Journal of Immunology Research

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Lead Guest Editor: Mingyi Zhao
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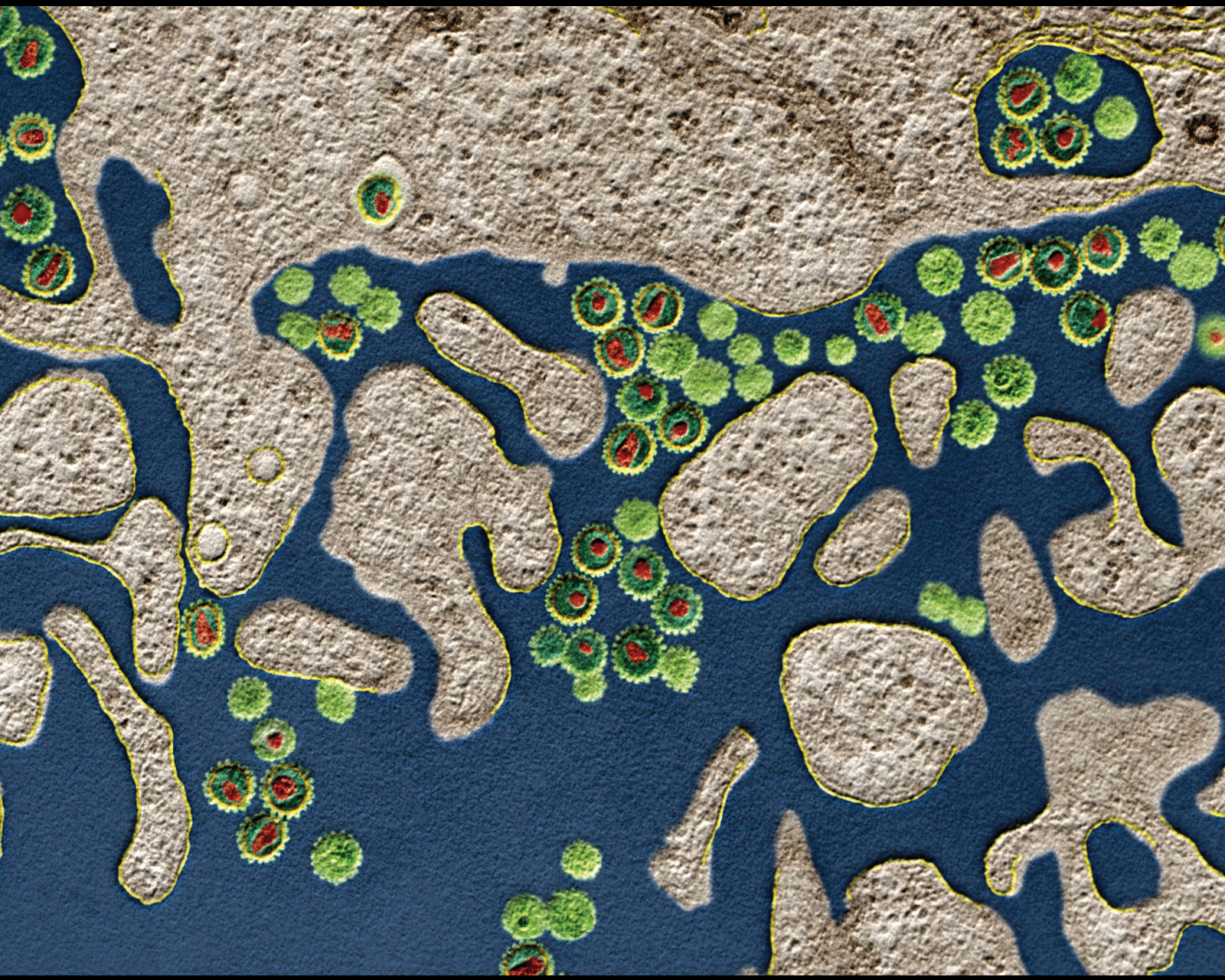

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Research Article

Imbalance of the Gut Microbiota May Be Associated with Missed Abortions: A Perspective Study from a General Hospital of Hunan Province

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Objective. To conduct a preliminary investigation that shows the possible correlation between the change of gut microbiota and missed abortions (MAs), which further provides a new potential insight for the prevention and therapy of MAs. *Method*. One hundred women, including 50 patients with MAs (case group) and 50 normal pregnant women (control group), were enrolled in the study. Fecal specimens were collected in the first trimester. Bacterial DNA was extracted, hybridized with primers of specific genes, and then detected by bacterial chip. The composition and the relative abundance of the gut microbiota were compared and analyzed. Furthermore, Kyoto Encyclopedia of Genes and Genomes enrichment analysis was used to explore the relative pathways. *Results*. (1) The α-diversity and β-diversity of the gut microbiota in patients with MAs were significantly lower than that those in normal pregnant women ($P < 0.05$). At the phylum level, *Firmicutes*, *Proteobacteria*, *Actinomycetes*, and *Bacteroidetes* accounted for the main proportion of intestinal flora in the 2 groups. Only *Actinobacteria* was high in the case group. Significant differences were found between the two groups at the phylum level ($P < 0.05$). *Prevotella*, *Lactobacillus*, and *Paracoccus* were significantly more abundant in the control group than in the case group at the genus level ($P < 0.05$). (2) KEGG pathway enrichment analysis found significant differences in 27 signaling pathways and metabolic pathways between the two groups of differentially expressed genes (all adjusted $P < 0.05$). (3) The positive rate of *M. hominins* (MH) detection in the control group was significantly higher in the MA group ($\chi^2 = 7.853$, $P = 0.004$). *Conclusion*. The high abundance of *Actinobacteria* in the MA group was the first time found and reported in the study. The dysbiosis of the gut microbiota correlates with MAs. This study provided insights into the potential change of gut microbiota of MAs and the potential underlying mechanisms through certain impaired lipid metabolism and aroused inflammation pathways. Comprehensive insights regarding gut microbiota may facilitate improved understanding and the development of novel therapeutic and preventive strategies for MAs.

1. Introduction

Miscarriage, common in pregnant women, can cause physical and psychological harm, such as excessive bleeding, infection, anxiety, depression, and posttraumatic stress disorder. Missed abortions (MAs) are a kind of miscarriage, which refers to the stagnation of embryonic or fetal development with no cardiac activity, still staying in the uterine

cavity for several days or weeks, and cannot be discharged naturally in time. It is defined as a pregnancy that fails to develop as a result of the cervix remaining closed [1]. MAs account for over 15% of spontaneous miscarriages and show an increasing trend [2]. Maternal factors, genetic and uterine abnormalities, endocrine and immunological dysfunctions, infections, and nutritional and environmental factors have been reported as risk factors for MAs [3–5]. However, approximately 30% causes of MAs remain unclear [6].

In recent years, an increasing number of studies have emerged indicating that the gut microbiota is involved in host endocrine metabolism, innate and acquired immunity maturation, epithelial cell injury modulation, and energy balance through various hormones or regulators [7–13]. The host provides nutrition and habitat for microbial communities and benefits from its symbionts that contribute to trophic functions, defensiveness, and metabolism [14]. In turn, the gut microbiota also plays a role in modulating the host response through metabolic activities [15, 16]. More and more evidence has revealed that the human gut microbiota is closely related to human health and diseases [17, 18], including obesity, type II diabetes, polycystic ovarian syndrome, and the processing of chronic infections [19–23]. This concept of gut microbiota disease provides a new exploration direction for the possible unknown risk and pathogenesis of MAs. To date, numerous investigations have reported that changes of the vaginal microbiota, such as bacterial imbalance, *Mycoplasma* infection, *Candida* infection, and viral infection, are associated with miscarriage in the first trimester [5, 24–27]. However, few studies have explored the potential relationship or deciphered the interplay between the gut microbiota and MAs.

This study hypothesized that the change of gut microbial communities might regulate the local gut microenvironment and the host metabolism, resulting in the possible outcome of MAs.

2. Materials and Methods

2.1. Study Participants. This study was approved by the ethics committee of the Third Xiangya Hospital of Central South University (IRB No. 2019-S460). From January to December 2019, fifty patients diagnosed as MAs in the first trimester (pregnancy within 12 weeks) without forwarding surgery were enrolled for the case group. For the control group, 50 pregnant women of similar age and a similar volume of menstrual flow to those of the case group before pregnancy were enrolled. They still had normal regular obstetric checkup results in the first trimester and had the normal pregnancy or birth outcome. All participants were enrolled and all informed consent was signed. According to the guidance or regulations of the Third Xiangya Hospital, the regular times and items of obstetric examination and treatment of pregnant women were implemented for all women in the 2 groups.

All participants satisfied the following inclusion criteria: (1) women aged 18–45 years who are healthy in the first-trimester pregnancy and had a history of normal menstrual cycles; (2) mentally competent participants able to understand the consent form and communicate with study staff; (3) no history of chronic diseases such as diabetes, enteritis, hepatitis, or tuberculosis; (4) no use of antibiotics in the lead up to the collection of specimens; and (5) no history of smoking, drinking, or toxic exposure. The exclusion criteria included (1) patients with cervical or intrauterine cancers or diseases, such as cervical cancer, genital tract malformations, submucosal fibroids, adenomyosis, and uterine adhesions; (2) patients with heart, liver, or kidney failure; (3) patients with inability to understand and cooperate with hospital staff due to mental impairment; (4) patients who withdrew from the study; and (5) patients with no signed informed consent or routine examinations uncompleted.

Fecal specimens from all participants were collected in the first trimester of pregnancy (before or in the 12^{th} week) and stored at -80°C for further investigations. The procedure of the study is showed in Figure 1.

2.2. Bacterial DNA Extraction and Labeling. Bacterial DNA was extracted from the fecal specimens using the Stool DNA Extraction Kit (Halgen, Ltd., Guangzhou, China) following the manufacturer's instruction. The primers F44 (RGTTYGATYMTGGCTCAG) and R1543 (GGNTACC TTKTTACGACTT) were used to amplify the V1–V9 regions of the 16S rRNA. Approximately 20–30 ng of the extracted DNA was used in a 50 μl polymerase chain reaction (PCR) according to the following conditions: an initial denaturing step at 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, and a final extension step of 72°C for 3 minutes. Agarose gel electrophoresis was performed to confirm the success of the PCR amplification. The DNA products amplified by PCR were directly labeled with a DNA labeling kit (Hagen Co., Ltd., Guangzhou, China) without purification. 5 μl PCR qualified DNA were loaded to the slide of Hagen array and mixed with hybridization buffer for hybridization according to the manufacturer's instruction.

2.3. Microarray Hybridization. Probes were selected from all the variable regions of the bacterial 16S rRNA. Each probe was designed to be approximately 40 bp in length. The hybridization mixture is contained 500 ng Cy5-labeled test sample DNA and 50 ng Cy3-labeled pool reference DNA for microorganism array (Halgen Ltd., Guangzhou, China). Add hybridization buffer to a final volume of 150 μl in the hybridization mixture, then heat to 100°C for 5 minutes, and cool on ice for 5 minutes. The mixtures were then placed in a slide of hybridization box and hybridized in an oven for 3.5 hours at 37°C. Slides were washed in 1x Saline Sodium Citrate (SSC), 0.25% Triton X-100, 0.25% sodium dodecyl sulfate (SDS), and 1x Dye Protector for 15 minutes at 63°C and then rinsed in 1x Dye Protector until water droplets cleared from the slides immediately following withdrawal from the solution. Slides were scanned promptly using a dual-channel scanner.

