis factor-alpha (TNF- α), interleukin-4 (IL-4), IL-6, IL-10 were detected by chemiluminescence immunoassay (CLIA) with the Siemens IMMULITE 1000 (Siemens Co. Ltd.) The

recommended reference range was as follows: IL-4 (≤ 8.56 pg/mL), IL-6 (≤ 5.4 pg/mL), IL-10 (≤ 12.9 pg/mL), TNF- α (≤ 16.5 pg/mL).

2.3 Extraction of Total DNA from the Microbiome

For microbiome samples from different sources, the most appropriate total DNA extraction method was selected, and DNA was quantified using Nanodrop. The quality of DNA extraction was verified by 1.2% agarose gel electrophoresis. Fluorescence quantification was performed using the Quant-iT PicoGreen dsDNA Assay Kit and Microplate reader (BioTek, FLx800). The Illumina TruSeq Nano DNA LT Library Prep Kit was used to prepare the sequencing library, which was then inspected using the Agilent Bioanalyzer with the Agilent High Sensitivity DNA Kit before sequencing.

2.4 Analysis of Sequence Data

QIIME2 software was utilized to denoise the DADA2 sequences, and R language script was used for statistical analysis of the length distribution of high-quality sequences in all samples. Species taxonomic annotation was performed using the Classify-sklearn and BROCC algorithms. The taxon map was constructed using the Linear Discriminant Analysis Effect Size (LEfse) package in Python and the R ggtree package.

2.5 Metabolite Sample Preparation

After the frozen samples were slowly thawed at 4°C, appropriate samples were added to a precooled methanol, acetonitrile, and water solution (2:2:1, v/v). After vortex mixing, the solution was ultrasounded at a low temperature for 30 minutes, left to stand at -20°C for 10 minutes, and then centrifuged at 4°C for 20 minutes. The supernatant was taken for vacuum drying. During mass spectrometry analysis, 100 μ L of an acetonitrile aqueous solution (acetonitrile: water = 1:1, v/v) was added to redissolve the samples. The samples were then vortexed and centrifuged at 4°C for 15 minutes, and the supernatant was taken for sample analysis.

2.6 LC-MS (Liquid Chromatography-Mass Spectrometry) Analysis

The samples were separated using an Agilent 1290 Infinity LC ultra-high-performance liquid chromatography (UHPLC) HILIC column. The column temperature was set at 25°C, with a flow rate of 0.5 mL/min and a sample size of 2 μ L. The mobile phase consisted of: A: water with 25 mM ammonium acetate and 25 mM ammonia water, B: acetonitrile. The gradient elution procedure was as follows: 0-0.5 min: 95% B; 0.5-7 min: B changed linearly from 95% to 65%; 7-8 min: B changed linearly from 65% to 40%; 8-9 min: B maintained at 40%; 9-9.1 min: B changed linearly from 40% to 95%; 9-12 min: B maintained at 95%. Samples were placed in an automatic injector at 4°C throughout the analysis. To avoid fluctuations in instrument detection signals, a random sequence of samples was used for continuous analysis. Quality control (QC) samples were inserted into the sample queue to monitor system stability and evaluate the reliability of the experimental data.

For the analysis of metabolic results, an AB Triple TOF 6600 mass spectrometer was used to collect primary and secondary spectra of the samples. The original data in Wiff format was converted into mzXML format using ProteoWizard, followed by peak alignment, retention time correction, and peak area extraction using XCMS software. Metabolite structure identification and data preprocessing were performed on the data extracted by XCMS, and the quality of experimental data was evaluated.

2.7 Statistical Analysis

After quality control of all samples, microbial community diversity was analyzed using Alpha diversity indicators, calculated and presented using QIIME (Version 2022.8) and R software (Version 4.2.0). Beta diversity was used to compare and analyze microbial community composition in different samples. For group difference analysis, multidimensional statistical analysis was performed to screen differential metabolites. Principal component analysis (PCA) was conducted using R software. A random forest model was then constructed to screen important species based on overall accuracy, and a nested stratified cross-test was performed to evaluate model performance. Finally, Spearman rank correlation analysis was used to assess the relationship between metabolites and gut microbes at the genus level.

3. Results

3.1 Summary data of Clinical Characteristics

A total of 55 individuals participated in this study. Group RA included 6 men and 40 women, aged between 21 and 73 years (mean age 54.23 ± 11.80 years). The disease duration in this group ranged from 1 to 12 years (mean duration 4.49 ± 3.35 years). Group healthy control (HC) comprised 1 male and 8 female subjects, aged between 33 and 65 years (mean age 53.11 ± 11.48 years). The study found significant differences in erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP), white blood cell (WBC) count, creatinine (Cr), IL-4, IL-6, IL-10, and TNF- α between the two groups ($P < 0.05$) (Table 1).