2.4. Data Analysis for Microorganisms. The relative abundance of each intestinal microbiota is proportional to the average value of its specific probe Cy5/Cy3 ratio. Therefore,

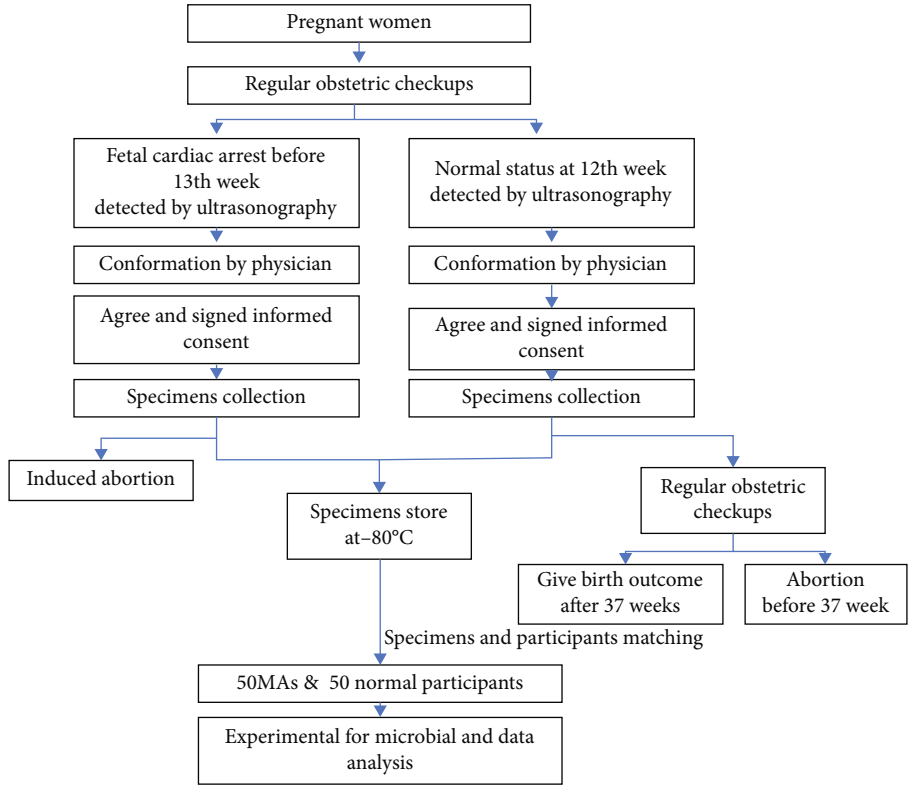

Figure 1: Procedures used in the study. Mas: missed abortions.

the type of intestinal microbiota could be determined by the ratio of each probe Cy5/Cy3. The sequencing program (Halgen, Ltd., Guangzhou, China) was used to detect the species and relative abundance of microorganisms in the fecal specimen. The alpha (α) and beta (β) diversity calculations and the QIIME (Quantitative Insights into Microbial Ecology) software default original parameters were used to analyze the general characteristics of the intestinal microbiota. The Wilcoxon rank-sum test was used to calculate the α-diversity within each group and the differences in β -diversity among groups. Principal coordinate analyses (PCoA) and nonmetric multidimensional scaling (NMDS) analyses were performed using the QIIME module and visualized in R (version 3.5.2). The α-diversity refers to the diversity of the microbial communities in the samples of the case and control groups. The species information index, Chao, abundance-based coverage estimator (ACE), Shannon, and Simpson indexes, were used for analysis. The Chao and ACE indexes, indicating the number of microbial communities in a single sample without involving the relative abundance of each bacterial community, represented the abundance of the communities in each sample. The Shannon and Simpson indexes were coaffected by sample richness and uniformity. The sample diversity was positively correlated with the Shannon index and negatively related to the Simpson index.

First, linear discriminant analysis (LDA) and the LEfSe (linear discriminant analysis effect size) software were used to screen the significant differential microorganisms between the case and control groups. Subsequently, enrichment analysis was performed to determine the Kyoto Encyclopedia of Genes and Genomes (KEGG, version 37) metabolic pathways related to the differentially expressed genes ($P < 0.05$), together with the metabolic pathways that were significantly altered under experimental conditions, including biochemical pathways and signal transduction pathways.

2.5. Statistical Analysis. Statistical analyses were performed using the SPSS 22.0 software. The nonparametric tests were performed using the Kruskal–Wallis test and the Wilcoxon test. The relative abundance between groups was compared, and differential species selection was performed with the LEfSe software using LDA. A corrected P value of <0.05 was considered as being statistically significant. To reduce the influence of overfitting, the leave-one-out test was used for cross-validation in the random clustering algorithm.

3. Results

3.1. Baseline Characteristics of the Study Population. The basic characteristics of the 100 participants (case and control groups) were analyzed and compared in this study. The average age of the case group and the control group was 31.7 ± 4.6 years and 30.9 ± 4.1 years, respectively. A similar volume of menstrual flow history was evaluated in 46 out of 50 (92%) women in the case group and 48 out of 50 (96%) women in the control group. Pregnant women with lower menstrual volume history were observed in 4 cases

(8%) of the case group and 2 cases (4%) of the control group. In the case group, 25 cases (50%) had a successful natural birth history, and 14 cases (28%) were cesarean section with live birth history. In the pregnancy history of the control group, 28 cases (56%) had a successful natural birth history and 15 cases (30%) were cesarean section with live birth history. There were no significant differences in any of the parameters of characteristics between the 2 groups ($P > 0.05$) except for the number of previous abortions and history of uterine cavity surgery (Table 1). These 2 factors are closely related to intrauterine adhesion (IUA), which commonly presents a reduction of menstrual flow. However, since there was no significant difference in the volume of menstrual flow between the two groups; all data were considered matched and comparable.

3.2. The Diversity of Gut Microbiota. Both the β-diversity and the α-diversity of the gut microbiota were investigated following successful validation of the sequencing quality (Figure 2). The results showed that the detection serial dilution curves of all subjects (in both case and control groups) gradually changed from a significant increase to a certain level and finally reached saturation.

3.2.1. β-Diversity of the Gut Microbiota. This result was calculated and recorded after 100 repeats of random sampling. The β-diversity demonstrated the significant differences between groups and can be compared using the algorithm of nonmetric multidimensional scaling (NMDS) and/or principal coordinate analysis (PCoA).

NMDS analyses showed the case and control groups could be clearly distinguished (pink and blue are grouped separately) in Figure 3. There was a significant deviation in the confidence ellipse between the 2 groups ($P = 0.003$), implying that the diversity of the case group and the control group was significantly separated ($P < 0.05$).

Combined with the QIIME software, the PCoA 2-dimensional and 3-dimensional maps of the β matrix were obtained (Figure 4). The results showed that the β-diversity of each sample in the 2 groups was similar, but the distance between the samples was relatively long. The confidence ellipse of the two groups has a significant deviation ($P = 0.001$), suggesting that the diversity of the case group and the control group was significantly separated ($P < 0.05$). The color area of the 2 sets of samples is small, indicating satisfactory repeatability for the sampling.

NMDS and PCoA results of the beta diversity analysis revealed that the gut microbiota in subjects with missed abortion clustered significantly compared to the controls.

3.2.2. The α-Diversity of the Gut Microbiota. From Sobs, Chao, ACE, and Shannon index, the comparison of α-diversity revealed that the diversity in the fecal specimen of the control group was significantly higher than that in the case group ($P < 0.05$), indicating a lower richness and evenness of gut microbiota in patients with missed abortion than in normal pregnant women (Figure 5 and Table 2).

Table 1: Characteristics of the case group and the control group.

General information	Case group	Control group	$t/z/\chi 2$	P value
Age (years)	31.7 ± 4.6	30.9 ± 4.1	-0.93	0.3568
Menstrual flow			0.7092	0.3997
No change	46 (92%)	48 (96%)		
Reduced	4 (8%)	2 (4%)		
Gravidity (times)			3.5088	0.061
0	8 (16%)	16 (32%)		
1	10 (20%)	19 (38%)		
≥2	32 (64%)	15 (30%)		
Parity (times)			0.3613	0.5478
0	25 (50%)	22 (44%)		
≥1	25 (50%)	28 (56%)		
Abortion (times)			18.5372	<0.0001
0	9 (18%)	30 (60%)		
1	24 (48%)	13 (26%)		
≥2	17 (34%)	7 (14%)		
Intrauterine operation			16.9779	<0.0001
No	9 (18%)	28 (56%)		
Yes	41 (82%)	22 (44%)		
Cesarean section			0.0486	0.8256
No	36 (72%)	35 (70%)		
Yes	14 (28%)	15 (30%)		

P value > 0.05 represents no significant difference; P value < 0.05 represents significant difference. Intrauterine operation: intrauterine surgeries such as abortion and hysteroscopic procedures. Cesarean section: a surgical procedure used to deliver a baby through incisions in the abdomen and uterus.

3.3. Composition of the Gut Microbiota. In this study, the gut microbiota was analyzed at 6 levels, including the phyla, class, order, family, genus, and species.

The highest abundance of gut microbiota for phylum categories in the 2 groups was *Firmicutes*, followed by *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*; all of them were not significantly different between the two groups except *Actinobacteria* (Figure 6). Meanwhile, a total of 8 phyla, only *Actinobacteria* was high in the case group, were found to have significant differences between the two groups at the phylum level ($P < 0.05$, Figure 7). The relative abundance of *Actinobacteria* and *Epsilonbacteraeota* was >1%, and the relative abundance of the other 6 phyla (*Planctomycetes*, *Tenericutes*, *Thermotogae*, *Spirochaetes*, *Verrucomicrobia*, and *Gemmatimonadetes*) was <1%. The abundance of *Fusobacteria*, *Elusimicrobia*, *Actinobacteria*, *Firmicutes*, and *Proteobacteria* was higher in the MA group ($P > 0.05$). *Firmicutes*/*Bacteroidetes* ratio has been suggested as an indicator of several pathological or obese conditions [28]. The ratio was 3.29 in the case group and 2.92 in the control group.

There were 168 genera of bacteria with significant differences ($P < 0.05$) between the 2 groups, including 11 genera with relative abundance > 1% (*Vibrio*, *Lachnoclostridium*, *Prey Prevotella*, *Roseburia*, *Bacteroides*, *Lachnospira*,

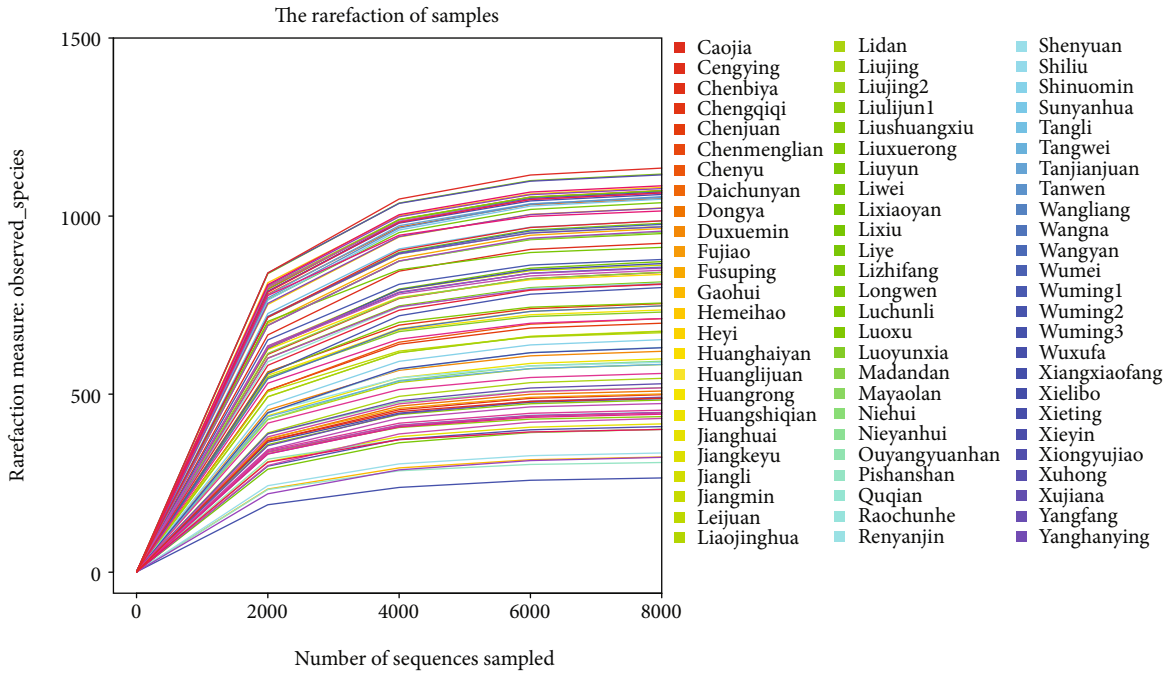

Figure 2: Dilution curve of each sample. The abscissa represents the number of sequences, and the ordinate represents the number of OTUs observed. According to the endpoint of the extension of the sample curve, the number of sequencing samples was found to correspond to the abscissa.

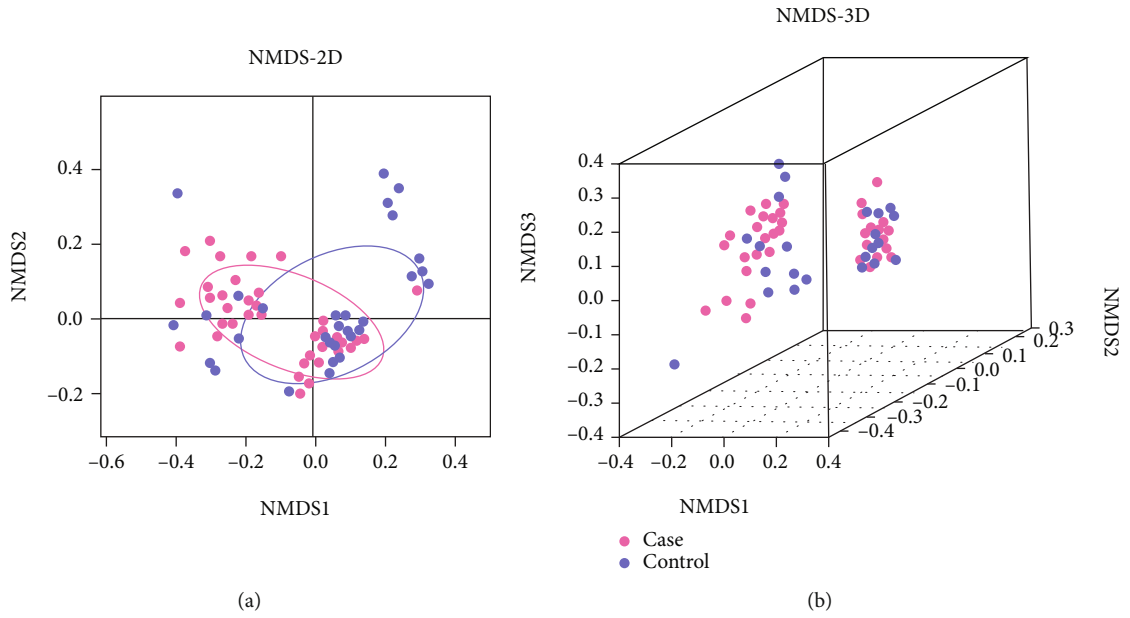

Figure 3: The analysis of NMDS by 2D and 3D models. (a) Two-dimensional map. (b) Three-dimensional map. The different β-diversity distance matrices were compared and analyzed from the original data. The 2-dimensional and 3-dimensional maps of the sample space location were obtained. It indicates an obvious difference between the case and control groups when the same color samples are grouped by circle. Each dot represented one sample by pink (case group) or blue (control group) in the map. NMDS: nonmetric multidimensional scaling.

Pseudomonas, *Lactobacillus*, *Leptotrichia*, *Parabacteroides*, and *Clostridium*). The relative abundances of *Prevotella* and Parabacteroides, belonging to *Bacteroidetes* phyla, were significantly higher in the control group than those in the MA group. However, the other bacterial genera, most of which belong to *Proteobacteria* and *Firmicutes* phyla, showed significantly higher relative abundances in the MA group ($P < 0.05$, Table 3).

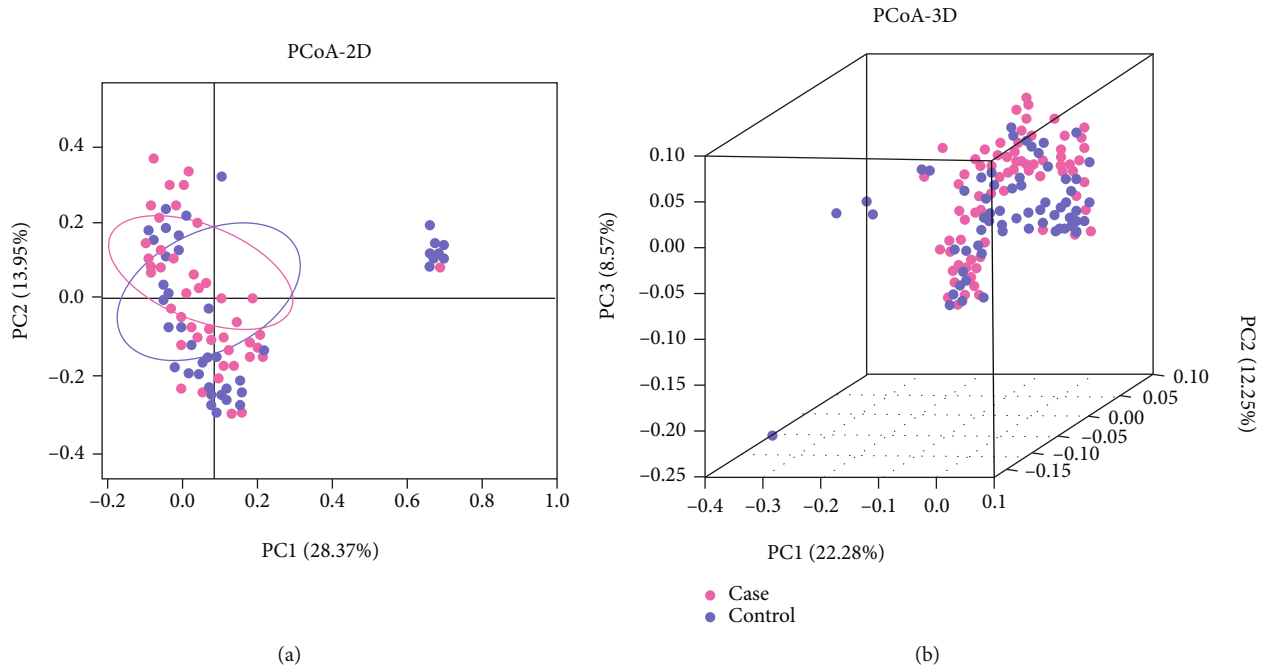

Figure 4: The analysis of PCoA by 2D and 3D models. (a) Two-dimensional map. (b) Three-dimensional map. PCoA map can be defined based on the distance matrix between samples to observe the differences of microbial populations between samples. If the abundance of the gut microbiota of the 2 samples is similar, the distance between the 2 points on the PCoA map will be closer. If the sample has good repeatability, the circle area is small; otherwise, the color area is large. Each dot represented one sample by pink (case group) or blue (control group) in the map. PCoA: principal coordinate analysis.

A detailed classification of the microbial flora was performed at the species level for samples in the 2 groups. More than 1000 species of bacteria were detected in the case group and the control group, among which 457 species showed significant differences ($P < 0.05$, Figure 8).

3.4. Potential Functional Pathways Were Predicted through the KEGG Database. The KEGG pathway enrichment analysis revealed that genes expressed by the differentially abundant microbial species were significantly enriched in 27 signaling pathways and metabolic pathways ($P < 0.05$). Most of the potential pathways for the control group were related to energy and nutrient metabolisms (glycan, vitamins, lipid, amino acids, and biosynthesis) and growth and development metabolism (translation, oxidation, catalysts, and nucleotide). Differentially expressed genes involved in pathways related to processing, lipid metabolism, and amino acid metabolism were more abundant in the control group than in the case group (Figure 9).

3.5. A Comparison of the Intestinal Mycoplasma. The positive detection rates of *Mycoplasma* and *M. hominis* (MH) were 96% (48 cases) and 38% (19 cases), respectively, in the case group and 98% (49 cases) and 66% (33 cases), respectively, in the control group. There was no statistical difference in the positive detection rate of *Mycoplasma* between the 2 groups ($\chi^2 = 0.344$, $P = 0.5$). However, the positive detection rate of MH was statistically higher in the control group than in the case group (66% vs. 38%; $\chi^2 = 7.853$, $P = 0.004$; Table 4). The average relative abundance of *Mycoplasma* and MH in the control group was significantly higher than that observed in the case group ($P < 0.05$, Table 5).

4. Discussion

Abortion is the common complicating disease during pregnancy, affecting about 15% of clinical pregnancies [29]. Recently, many clinical studies indicated that vaginal bacterial composition in the first-trimester miscarriage was associated with reduced abundance of *Lactobacillus* spp., which will lead to the activation of inflammatory pathway and premature delivery [24, 30, 31]. Liu et al. reported that the vaginal bacterial species richness and diversity of women with MAs were higher than those in normal pregnant women in Shanghai, China [32]. The causes of preterm birth and abortion may not be limited to the microbiota in the reproductive tract or local immune response, but rather integrated with maternal systemic immunity.