Table 1
Comparison of clinical data between the two groups

Group	RA(N = 46)	HC(N = 9)	t/z/x2	P
Gender	male = 7, female = 39	male = 1, female = 8	-	> 0.05
Age	54 ± 11.82	53.11 ± 11.48	-	> 0.05
DAS28 score	4.25 ± 0.55	0.00	-	< 0.001
VAS score(cm)	5.72 ± 1.36	0.00	-	< 0.001
ESR(mm/H)	50.00(32.00–83.00)	8.00(4.00–14.00)	-4.720	< 0.001
CRP(mg/L)	25.70(10.30–39.60)	3.02(2.14–3.89)	-4.384	< 0.001
RF(IU/ml)	176.00(59.90–396.00)	10.40(5.40–15.10)	-4.719	< 0.001
Anti-CCP antibody(RU/L)	116.70(64.20–500.00)	2.97(1.47–3.79)	-4.541	< 0.001
WBC(10 ⁹ /L)	6.85(3.61–13.83)	4.77(3.57–7.04)	2.113	0.042
RBC(10 ¹² /L)	4.02(2.97–4.85)	4.35(3.89–5.01)	-1.649	0.109
HGB(g/L)	117.34(87.00-137.00)	124.50(115.00-135.00)	-1.351	0.186
PLT(10 ⁹ /L)	252.24(130.00-433.00)	260.17(175.00-332.00)	-0.255	0.800
ALT(U/L)	17.24(0.80–59.20)	23.67(14.00–33.00)	-1.222	0.230
AST(U/L)	18.86(7.60–38.00)	20.67(13.00–32.00)	-0.560	0.579
Cr(umol/L)	48.69(29.60–72.80)	65.33(47.00–80.00)	-3.789	0.001
Number of tender joints	7.68 ± 2.41	0.00	-	< 0.001
Number of swollen joints	6.74 ± 2.43	0.00	-	< 0.001
HAQ score	13.38 ± 4.43	0.00	-	< 0.001
IL-4(pg/ml)	4.58 ± 2.20	6.21 ± 0.96	-2.881	0.01
IL-6(pg/ml)	8.41 ± 4.19	1.94 ± 1.36	3.705	0.001
IL-10(pg/ml)	14.85 ± 5.77	8.00 ± 4.16	2.751	0.01
TNF-α(pg/ml)	21.06 ± 6.56	8.78 ± 4.72	4.338	< 0.001
Abbreviations: DAS28 Score: Disease activity score 28; VAS score: Visual analogue score; ESR: Erythrocyte sedimentation rate; CRP:C-reactive protein; RF: Rheumatoid factors; HAQ: health assessment question score; HC: Healthy control.				

3.2 Gut Microbial Profiles of RA Patients

Rank-abundance curves are valuable tools for illustrating species abundance and community evenness. As depicted in Fig. 1A, the flatness of the broken lines in the abundance grade curve indicated the evenness of community composition, suggesting that the sequencing data in this study were adequate. To thoroughly evaluate microbial community richness, the Beta diversity index was introduced. Beta diversity not only measures differences between communities but also describes the number of species. The closer the distance between two points, the smaller the difference in community composition between the samples. Both unweighted and weighted PCoA plots demonstrated that RA had a significant impact on the diversity of gut microbiota (Fig. 1B).

3.3 Alterations in Microbial Composition Associated with RA

The number of unique OTUs (Operational Taxonomic Units) in RA and HC were 22,990 and 3,220, respectively. The common OTU number between the two groups was 2,431. This indicates a significant difference in species composition between healthy individuals and RA patients, with RA patients showing a much higher unique OTU number (Fig. 2A).

In evaluating the abundance difference at different taxonomic levels, the most abundant phyla were Bacteroides, Firmicutes, and Proteobacteria. At the genus level, Bacteroides, Prevotella, and Faecalibacterium were enriched in the gut (Fig. 2B).

To study species differences between RA and HC groups, a heatmap was drawn based on species richness. The analysis revealed higher abundance of Bacteroides, Faecalibacterium, Megamonas, and Dialister in the RA compared to HC group (Fig. 2C). Further, using LEfSe analysis (Linear discriminant analysis Effect Size), 27 significantly discriminant taxa ($LDA > 3$, $P < 0.05$) were identified. Sutterella, Synechococcus, and Oceanicaulis were the main taxa enriched in healthy individuals, whereas Turicibacter was more abundant in RA patients (Fig. 3A). Figure 3B depicts the taxonomic hierarchy from phylum to genus.

Random forest analysis was employed to distinguish between the RA and HC groups. This algorithm is particularly suitable for microbial community data owing to its ability to handle discrete and discontinuous distributions effectively. The analysis at the genus level was based on OTU characteristics. The most important species were identified as markers of differences between the groups from the flora's importance score (Fig. 3C). Twenty important genera were selected and a 10-fold cross-validation was performed to score the model's predictive ability. The accuracy of the model prediction results was also obtained.

In Fig. 3D, the vertical axis represents the actual label of the sample, and the horizontal axis represents the predicted label by the classifier. The shade of color in each row grouping indicates the proportion of labeled samples predicted. Darker colors on the main diagonal and lighter colors outside it signify higher prediction accuracy. The values in the top half of each row represent the proportion of samples correctly or incorrectly classified into each group. The last three rows represent the overall prediction accuracy,

baseline accuracy, and their ratio. The model's overall accuracy was 0.818, indicating that a higher overall prediction accuracy improves the relative baseline error rate, hence demonstrating the high accuracy of the model.

3.4 Differentially Abundant Metabolites Between the RA and HC Groups

In this study, non-targeted metabolomics was applied to detect changes in all small molecule metabolites in organisms without bias. Principal Component Analysis (PCA), an unsupervised data analysis method, reduces the dimensions of original data using two selected comprehensive variables. PCA can also observe the overall distribution trend and degree of difference between groups. The PCA score chart is shown in Fig. 4A.

Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) is a supervised discriminant analysis method. By applying partial least squares regression, it models the relationship between metabolite expression and sample class. In both positive and negative ion modes, RA and HC groups showed clear separation. The OPLS-DA model score chart is shown in Fig. 4B, indicating the model's ability to distinguish the two groups.

The model's validity was tested using a permutation test ($n = 200$). The test showed that R^2 and Q^2 of the stochastic model declined gradually with decreased permutation retention, indicating no overfitting and confirming the model's reliability. The permutation test diagram is shown in Fig. 4C.

3.5 Screening and Identification of Differential Metabolites in RA Patients

This project utilized a local self-built standard product database search. Metabolite structures were identified by matching information on retention time, molecular mass (error < 10 ppm), second-order fragmentation spectrum, and collision energy in the local database. The identification results were then strictly checked and confirmed manually, achieving an identification level above Level 2. The identified metabolites mainly included: Lipids and lipid-like molecules (28.1%), Organic acid derivatives (20.6%), Organo heterocyclic compounds (6.45%), Benzenoids (8.91%), Other markers.

3.6 Variable Importance for the Projection (VIP)

The VIP obtained from the OPLS-DA model measures the influence of metabolite expression patterns on group discrimination, helping explore differential lipid molecules of biological significance. Metabolites with $VIP > 1$ are considered significant for model interpretation. Metabolomics typically use strict criteria of OPLS-DA $VIP > 1$ and $P\text{-value} < 0.05$ for identifying significant differences, which this study follows. Significant differential multiples are visually represented in Fig. 5A.

From the screening, the following metabolites were found to be upregulated: Lysine-Proline peptides (Lys-Pro), Kanamycin A, Tauroolithocholic Acid Sulfate, Probucol, Neohesperidose, and L-Leucine. The

downregulated metabolites included Neohesperidin, Narirutin, Didymin, and Naringin.

Metabolites with high abundance in RA patients were identified as potential biomarkers. L-Leucine and Tauroolithocholic Acid Sulfate were selected as candidate biomarkers for RA (Figs. 5C and 5D). The areas under the curve (AUCs) were 0.796 and 0.772, respectively, indicating that the diagnostic model has relatively high accuracy and can effectively distinguish RA patients from healthy controls. However, further validation with an expanded sample size is necessary.

Using the abundance data of metabolic pathways, we aimed to identify pathways with significant differences between RA patients and healthy volunteers. The metagenomeSeq method was applied, revealing that the mTOR signaling pathway differed significantly between the two groups (Fig. 5B).

3.7 Correlation Analysis of Intestinal Differential Metabolites and Flora

Spearman correlation analysis was conducted to explore associations between intestinal differential flora and fecal differential metabolites at the genus level. Clustering heat maps were created to visualize the correlation coefficients (Fig. 6A, B). In these maps, red indicates a strong positive correlation, while blue signifies a strong negative correlation.

Figure 6A highlights a positive correlation between Gemmiger, Barnesiella, and brassicasterol. Lys-pro is positively correlated with Bacteroides, indicating its potential impact on the final concentration of these metabolites and the function of the intestinal barrier. There is a strong positive correlation between Diphenhydramine and Bilophila.

Figure 6B shows that Bilophila has a strong positive correlation with Deoxyinosine. The abundance of anaerostipes is low in RA patients and negatively correlated with Probuco. Tauroolithocholic acid sulfate is highly enriched in RA patients, with Alistipes, Parabacteroides and Odoribacter negatively correlated with it. These findings suggest that these bacteria could influence the progression of RA by affecting tauroolithocholic acid sulfate levels. Additionally, metabolites such as Probuco, Neohesperidose, Kanamycin a, and L-Leucine show a strong negative correlation with Alistipes.

3.8 Correlation between Intestinal Flora/Metabolites with Clinical Indicators and Cytokines

Spearman correlation analysis was employed to explore the relationship between fecal differential metabolites and gut microbiota with clinical indicators and cytokines. The correlation coefficients were visualized using cluster heatmaps (Figs. 7A, B, C, D). In the heatmaps, red indicates a strong positive correlation, while blue indicates a strong negative correlation.

In terms of clinical indicators (Fig. 7A, C), Alistipes and Parabacteroides were significantly negatively correlated with Disease Activity Score 28 (DAS28), anti-cyclic citrullinated peptide (anti-CCP), and RF; among them, Parabacteroides was also significantly negatively correlated with CRP. Bilophila was

significantly negatively correlated with DAS28, RF, and ESR, and positively correlated with creatinine (Cr). Tauroolithocholic Acid Sulfate and Probiucol were significantly positively correlated with DAS28, RF, and ESR, with the former also showing significant negative correlations with Cr and hemoglobin (HGB). Neohesperidose was significantly positively correlated with DAS28 and RF, and negatively correlated with RBC; L-Leucine was significantly positively correlated with DAS28, anti-CCP, and RF.

In terms of cytokines (Fig. 7B, D), *Bilophila* was significantly negatively correlated with IL-6, IL-10, and TNF- α . L-Leucine and Lys-Pro were significantly positively correlated with IL-6, IL-10, and TNF- α , while Tauroolithocholic Acid Sulfate was significantly positively correlated with IL-6 and TNF- α ; Neohesperidose was significantly positively correlated with IL-10 and TNF- α ; Probiucol was significantly negatively correlated with IL-4 and significantly positively correlated with IL-10.