The gut microbiota is a key factor in the formation and regulation of immune response [33–35]. To further understand the possible relationship between host gut microbiota of women with missed abortions and normal pregnancy, we conducted a clinical study in Hunan, China, and put forward preliminary insights. In our study, the α-diversity of gut microbiota in the control group was significantly higher than that of the MAs ($P < 0.05$). The result was the same as that of the reduction of bacterial diversity in the fecal microbiota of miscarriage patients [36]. The decrease in α-diversity was still found strongly associated with the development of

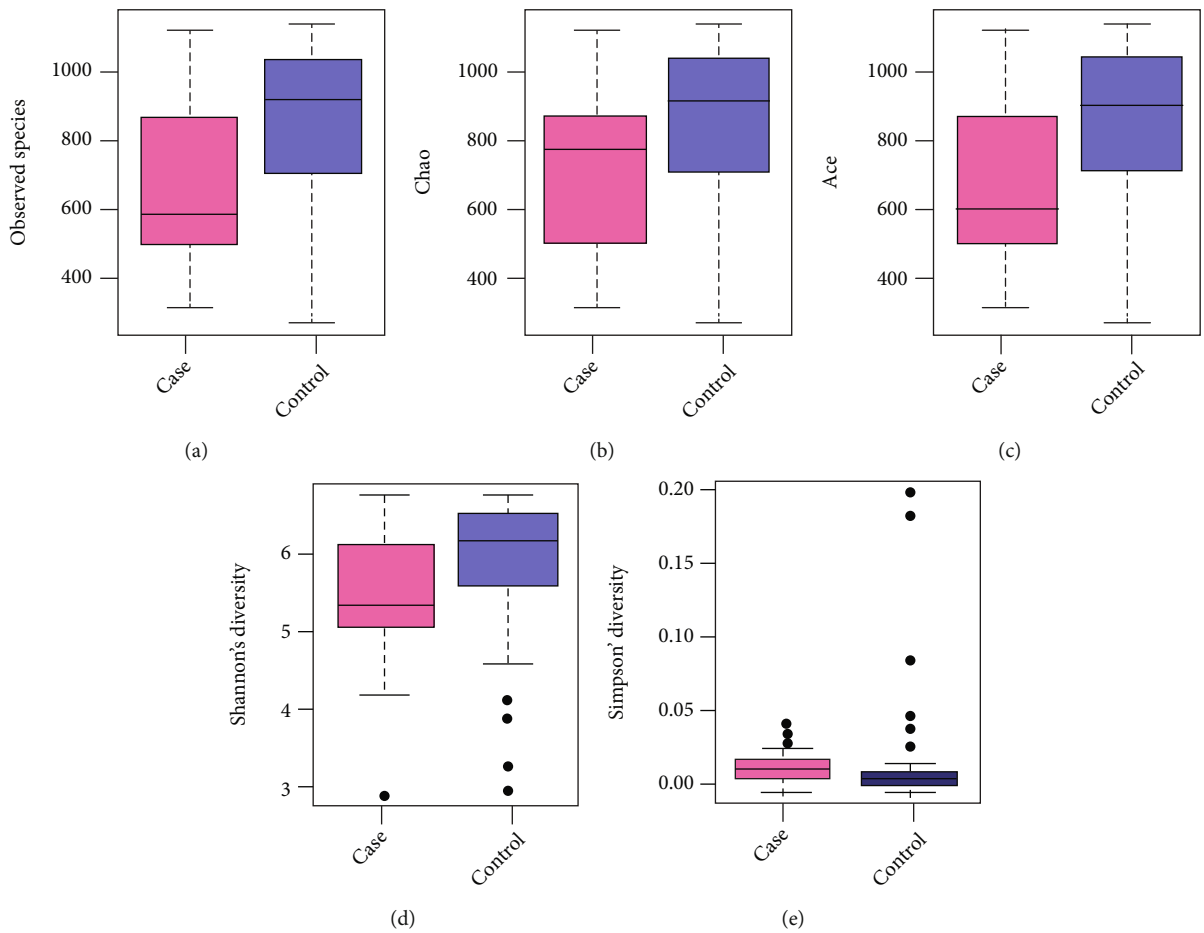

Figure 5: The α-diversity box plots of the 2 groups: (a) the Sobs; (b) Chao; (c) ACE; (d) Shannon; (e) Simpson is a type of α-diversity index. Each dot represented one sample by pink (case group) or blue (control group) in the map. Sobs: number of observed species; ACE: abundance-based coverage estimator.

Table 2: The statistics of the α-diversity analyses.

α-Diversity	Average (case)	SD value (case)	Average (control)	SD value (control)	P value
Sobs	663.48000	231.67115	837.96000	232.63302	0.00077
Chao	663.48000	231.67115	837.96000	232.63302	0.00077
ACE	663.48000	231.67115	837.96000	232.63302	0.00077
Shannon	5.49967	0.70135	5.89128	0.90201	0.00185
Simpson	0.01692	0.03893	0.01296	0.01042	0.00576

Sobs, Chao, ACE, Shannon, and Simpson are types of α-diversity indexes. P value > 0.05 represents no significant difference; P value < 0.05 represents significant difference. Sobs: number of observed species; ACE: abundance-based coverage estimator; SD: standard deviation.

spontaneous preterm birth [37]. In conclusion, the results of gut microbiota showed that the α- and β-diversities of women with missed abortions and miscarriage were lower than those of normal pregnant women. Besides, not only the NMDS but also the PCoA predicted the gut microbiota to be significantly different between the case group and the control group ($P < 0.05$), which suggested that the composition of the gut microbiota might have been disturbed, shifted, and rebalanced between the genesis or progression of MAs. This implication is highly consistent with our initial hypothesis, and it was further reinforced by the gut microbiota composition analyses, mainly at the phylum level.

Gut microbiota can digest dietary polysaccharides that cannot be digested by the host into monosaccharides and short-chain fatty acids for intestinal absorption and conversion into lipids in the liver. The greatest change in the gut microbiota occurs in the increase of some key bacteria, which is similar to the high level of *Firmicutes*, butyrate producers, in obese patients [38]. The increase of *Bacteroides* was significantly associated with weight loss but not with total calorie intake. In the first trimester, the gut microbiota pattern is similar to that of healthy nonpregnant women in many aspects, indicating that *Firmicutes* (mainly *Clostridiales*) is dominant over *Bacteroidetes* in our study [39]. In

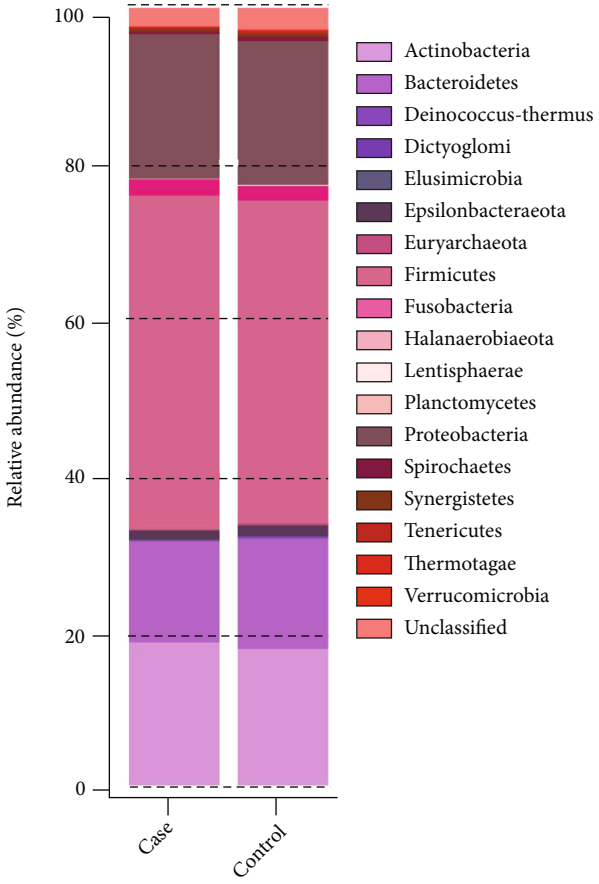

Figure 6: A column chart shows the relative abundances of species at the phylum level.

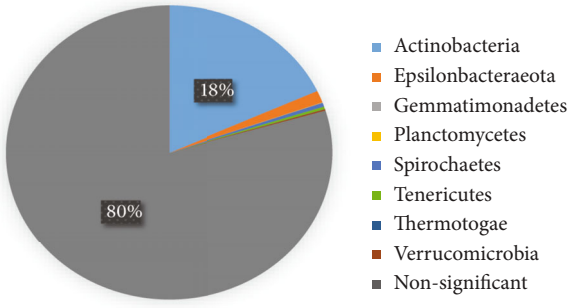

Figure 7: The proportion of 8 significant different phyla categories between the 2 groups. *Actinobacteria* (18.15%), *Epsilonbacteraeota* (1.32%), *Spirochaetes* (0.43%), *Tenericutes* (0.30%), *Gemmatimonadetes* (0.02%), *Planctomycetes* (0.04%), *Thermotogae* (0.01%), and *Verrucomicrobia* (0.14%).

our study, significant differences were found in 8 phyla between the 2 groups ($P < 0.05$). In the capacity for energy harvest, *Firmicutes* were significant enriched in the case group in our study. However, both *Firmicutes* and *Bacteroidetes* were enriched in the miscarriage group compared to the control group in Liu et al.'s study [36]. A previous study

Table 3: A comparison of the differential bacterial genera between the 2 groups.

Differential bacteria	Case	Control	*P* value
Vibrio	1.1886	0.9978	0.0187
Clostridium	1.3399	0.9580	0.0010
Prevotella	0.7007	1.4912	0.0112
Roseburia	1.5566	1.1781	0.0044
Bacteroides	4.4468	3.9660	0.0367
Laospirillum	1.2569	0.8197	0.0011
Pseudomonas	1.2058	1.0328	0.0463
Lachnospira	1.2746	2.0662	0.0046
Ciliates	1.5743	1.1802	0.0406
Parabacteroides	1.1873	1.4232	0.0399
Bacillus	1.1831	0.3344	0.0005

P value > 0.05 represents no significant difference; *P* value < 0.05 represents significant difference.

also reported that the increased ratio of the *Firmicutes* to *Bacteroidetes* is related to chronic inflammation [21]. The analysis of the *Firmicutes*/*Bacteroidetes* ratio indicated that the change of pathophysiology of obesity occurred in patients with missed abortions, which were similar to those in the miscarriage study. On the other hand, the *Firmicutes*/*Bacteroides* ratio (case : control) in our study (3.29 : 2.92) was higher than that in the miscarriage study (0.65 : 0.80) [36]. The results showed that the nutritional supply of pregnant women was significantly different among regions. Compared with the control group, the IL-2, IL-17A, IL-17, TNF-α, and IFN-γ levels of serum were increased significantly in the miscarriage group [36]. Although no serological test was performed in our study, it can be inferred from the analysis and comparison above that missed abortion will be highly affected by an immune response and inflammatory reactions.

To support the fetus's growth, the mother's communities of gut microbiota change toward energy production and storage from the first to third trimesters of pregnancy. At the phylum level, *Firmicutes* have been associated with increased energy storage, whereas *Proteobacteria* and *Actinobacteria* protect mother and fetus from external infections. *Actinomycetes* have unparalleled metabolic diversity, and their secondary metabolites can be used as clinical antibiotics, anticancer compounds, immunosuppressants, and so on [33]. During the chronic inflammation (aging process) of female mice, the abundance of *Actinobacteria* and *Firmicutes* increased, while the *Proteobacteria* decreased. In our study, the abundance of *Actinobacteria*, *Firmicutes,* and *Proteobacteria* increased in patients with missed abortions. Only *Actinobacteria* was found significantly higher in MAs than in control groups, but the finding cannot be observed in miscarriage and premature delivery groups [36, 37]. This is the first report of the high abundance of *Actinobacteria* found in the feces of pregnant women with missed abortions. The reasonable explanation is that mothers need more mechanisms to protect themselves from infection caused by

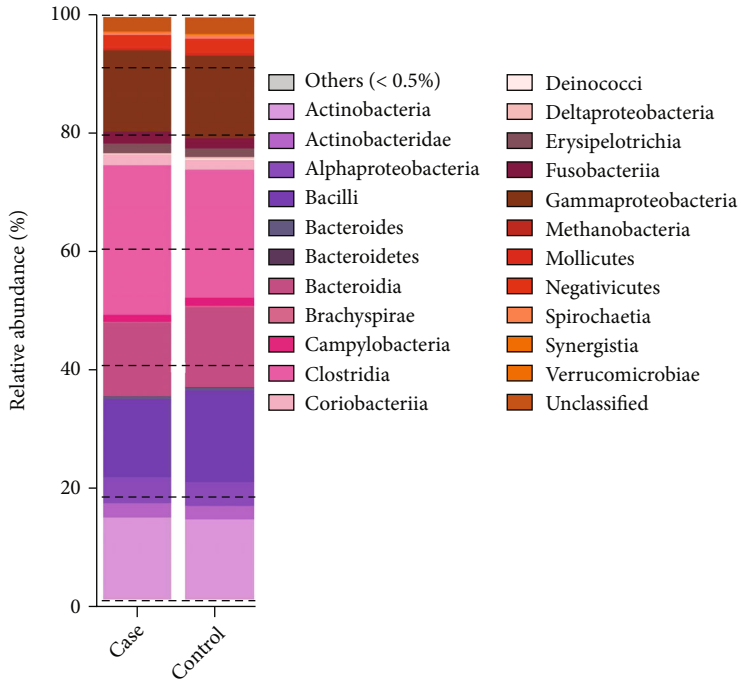

Figure 8: A column chart shows the relative abundances of species in the 2 groups at the genus level. The *P* values of the relative abundance of the intestinal microbiota of the 2 groups are the values after FDR correction.

embryonic stagnation or stillbirth. The increase of *Actinobacteria* in the gut of the mother illustrates one of the possible processes to protect the mother far from infection.

Proteobacteria may be the possible microbial signature of metabolic disorders and inflammatory bowel disease. The higher intake of monounsaturated fat, cholesterol, and fat-soluble vitamins was associated with increased *Proteobacteria*. Most clinical studies have focused on the increase of *Proteobacteria* in the gut microbiota of patients with inflammatory bowel diseases. Our study found that the relative abundance of *Proteobacteria* in the MA group was 3% higher than that in the control group. Liu et al. showed *that Proteobacteria* was enriched in the normal group, not the miscarriage group [36]. The difference in the 2 studies can be explained that the increase of *Proteobacteria* might be related to embryonic stagnation or different inflammatory genesis to protect the mother. This might be the first attempt to explore the relationship between gut *Proteobacteria* and the MAs. The abundance of *Proteobacteria* was related to the disease progression of the genital and respiratory systems, but it may also be related to the gut system for MAs. From the study, the abundance of *Proteobacteria* and *Actinobacteria* was found in the MA group, which can be inferred as a possible mechanism to protect mothers from internal infection caused by dead fetuses. In conclusion, this is the first time that the simultaneous increase of *Proteobacteria* and *Actinobacteria* has been found in pregnant women, which can speculate that the increase of specific intestinal flora may be related to the avoidance of infection, immunity, and inflammation.

Two significant abundances of bacterial genera, *Prevotella* and *Parabacteroides*, belonged to *Bacteroidetes* phyla. The other 9 significant abundances of bacterial genera in the case group were classified in *Firmicutes* (5 phyla), *Proteobacteria* (2 phyla), and *Bacteroidetes* (1 phylum). Therefore, there is more energy metabolizing relative gut microbiota found in the gut of normal pregnant women, while more energy accumulation and defense-related bacteria were found in women with missed abortions. The conclusion from gut microbiota is similar to the results of the KEGG pathway (Table 3). Gut microbial dysbiosis is a risk factor in the development of inflammation [40, 41]. The potential mechanism of our findings might be addressed as the disturbed gut microbiota accumulating secretions such as the hepatic fibroblast growth factor 21 (FGF21) through the gut-liver axis or bile metabolism leads to the impaired lipid metabolism [34, 42], triggers the intestine inflammation via the NF-KB pathway activation [35, 43], further damages the intestine barrier [43], destroys the energy balance, and finally alters the systematic homeostasis [44]. To the best of our knowledge, this is the first study to investigate the association of gut microbiota and lipid/inflammation metabolism in MA patients. This may contribute to the interplay between the host and gut microorganisms in terms of metabolism modulation.

The gut microbiota of the patients in this study was further investigated at the species level. Out of 1000 bacteria species examined, 457 were found to be significantly different between the two groups ($P < 0.05$). However, the relative abundance of bacterial species was less than 0.1% of the total species in the gut. We infer that changes in species of gut microbiota do not affect outcomes and prognosis in our study.

The relative abundance of vaginal *Lactobacillus* was significantly increased in the control group compared to the

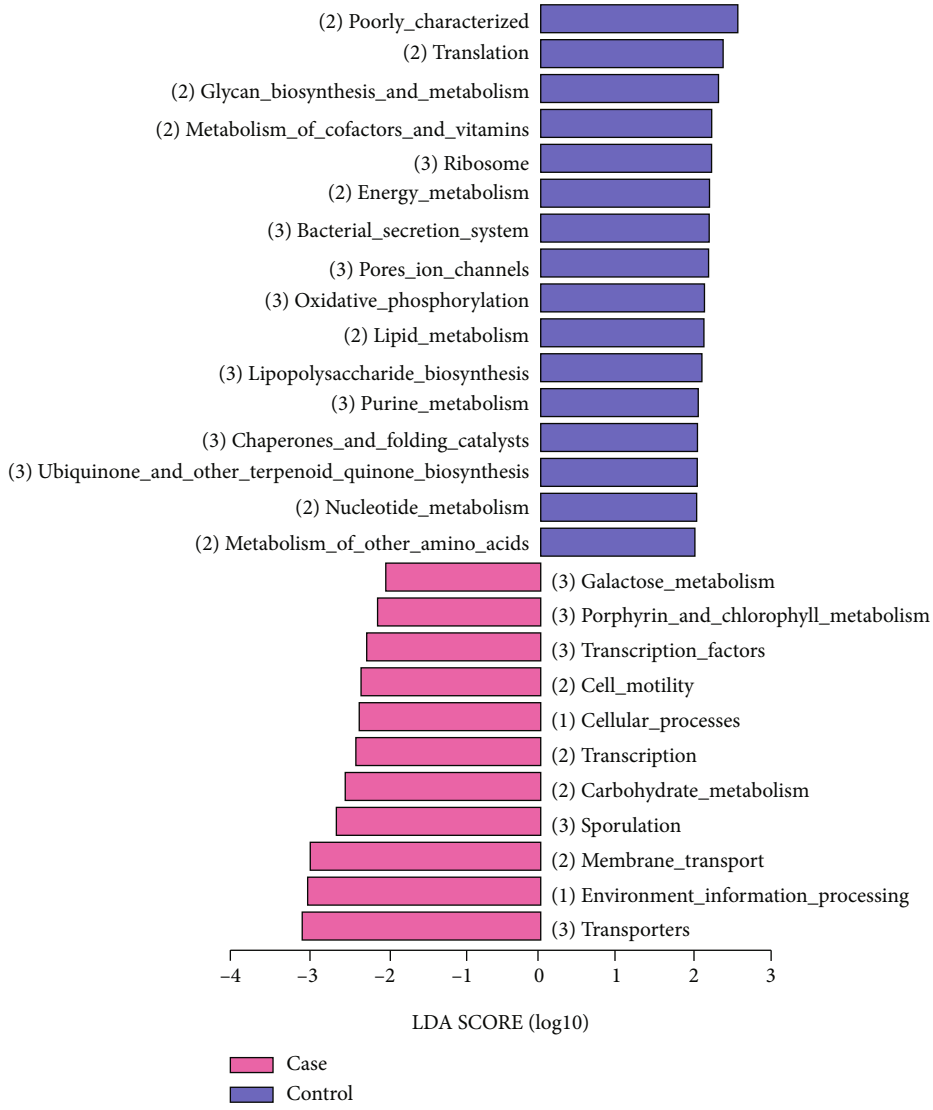

Figure 9: Screening of the differential functions using the KEGG pathway. The abundant signal pathways in the case group are represented by pink bars, and the abundant signal pathways in the control group are represented by blue bars.

Table 4: A comparison of the positive detection rate of *Mycoplasma* between the 2 groups.

Mycoplasma	Case group [*n* (%)]	Control group [*n* (%)]	χ^2	*P* value
Mycoplasma	48 (96%)	49 (98%)	0.3440	0.5000
Mycoplasma hominis	19 (38%)	33 (66%)	7.8530	0.0040

P value > 0.05 represents no significant difference; *P* value < 0.05 represents significant difference.

Table 5: A comparison of the average relative abundance of *Mycoplasma* between the 2 groups.

Mycoplasma	Case (AVG ± SD)	Control (AVG ± SD)	*P* value
Mycoplasma	0.2306 ± 0.1714	0.3009 ± 0.1592	0.0360
Mycoplasma hominis	0.0202 ± 0.2873	0.0392 ± 0.3628	0.0040

Note: *P* value > 0.05 represents no significant difference; *P* value < 0.05 represents significant difference. AVG: average; SD: standard deviation.

risk of early pregnancy miscarriage, which was supported by the 2007 study by Nelson et al. [32, 45]. It was also reported that the decrease in the abundance of vaginal *Lactobacillus* spp. drives the activation of inflammatory pathways, thereby reducing endometrial receptivity and implantation. Lower *Lactobacillus spp.* might increase the richness and diversity of potential pathogens, such as *Prevotella* and *Mycoplasmas* in the vaginal bacterial ecosystem [46]. However, our data of gut microbiota suggested that the relative abundance of *Prevotella*, *Mycoplasma*, and MH was significantly higher in the control group compared to the MA group. This may be the differences in disease correlation caused by the location of microbiota communities or differences in the metabolic mechanisms of different anatomical systems [47].

There were certain limitations in this investigation exploring the potential correlation between the gut microbiota and

the MAs. First, the relatively small sample size in this study may not represent the general population. Second, the gut microbial composition depends on perturbations, such as dietary habits, antibiotics, age, sanitation, hygiene, geography, climate, environment exposure, and health conditions [48–55]. We did not obtain a complete questionnaire on participants' dietary habits, so we were unable to determine whether the diet was a factor in the gut bacterial imbalance. In addition, we cannot rule out other etiologies of miscarriage patients, such as genetic variation. Then, the related serum biomarkers of the MAs were not collected, tested, and compared. Therefore, we found the possible metabolic pathways but did not further evidence for the relevant metabolites of potential pathways. Therefore, a more extensive and multicentered study with detailed questionnaires, metabolites, and genetic variation would benefit future investigations. However, this is the first clinical study to provide significant findings and new insights into the imbalance of gut microbiota associated with missed abortion.