4. Discussion

Our study demonstrated significant differences in the intestinal microbiome and metabolites of RA patients compared to healthy individuals, characterized by the following: (1) Microorganism Abundance Changes: *Turicibacter* was identified as the major upregulated microbiome in RA patients using various analysis methods. (2) Fecal Metabolite Levels: RA patients exhibited increased levels of metabolites such as Lys-Pro, tauroolithocholic acid sulfate, and L-Leucine, while showing relatively lower levels of metabolites like sucralose and ADP-glucose. In addition, we conducted a correlation analysis, including the following points: (1) Correlations Between Microbiota and Metabolites: The intestinal microbiota and metabolites in RA patients displayed varying degrees of positive and negative correlations. (2) Correlation between intestinal flora and metabolites with clinical indicators. (3) Correlation between intestinal flora and metabolites with cytokines respectively.

Bacteria and the human body exist in a symbiotic state, with intestinal flora participating in the regulation of multiple host metabolic pathways. This interactivity creates a host-microbiome metabolic and immune regulation pathway (15, 16). According to the “gut-joint axis” concept, RA is thought to result from the interaction between intestinal mucosal immunity and abnormal local flora, which subsequently affects the synovial membrane and joint sites (17, 18). Therefore, changes in microflora composition are considered an important factor in the development of RA.

We compared the differences in the number and abundance of intestinal flora between patients with RA and healthy individuals, identifying dominant flora such as *Aliihoeflea*, *Gemmiger*, *Bilophila*, *Sutterella*, *Nesterenkonia*, *Oceanicaulis*, *Prevotella*, *Phascolarctobacterium*, and *Alistipes*. Studies have shown that *Gemmiger* is a significant feature for distinguishing Crohn’s disease from healthy individuals (19). *Bilophila* has been associated with prevalent symptomatic hand osteoarthritis and is enriched in fecal samples from patients with active Behcet’s disease (20). We also found that *Bilophila* was significantly negatively correlated with IL-6, IL-10, and TNF- α , which is consistent with previous research (21). As a butyrate-producing bacterium, *Bilophila* can reduce inflammatory cytokine synthesis and increase anti-inflammatory cytokine secretion in colon cells. Other studies have confirmed that *Sutterella* can secrete

IgA protease, degrading IgA in the intestinal mucosa, which increases mucosal susceptibility and facilitates pathogen invasion of intestinal epithelial cells (22). Pattern recognition receptors (PRRs) of *Sutterella* are activated to promote IL-8 secretion and induce inflammatory reactions (23). There are few previous studies on *Turicibacter*, which is significantly different in the intestinal tract of RA patients. This paper may provide new directions for future research on the intestinal bacteria of RA. Previous research has reported increased abundance of *Prevotella* in patients with high levels of inflammatory cytokines, suggesting a link between abnormal intestinal flora and the inflammatory response in RA (24–27). *Alistipes* is an opportunistic pathogen that may aggravate intestinal inflammation (28). In our study, we found a significant negative correlation between *Alistipes* with DAS28, anti-CCP antibody, and RF. However, there are no relevant reports on this in rheumatoid arthritis. *Phascolarctobacterium* can produce short-chain fatty acids through fermentation in the human intestinal tract, which plays a crucial role in protecting the intestinal mucosal barrier and reducing inflammation (29). In our study, we found that *Parabacteroides* was significantly negatively correlated with important evaluation indicators in rheumatoid arthritis, including DAS28, anti-CCP antibody, RF, and CRP, which is consistent with previous research (30).

In the study of metabolic products, we found that Tauroolithocholic Acid Sulfate was significantly positively correlated with DAS28, RF, and ESR, and was also significantly negatively correlated with Cr and HGB. Although the relationship between Tauroolithocholic Acid Sulfate and the activity and inflammation of RA is not yet clear, existing research has shown that elevated bile acid levels in RA patients can reduce DAS28, ESR, CRP, RF, and anti-CCP antibody levels. XiaoHe Wang et al. found that neohesperidin can reduce the levels of cytokines such as IL-6, IL-8, and TNF- α in RA-FLS, and decrease the accumulation of reactive oxygen species (ROS) induced by TNF- α , suggesting its potential therapeutic value (32). Our research also revealed a significant positive correlation between neohesperidin and both IL-10 and TNF- α .

We also found a close correlation between many metabolites, clinical indicators, and cytokines, such as Probucol, Neohesperidose, L-leucine, Lys-Pro, taurocholic acid sulfate, etc. It is particularly noteworthy that L-leucine was found to be significantly positively correlated with DAS28, anti-CCP antibody, and RF. L-leucine and Lys-Pro were significantly positively correlated with IL-6, IL-10, and TNF- α . However, current research on the relationship between Probucol, Neohesperidose, L-leucine, Lys-Pro, taurocholic acid sulfate, and RA remains limited.

The above results indicate that these gut microbiota and fecal differential metabolites are closely related to disease activity and immune disorders in RA, and may play an important role in the occurrence and development of RA, serving as potential biomarkers.

Although many meaningful metabolites have been discovered, there is still a lack of representative markers for rheumatoid arthritis (RA). Therefore, through receiver operating characteristic (ROC) curve analysis, we found that L-leucine and taurocholic acid sulfate, which exhibit significant metabolic differences, may serve as potential biomarkers for early diagnosis. However, due to the lack of detailed

biological information regarding these metabolites, the mechanisms associated with the pathogenesis of rheumatoid arthritis (RA) still require further investigation.

Finally, we analyzed the differential metabolic pathways, among the differential metabolic pathways, the most representative is the mammalian target of rapamycin (mTOR) signaling pathway. The mTOR pathway regulates various biological processes, including cell growth, proliferation, apoptosis, and metabolism (33, 34). The mTOR pathway is divided into two complexes: mTORC1 and mTORC2. Amino acids, particularly leucine, play a crucial role in mTORC1 activation. When leucine is abundant, secretion associated Ras related GTPase 1B (SAR1B) binds to leucine, dissociates from GATOR2, and activates mTORC1, subsequently initiating metabolic and inflammatory signaling pathways (35). The results of this study suggest that the occurrence and progression of RA may be closely related to the activation of the mTOR signaling pathway by leucine, which regulates the activities of various cells and inflammatory factors.

Currently, there are limited reports on the relationship between RA and leucine. Based on previous and current research findings, we hypothesize that the microbiota may be involved in amino acid metabolism and fermentation, affecting the permeability of the intestinal mucosa and increasing human susceptibility to diseases. Furthermore, they may activate the mTOR signaling pathway related to amino acids, leading to the release of inflammatory factors and the onset of diseases. This may provide new avenues for future research and clinical diagnosis and treatment.

The limitations of this study include a relatively small sample size and significant population differences between the RA and healthy groups, primarily due to the lower incidence of RA in men. Additionally, since all participants were recruited from a single hospital, our conclusions would be more convincing with a larger, more diverse sample pool in future studies. More RA populations need to be included to confirm our findings. Furthermore, the exact effect of L-leucine on RA is not yet clear and requires further research.

5. Conclusion

In this study, we provided a comprehensive metabolomic and microbial profile comparing the RA group and the healthy group. Utilizing a non-targeted metabolomics approach based on LC-MS, we successfully distinguished RA patients from healthy individuals. Furthermore, we discovered the relationships between clinical indicators, cytokines, microbiota, and metabolites. Among these, L-leucine was identified as a potential gut-specific metabolite associated with RA. In future research, we will conduct further research to validate these findings and elucidate their specific associations with RA.

Abbreviations

RA: Rheumatoid arthritis

LC-MS: Liquid chromatography-mass spectrometry

HC: Healthy controls

Lys-Pro: Lysine-Proline peptides

mTOR: Mechanistic target of rapamycin

AIA: Adjuvant-induced arthritis

EULAR: European League Against Rheumatism

IBD: Inflammatory bowel disease

CLIA: Chemiluminescence immunoassay

UHPLC: Ultra-high-performance liquid chromatography

TNF- α : Tumor necrosis factor-alpha

IL-4: Interleukin-4

QC: Quality control

PCA: Principal component analysis

ESR: Erythrocyte sedimentation rate

CRP: C-reactive protein

RF: Rheumatoid factor

anti-CCP: Anti-cyclic citrullinated peptide

WBC: White blood cell

Cr: Creatinine

VAS score: Visual analogue score

DAS28: Disease Activity Score 28

OTUs: Operational Taxonomic Units

OPLS-DA: Orthogonal Projections to Latent Structures Discriminant Analysis

VIP: Variable Importance for the Projection

AUC: Areas under the curve

HGB: Hemoglobin

ROC: Receiver operating characteristic

SAR1B: Secretion associated Ras related GTPase 1B

ROS: Reactive oxygen species

Declarations

Author contributions

Conception and design: Rui Liu.

Methodology and interpretation: Xinxin Wang, Dawei Cui.

Data collection: Na Li, Ming Chen.

Statistical analysis: Ming Chen.

Writing – Original Draft: Haifeng Yun.

Writing – Review & Editing: Dawei Cui, Guoxing Zhang.

All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This project was approved by the Ethics Committee of the Suzhou TCM Hospital Affiliated to Nanjing University of Chinese Medicine (2019-010), and all study protocols complied with the Declaration of Helsinki. All involved participants provided written informed consent.

Consent for publication

Not applicable.

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Competing interests

All authors declared no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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Figures

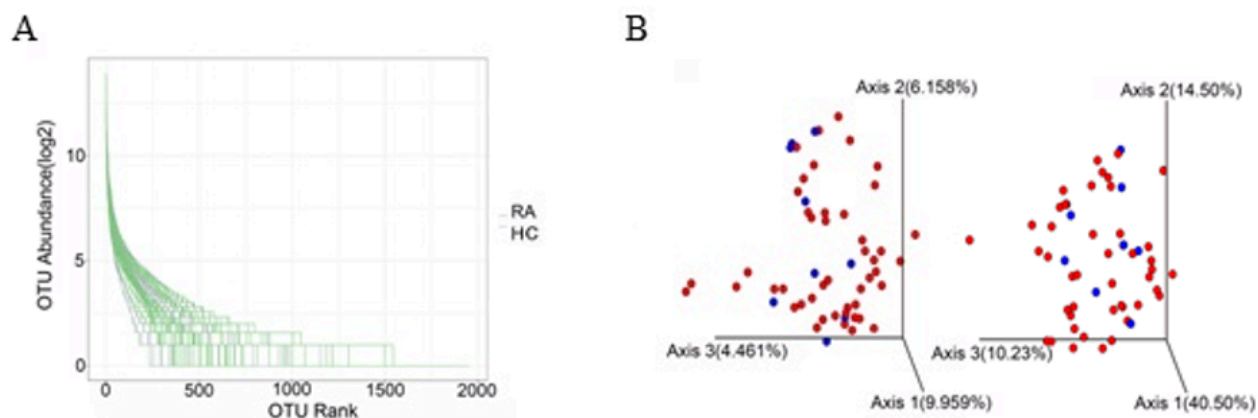


Figure 1

Comparison analysis of species diversity and relative abundance. (A) Curve of each sample with the sequencing depth. (B) PCoA Plot base of the relative abundance.