In conclusion, we demonstrated an association between gut bacterial dysbiosis and MAs. An enormous diversity of gut flora has a symbiotic relationship with the health of an individual. It is the first report that the protection bacteria, *Proteobacteria* and *Actinobacteria*, have significant abundance in MAs but not in patients with miscarriage or premature delivery. It could be the internal mechanism to protect the mother far from infections. This study provided insights into the potential change of gut microbiota of MAs and the potential underlying mechanisms through certain impaired lipid metabolism and aroused inflammation pathways. Understanding the interaction between gut microbiota and MAs will contribute to the conception and realization of novel diagnostic, therapeutic, and preventive strategies for MAs. Moreover, the structural manipulation of gut microbiota communities may be a promising target for regulating metabolic balance in patients with missed abortions.

Data Availability

The patients' data used to support the findings of this study are documented in our department.

Ethical Approval

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

D Xu and Y Liou were responsible for conception and design; D Xu was responsible for administrative support; B Gao, X Zhao, and X Liu were responsible for provision of study materials or patients; B Gao, X Zhao, and X Liu were responsible for collection and assembly of data; B Gao, A Zhang, H Huang, and Y Liou were responsible for data analysis and interpretation. All authors were responsible for preparation of the manuscript. All authors were responsible for the final approval of the manuscript. Bingsi Gao and Xingping Zhao are co-first authors.

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Research Article

Elderly Patients with Mild Cognitive Impairment Exhibit Altered Gut Microbiota Profiles

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Background. As a transitional state between normal aging and Alzheimer's disease (AD), mild cognitive impairment (MCI) is characterized by a worse cognitive decline than that of natural aging. The association between AD and gut microbiota has been reported in a number of studies; however, microbial research regarding MCI remains limited. *Methods*. This study examined 48 participants, of whom 22 were MCI cases and 26 were normal control cases. Fecal samples were collected for 16S ribosomal RNA (rRNA) quantitative arrays and bioinformatics analysis. *Results*. A principal coordinates analysis (PCoA) and nonmetric multidimensional scaling (NMDS) both demonstrated that the microbial composition of participants with MCI deviated from that of healthy control participants. Multiple bacterial species were significantly increased (e.g., *Staphylococcus intermedius*) or decreased (e.g., *Bacteroides salyersiae*) in samples from the MCI group. *Conclusion*. The composition of gut microbiota differed between normal control and MCI cases. This is the first study to identify a signature series of species in the gut microbiota of individuals with MCI. The results provide a new direction for the future development of an early diagnosis and probiotic regimen.

1. Background

Mild cognitive impairment (MCI) is regarded as the transitional state between normal aging and Alzheimer's disease (AD) [1]. MCI is a complicated syndrome that is characterized by a cognitive decline greater than that of natural aging, but which does not dramatically interfere with daily life [2]. Epidemiological studies have suggested that the prevalence of MCI is nearly 20% in those older than 65 years old [3]. Despite the seemingly normal status of some MCI patients, several clinical studies have found that most MCI patients will eventually develop AD [4].

Emerging evidence suggests that the disruption of the gut microbiome could undermine mental health. Notably, Zhuang et al.'s clinical research showed that the series of bacteria taxa (e.g., *Bacteroides*, *Ruminococcus*, and Actinobacteria) in AD patients differed from that of control subjects [5]. Vogt et al. identified significant differences in the abundance of Firmicutes (phylum), Bacteroidetes (phylum), and *Bifidobacterium* (genus) in the microbiota of AD cases. Further, correlations have been found between the abundance of certain bacterial genera and biomarkers of AD in cerebrospinal fluid [6]. In AD animal models, the gut microbiome has also been found to be correlated with impaired spatial learning and memory [7]. Li et al. documented similar changes in the gut microbiome among MCI and AD cases [8]. However, there is still very limited evidence concerning the specific abnormalities of gut microbiota in MCI cases compared to those in normal control cases.

In the present study, potential alterations in the gut microbiota of cognitive impairment patients were investigated with 16S ribosomal RNA (rRNA) quantitative microarray, a novel

high-throughput biotechnology that quantifies various bacteria taxa without conventional culture-based procedures [9, 10]. We also examined whether the composition of microbiota was correlated to certain mental status parameters of cognitive impairment.

2. Methods

2.1. Study Design and Sample Collection. MCI ($n = 22$) and control ($n = 26$) participants were recruited from The Third Xiangya Hospital of Central South University. MCI was diagnosed by the Geriatric Department of The Third Xiangya Hospital of Central South University, China. This study was approved by the Ethics Committee of The Third Xiangya Hospital of Central South University and performed in accordance with the relevant guidelines and regulations. Written informed consent was obtained before the study from the participants or guardians on behalf of those participants with impaired cognition. The exclusion criteria for the study were as follows: use of antibiotics within the last 6 months, large doses of probiotics consumed in the last 3 months, current gastrointestinal disorders (e.g., chronic diarrhea, inflammatory bowel disease, or infectious gastroenteritis), and/or major gastrointestinal surgery in the past 3 years. Fresh stool samples were collected and saved in sampling tubes with preservative solution. The tubes and preservative solution were provided by Halgen Ltd. (Zhongshan, China).

2.2. DNA Extraction and Labeling. Bacterial DNA was extracted from the stool samples using the Stool DNA Extraction Kit (Halgen Ltd.) in accordance with the procedures described in the product instruction manual. Following previously published protocols [11], a universal primer pair was used to amplify the DNA in the V1-V9 regions of the 16S rRNA gene. Agarose gel electrophoresis was run to check the success of the PCR amplification.

2.3. Microarray Hybridization. Here, again, previous protocols [11] were followed to carry out the hybridization between samples and probes on the microarray (3.5 h at 37°C). Immediately after hybridization, the microarray was washed in 2x SSC, 0.25% Triton X-100, 0.25% sodium dodecyl sulfate (SDS), and 1x Dye Protector (Halgen Ltd.) for 15 mins at 63°C and then rinsed in 1x Dye Protector. Finally, a dual-channel scanner was used to quantify the intensity of hybridization.

2.4. Data Analysis. Alpha diversity was calculated using QIIME software [12] (and its default parameters). The differences of the alpha diversities between the groups were calculated using a Wilcoxon rank-sum test. A principal coordinates analysis (PCoA) and nonmetric multidimensional scaling (NMDS) were performed by QIIME modules and visualized by R package (version 3.5.2). To detect any statistical differences in the beta diversity metrics between the groups, a permutational multivariate analysis of variance (PERMANOVA) was used in the vegan package in R. A linear discriminant effect size (LEfSe) [13] analysis was performed to analyze any differences in the bacterial species between the groups. The P value for each species was calculated using a Kruskal-Wallis test and Wilcoxon test. Unsupervised random forest clustering and receiver operating characteristic curve (ROC curve) proportional hazards statistics were also determined using R. Cross-validations were performed by a leave-one-out test in random forest clustering to reduce the effect of overfitting.

3. Results

3.1. Demographic Data of Study Participants. A total of 48 participants (comprising 22 MCI cases and 26 control cases) were recruited from The Third Xiangya Hospital of Central South University. The gut microbiota of fecal samples collected by clinicians were analyzed (see Methods). As Table 1 shows, the MCI and control groups did not differ with respect to the female-to-male ratio, body mass index (BMI), education, major preexisting conditions, or physiological variables, and only a minor difference in average age was observed ($P = 0.046$). However, there were significant differences ($P < 0.01$) between the two groups in terms of mental state and cognitive function as measured by Mini-Mental State Examination scores and the Index for Activities of Daily Living.

3.2. MCI Cases Harbored an Altered Gut Microbiota. Compositional analysis revealed the presence of 19 phyla across all samples; however, only 7 of the 19 phyla were above the mean value of 1% of the total abundance (see Figure 1(a)). The relative abundance of *Bacteroidetes* was found to be lower in MCI cases than in control cases. Conversely, *Fusobacteria* were significantly more abundant in MCI cases than in control cases.

The analysis of alpha diversity (see Figure 2) included the calculation of Chao, ACE, Shannon, and Simpson indices; however, no significant difference between the MCI and control groups was detected ($P > 0.05$). The analysis of beta diversity, including the PCoA and NMDS, demonstrated that the gut microbiota profiles of the MCI cases clustered apart from those of control subjects (see Figure 3; PERMANOVA $P = 0.048$). Such separation indicated that MCI-related changes may occur in certain bacterial taxa.

3.3. Association between Bacterial Abundance and Cognitive Status. Given the MCI-related alterations in gut microbiota, an in-depth analysis was undertaken using the LEfSe analysis (see Methods). A series of bacterial taxa were identified as displaying a differential abundance between the MCI cases and normal controls (see Figure 4(a) and Table S1). The 16S rRNA microarray revealed the significant enrichment of 9 species (e.g., *Staphylococcus intermedius*, Figure 4(b)) and the attenuation of 25 species (e.g., *Bacteroides salyersiae*; see Figure 4(c)) among the MCI cases, particularly at the species level.

4. Discussion

MCI has important implications for the health of the elderly, as individuals with a history of MCI are more likely to develop AD in the long term [14, 15]. A number of studies

Table 1: Baseline characteristics of study subjects.