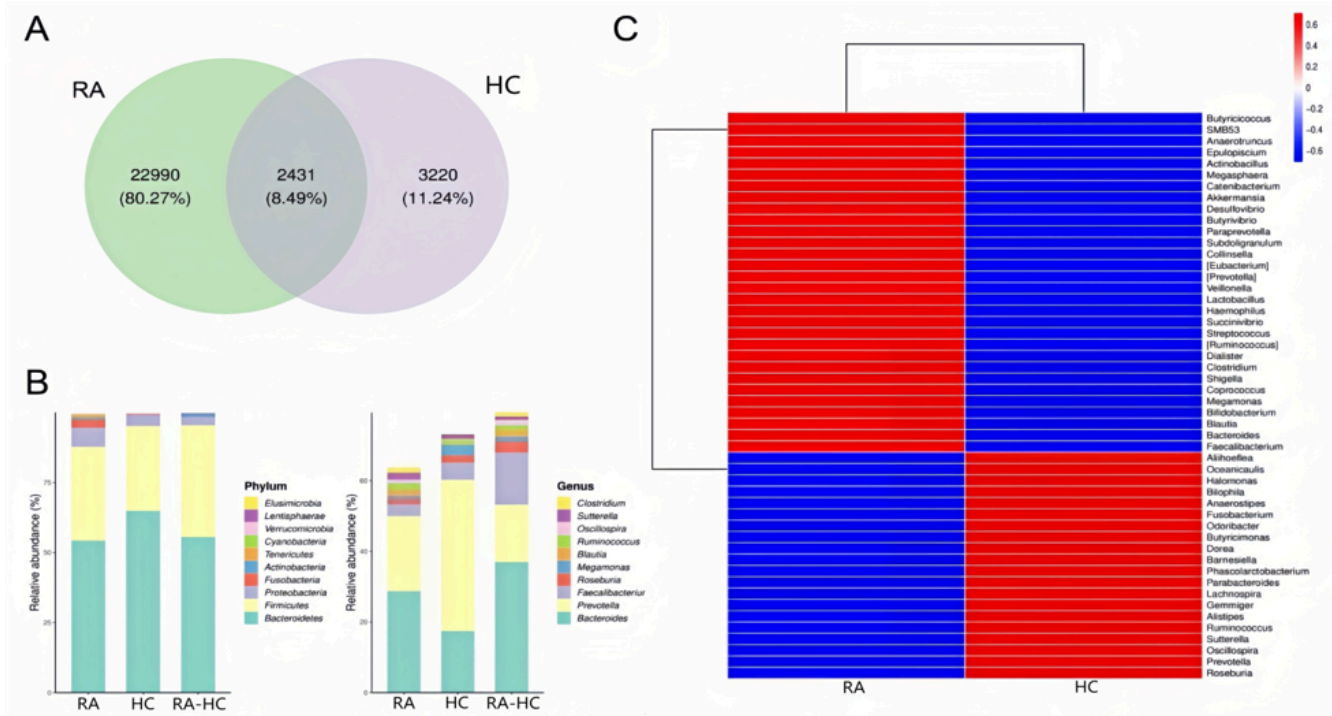


Figure 2

The composition, quantity and abundance of microorganisms. (A) Venn diagram of the sample group. (B) Bar chart of relative abundance. (C) Species richness heatmap. RA: Rheumatoid arthritis; HC: Healthy control.

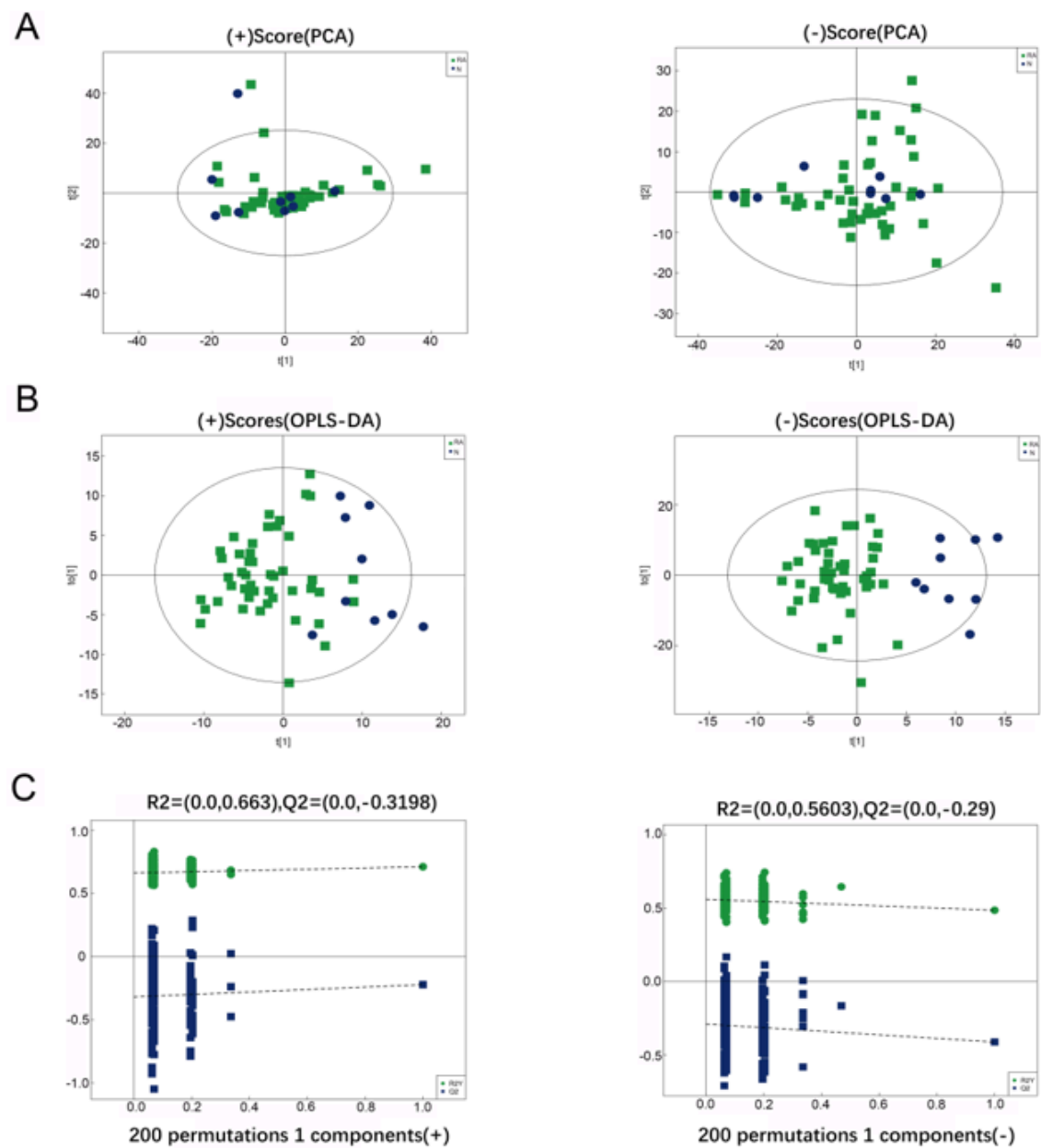


Figure 4

Multivariate statistical analysis. (A) Principal component analysis. (B) Orthogonal Projections to Latent Structures Discriminant Analysis. (C) Permutation test diagram of the OPLS-DA model.