	MCI case ($n = 22$)	Control ($n = 26$)	P value
Age (yrs, mean ± SD)	71.45 ± 8.03	67.31 ± 5.27	0.046
Sex (female/male)	14/8	19/7	0.543
BMI (kg/m^2, mean ± SD)	23.78 ± 3.98	22.05 ± 5.10	0.194
Education			0.87
Illiteracy	5	7	
Elementary school	11	11	
High school	6	8	
MMSE score (mean ± SD)	15.55 ± 4.50	23.96 ± 2.84	7.81×10^{-9}
Hamilton Depression Rating Scale (HAM-D, mean ± SD)	5.09 ± 4.85	4.08 ± 3.63	0.424
Index for Activities of Daily Living (ADL, mean ± SD)	25.95 ± 8.14	21.04 ± 1.28	0.01
Major preexisting conditions			
Cerebrovascular diseases	9	9	0.654
Cardiopathy	5	4	0.781
Hypertension	2	5	0.561
Diabetes	3	1	0.485
Respiratory tract diseases	6	5	0.509
Genital diseases	7	10	0.632
Physiological data			
Blood glucose (mmol/L)	5.50	4.82	0.078
TAG (mmol/L)	1.93	1.59	0.362
TC (mmol/L)	4.63	4.59	0.903
HDL-cholesterol (mmol/L)	1.21	1.08	0.379
LDL-cholesterol (mmol/L)	2.25	2.34	0.693

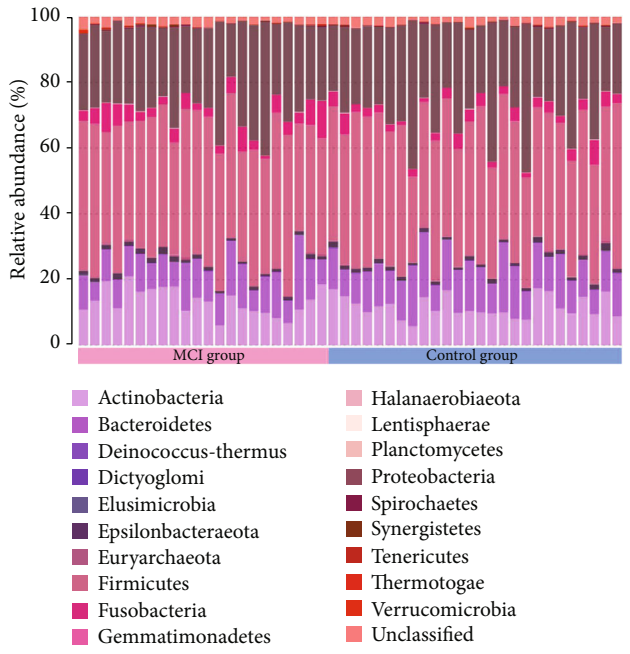


Figure 1: Gut microbiota composition. Proportion of the different phyla (represented by different colors) detected in the two groups.

have provided compelling evidence that dysbiosis plays an important role in the pathogenesis of AD [16] and MCI [17]. However, to date, there remains a lack of extensive research on the correlation between gut microbiome and MCI. In the present study, we examined gut dysbiosis in MCI cases. We found that the relationship between bacterial taxonomic profiles and MCI was not characterized by altered alpha diversity. However, a beta diversity analysis visualized a distinction between MCI and control groups that suggested an abnormal depletion of certain bacterial taxa in the MCI cases. Notably, the reduction of *Bacteroides salyersiae* and *Bacteroides gallinarum* in MCI cases was in line with previous research on AD. Zhuang et al. reported the depletion of the *Bacteroides* in AD cases but did not specify the depleted species by conventional 16S rRNA sequencing [5]. *Bacteroides fragilis*, another species of *Bacteroides*, has also been reported to be decreased in patients with cognitive impairment and brain amyloidosis [18]. Our findings further corroborate the relevance of the *Bacteroides* genus in the gut microbiota in relation to neurodegenerative diseases and identified two more species that can be used as potential biomarkers in the early detection of MCI or AD [19].

Conversely, the enrichment of certain taxa in MCI cases was also found to be related to neurodegeneration. For example, *Staphylococcus intermedius* and *Staphylococcus lentus* of the *Staphylococcus* genus were significantly more abundant in the MCI group than in the control group. A

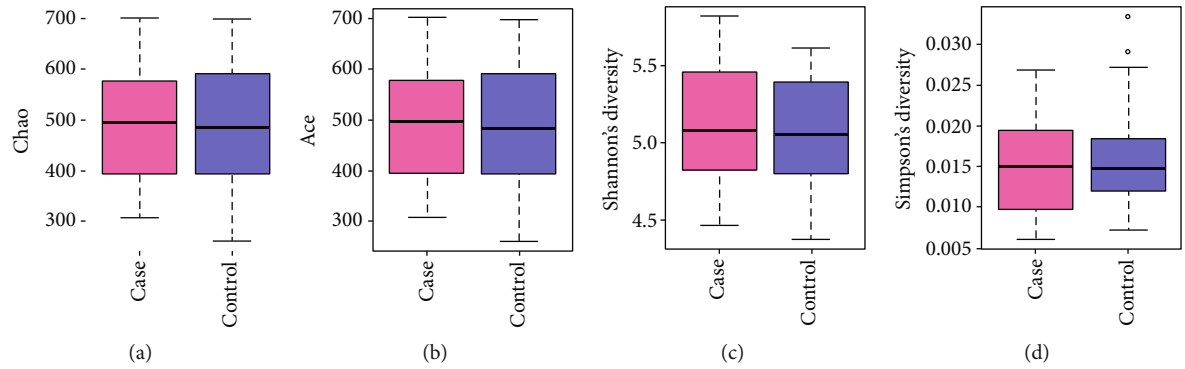


Figure 2: Alpha diversity in the MCI and control groups as represented by Chao index (a), ACE index (b), Shannon diversity (c), and Simpson diversity (d).

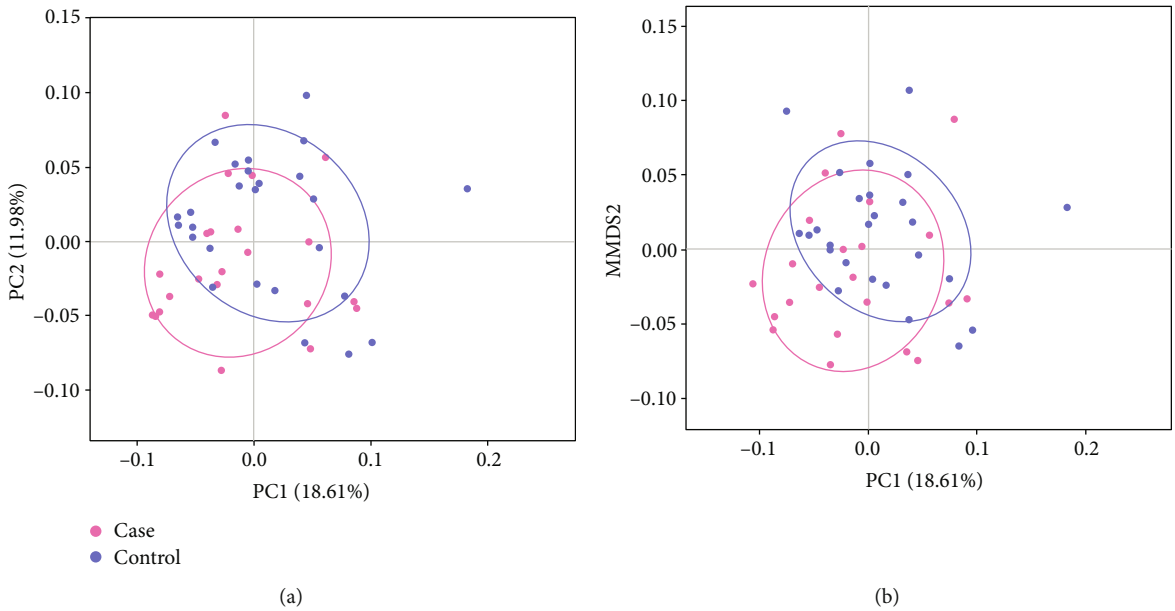
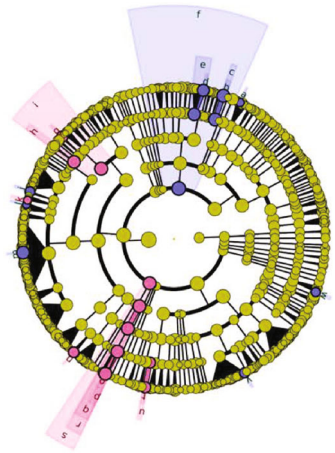


Figure 3: Analysis of beta diversity based on PCoA (a) and NMDS (b) with weighted UniFrac distance. MCI cases and control subjects are denoted with pink and blue nodes, respectively.

number of studies have suggested that *Staphylococcus* is involved in the generation of extracellular amyloid fibers [20] through multiple mechanisms, including the regulation of phenol soluble modulins (PSMs). The PSMs produced by *Staphylococcus* have been documented to form amyloid fibers in biofilms [21]. To date, most published research has noted the relevance of *Staphylococcus aureus*; however, our results identified two other species of the same genus, thus expanding the scope for investigating the role played by *Staphylococcus* in neurodegeneration.

Our study is notable, as it has certain technical advantages. First, unlike other studies on AD, the present research initiatively investigated MCI as a separate phenotype, thus providing unique insights into the progression of MCI to AD. Second, unlike 16S rRNA sequencing that only provides genus-level data [22], the 16S quantitative microarray technology enabled us to scrutinize MCI-related alterations in the gut microbiome at the species level. In addition to shedding light on the role played by the brain-gut axis in the process of neurodegeneration, our findings might also promote the development of more precise diagnostic methods for MCI that are based on gut microbiota signatures.

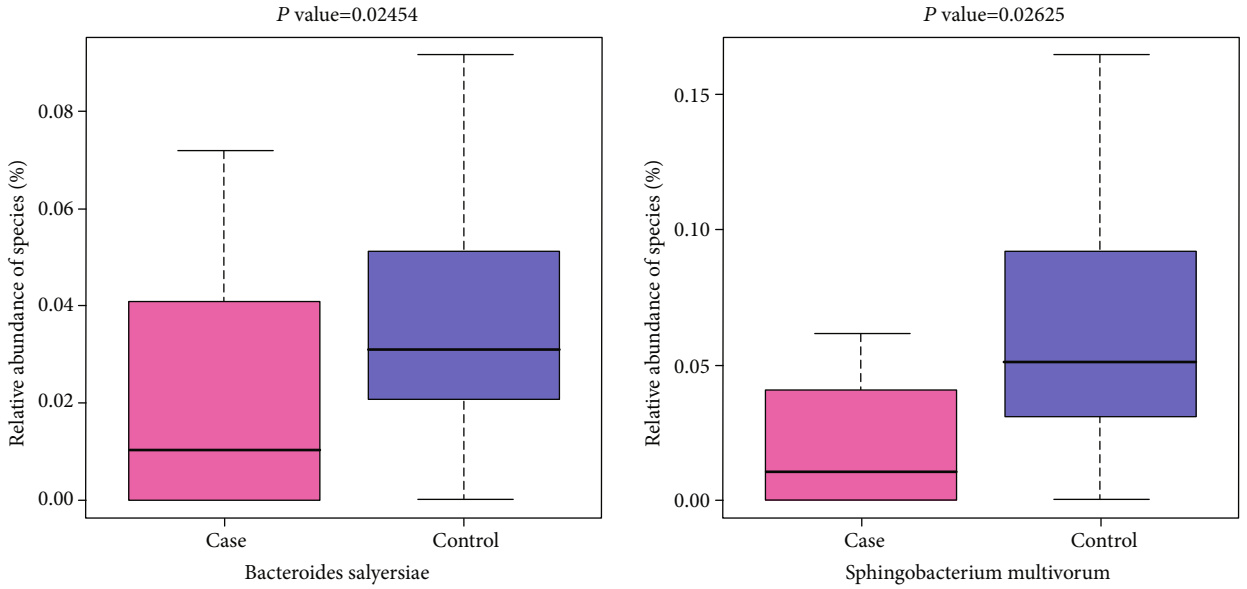
However, a few limitations of our study should also be taken into consideration. First, due to the complex process of participant enrollment and the application of our strict exclusion criteria, the size of the sample was restricted. Due to the relatively low sample size, the occurrence of beta error cannot be completely excluded, particularly as statistical significance was not reached. This might explain the discovery of some differentially abundant taxa in our MCI samples that were not discussed in previously published results on AD patients. Second, since gut microbiota is associated with a variety of diseases, although several gastrointestinal disorders have been considered in patient enrollment, our sample size restricted the ability to adopt more comprehensive exclusion criteria. Third, this study lacks information on diet of individual participants, while recently several studies suggested the important role of diet in



(a) Gordonibacter
(b) Bateroides
(c) Bateroidaceae
(d) Alloprevotella
(e) Prevotellaceae
(f) Bacteroidetes
(g) Lactobacillus
(h) Streptococcaceae
(i) Lactobacillales
(j) Anoxynatronum
(k) Pseudoramibacter
(l) Ezakiella
(m) Lachnospira
(n) Phascolarctobacterium
(o) Leptotrichia
(p) Leptotrichiaceae
(q) Fusobacteriales
(r) Fusobacteriia
(s) Fusobacteria
(t) Methylobacterium
(u) Beijerinkiaceae
(v) Uncultured
(w) Lysobacter

Case
Control

(a)



(b)

Figure 4: Continued.