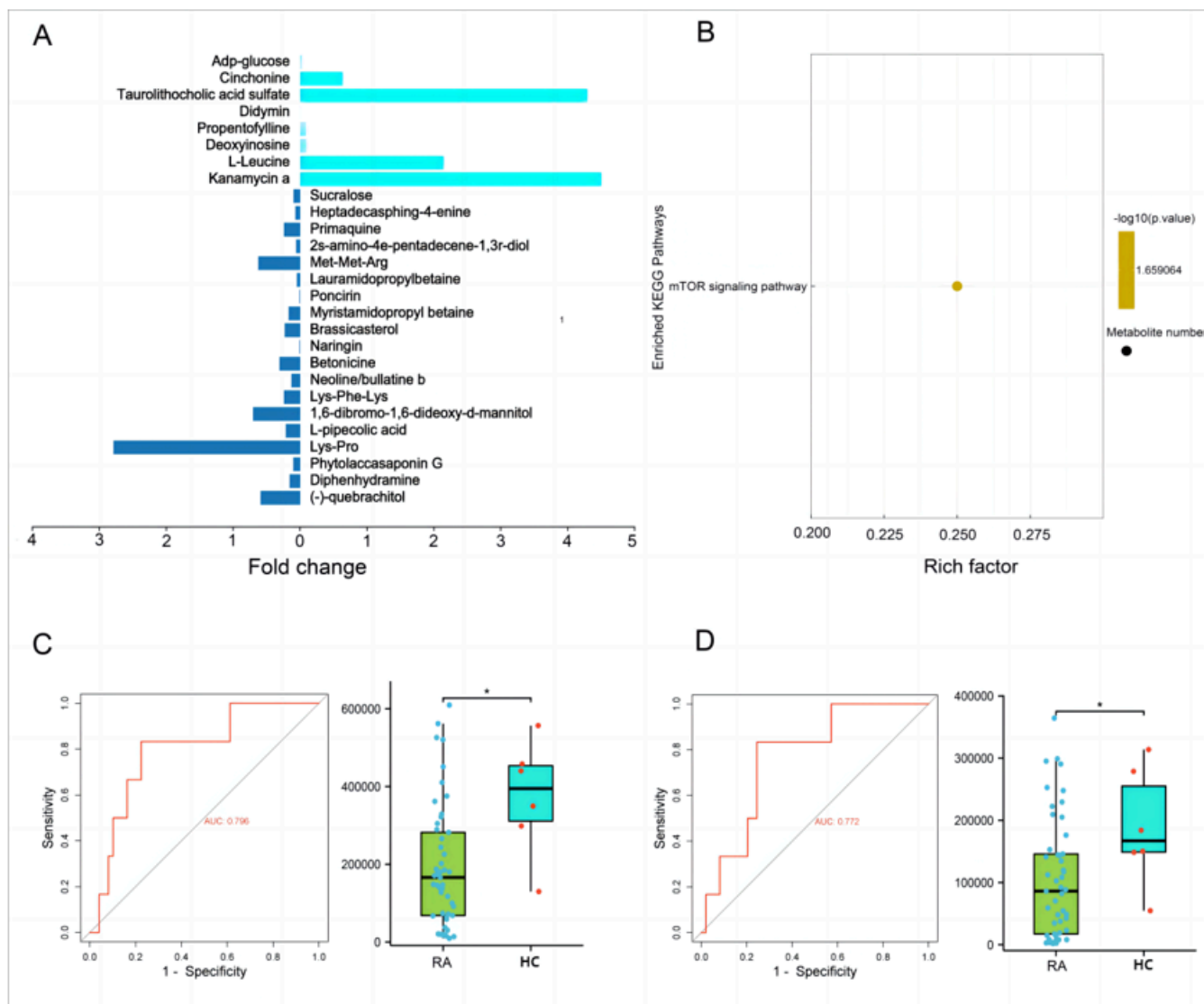


Figure 5

Differential metabolites signaling pathways and potential markers. (A) Metabolites with significant differences. (B) Differential metabolic pathways. (C) ROC curve analysis of L-Leucine. (D) ROC curve analysis of tauro lithocholic acid sulfate.

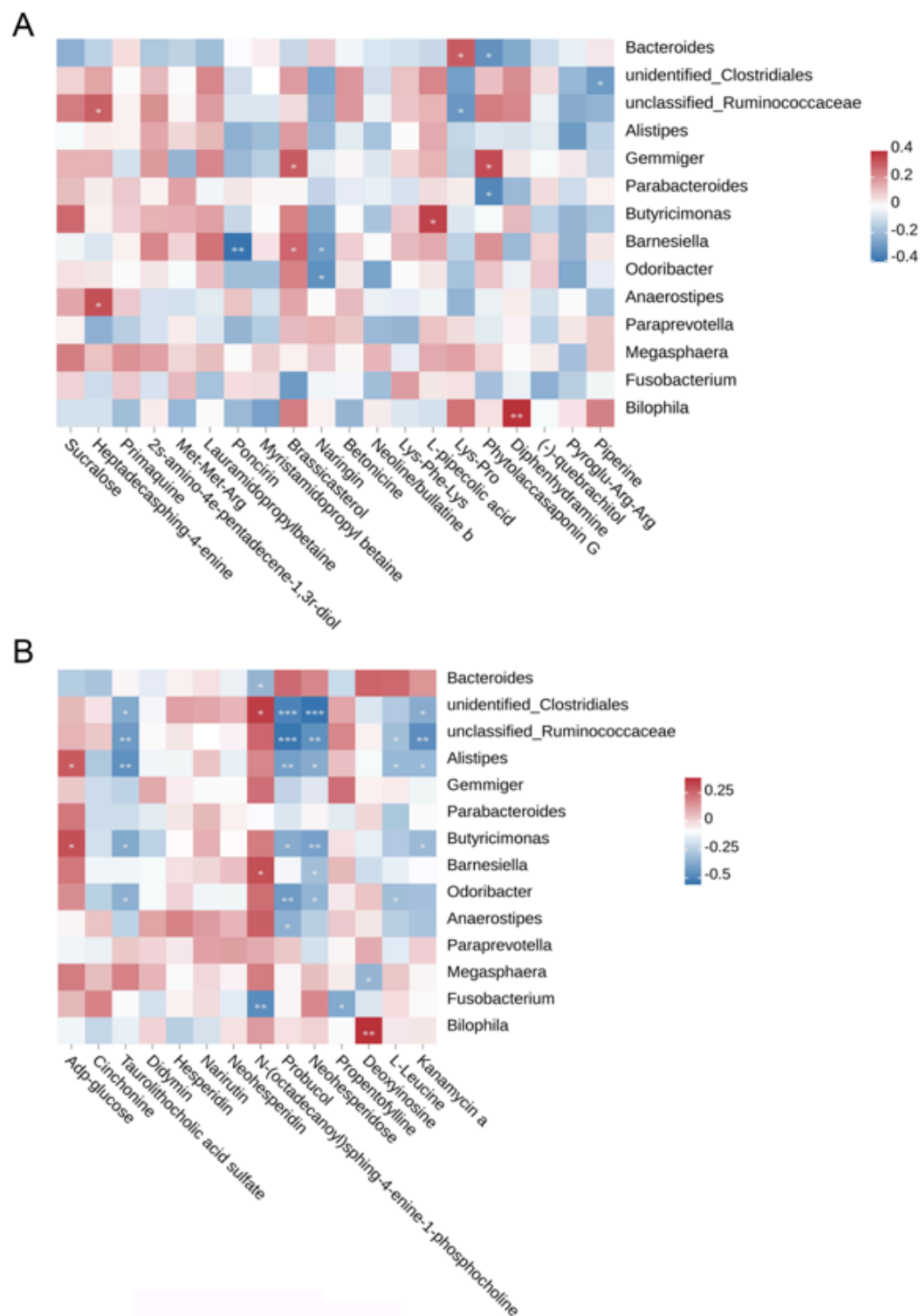


Figure 6

Correlation heatmaps of metabolites and microorganism.(A) Positive ion mode. (B) Negative ion mode. Red means positive correlation, and blue means negative correlation; * $P < 0.05$, ** $P < 0.01$.