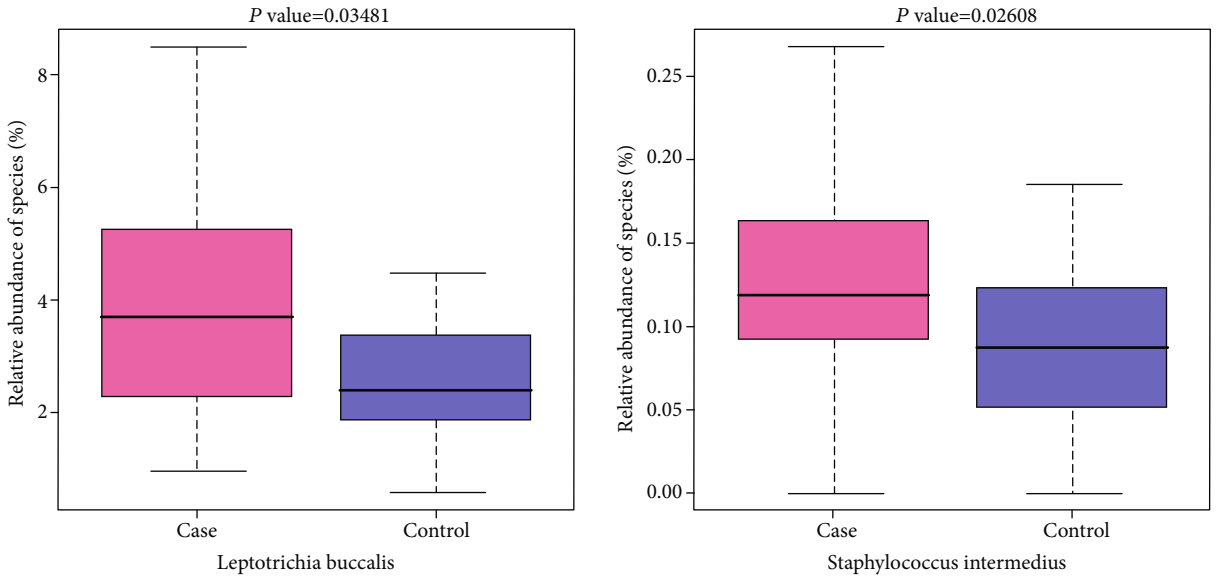


Figure 4: Bacterial taxa differentially represented in samples from MCI cases compared to control cases. (a) Cladograms generated by LEfSe software show the differences. Nodes in pink and blue indicate taxa that were enriched in the MCI and control groups, respectively. (b) Representative species with decreased abundance in the MCI group. (c) Representative species with increased abundance in the MCI group.

shaping gut microbiome. As a result, potential dietary differences may still affect the results to some extent. Fourth, we observed a barely detectable age difference between case and control groups, which may slightly affect the results of statistical analysis. In addition, as all of the participants were recruited from the same hospital, potential regional variations among gut microbiota could not be evaluated. We intend to conduct further multicenter clinical research with a larger sample size to more thoroughly investigate gut microbiota among MCI subjects across different regions.

In conclusion, the present study provided new evidence of abnormalities in the gut microbiota of MCI cases in relation to those of control subjects. Our results can be used to guide the development of a microbiota-based diagnosis in the early detection of MCI and subsequent AD. Additionally, the new-found alterations in the bacteria of MCI cases may provide clues for a probiotic regimen that could alleviate age-associated cognitive decline.

Data Availability

The datasets used and/or analyzed in the current study are available from the corresponding authors on reasonable request.

Ethical Approval

The Ethics Committee of The Third Xiangya Hospital of Central South University approved the study.

Consent

Written informed consent was obtained from all of the participants.

Disclosure

Our manuscript can be viewed as a preprint at https://www.researchsquare.com/article/rs-74865/v1.

Conflicts of Interest

The authors have no competing interests to declare.

Authors' Contributions

QP, KW, and QT designed the study; QP, YL, and KG collected samples and conducted experiments; QP, MX, YG, KW, DX, and QT wrote the manuscript.

Acknowledgments

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Supplementary Materials

Table S1: bacterial species with significant change of relative abundance between the MCI and control groups. *(Supplementary Materials)*

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Research Article

Correlation Analysis between Gut Microbiota and Metabolites in Children with Systemic Lupus Erythematosus

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Systemic lupus erythematosus (SLE) is an autoimmune-mediated diffuse connective tissue disease characterized by immune inflammation with an unclear aetiology and pathogenesis. This work profiled the intestinal flora and faecal metabolome of patients with SLE using 16S RNA sequencing and gas chromatography-mass spectrometry (GC-MS). We identified unchanged alpha diversity and partially altered beta diversity of the intestinal flora. Another important finding was the increase in Proteobacteria and Enterobacteriales and the decrease in Ruminococcaceae among SLE patients. For metabolites, amino acids and short-chain fatty acids were enriched when long-chain fatty acids were downregulated in SLE faecal samples. KEGG analysis showed the significance of the protein digestion and absorption pathway, and association analysis revealed the key role of 3-phenylpropanoic acid and *Sphingomonas*. *Sphingomonas* were reported to be less abundant in healthy periodontal sites of SLE patients than in those of HCs, indicating transmission of oral species to the gut. This study contributes to the understanding of the pathogenesis of SLE disease from the perspective of intestinal microorganisms, explains the pathogenesis of SLE, and serves as a basis for exploring potential treatments for the disease.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune-mediated diffuse connective tissue disease characterized by immune inflammation [1]. The presence of antinuclear antibodies in the serum and multisystem involvement are the two main clinical features of SLE, and the incidence rate of SLE in women is significantly higher than that in men [2]. Lupus nephritis (LN) occurs when SLE is complicated by renal damage. Approximately 60% of SLE patients suffer from LN complications that cause a higher mortality than SLE patients without LN [3]. At present, the aetiology and pathogenesis of SLE and LN are still unclear [4]. Studies have shown that the occurrence and development of some autoimmune diseases, such as inflammatory bowel disease [5, 6], rheumatoid arthritis [7, 8], and SLE [9, 10], are correlated with changes in intestinal microecology. Some studies found that the intestinal microbes of patients or animals with SLE were different from those of the control group through experimental models and clinical models [11–13], and the

reconstruction of intestinal microecology through diet and drug regulation could help improve the severity of the disease [14]. There are many hypotheses on the interaction mechanism between intestinal microbes and the host and its relationship with SLE, but there are no mature theories yet. Currently, an increasing number of studies have shown that metabolites of the intestinal flora, such as short-chain fatty acids (SCFAs), free fatty acids (FFAs), amino acids, and arachidonic acid, correlate with autoimmune reactions [14–16]. However, the relationship between microbial metabolites and SLE remains unknown, and previously published studies are limited to adult patients. In this study, we collected the intestinal flora and metabolite data of 33 SLE children and 28 healthy controls (HCs) to clarify the pathogenesis of the disease from the perspective of intestinal microorganisms as much as possible, explain the pathogenesis of SLE, and serve as a basis for exploring potential treatments for the disease.

2. Material and Methods

2.1. Ethical Statement. All enrolled patients and healthy controls understood the sampling process and study plan in detail before sampling and signed informed consent forms. Ethical approval for this study was obtained from the Ethics Committee of the Second Xiangya Hospital of Central South University.

2.2. Study Subjects. A total of 28 healthy controls and 33 SLE patients were enrolled from the Second Xiangya Hospital of Central South University between December 2018 and April 2019. The diagnostic criteria of SLE were consistent with SLE classification criteria 2 revised by American College Rheumatology (ACR) in 1997 [17]. SLE patients were given hormone and/or immunosuppressive therapy. None of the selected subjects had applied or taken antibiotics, probiotics, symbiotic, or other microecological preparations for at least 3 months before sampling.

2.3. 16S rRNA Microbiota Analysis. Total genomic DNA from samples was extracted using the CTAB/SDS method. 16S/18S rRNA genes were amplified using primers including 16S V4-V5 : 515F-907R, 18S V9 : 1380F-1510R, and ITS1 : ITS1F-ITS2R. All PCRs were carried out in 30 μl reactions with 15 μl of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM forward and reverse primers, and approximately 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 60 s then 5 min. Electrophoresis was performed on a 2% agarose gel. PCR products were purified with GeneJET Gel Extraction Kit (Thermo Scientific). Sequencing libraries were generated using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA), and the library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina MiSeq platform.

A total of 5507169 sequences were used for analysis. Sequence analysis was performed by the UPARSE software package using the UPARSE-OTU and UPARSE-OTUref algorithms. In-house Perl scripts were used to analyse the alpha diversity and beta diversity. Cluster analysis was preceded by principal component analysis (PCA). QIIME calculates both weighted and unweighted UniFrac distances. We used unweighted UniFrac distance for principal coordinate analysis (PCoA) and unweighted pair group method with arithmetic mean (UPGMA) clustering. Metastats software was utilized to confirm differences in the abundances of individual taxa between the two groups, and LEfSe was used for the quantitative analysis of biomarkers within different groups. To identify differences in microbial communities between the two groups, ANOSIM [18] and MRPP (multiresponse permutation procedure) [19] were performed based on Bray-Curtis dissimilarity distance matrices.

2.4. GC-MS Analysis. The instrumental status and balance as well as the stability of the gas chromatography-mass spectrometry system were evaluated using QC samples. Samples (60 mg) were mixed with 200 μg water and vortexed for 60 s before being added to 800 μl methanol acetonitrile (Merck, 1499230-935) solution (1 : 1, v/v), followed by vortexing for 60 s and low-temperature ultrasound for 30 min twice. Samples were stored at -20°C for 1 h to precipitate protein. Finally, the samples were centrifuged for 20 min at 14000 RCF at 4°C, and the supernatant liquor was collected for freeze drying and stored at -80°C.

For GC analysis, the samples were separated by an Agilent 1290 Infinity LC ultra-high-performance liquid chromatography (UHPLC) HILIC column at 25°C with a flow rate of 0.3 ml/min. The mobile phase was the composite of A (water, 25 mm ammonium acetate, 25 mm ammonium) and B (acetonitrile). Gradient elution procedures were as follows: B was 95% in 0-1 min, then changed linearly from 95% to 65% in 1-14 min and 65% to 40% in 14-16 min, and maintained at 40% in 16-18 min before changing linearly from 40% to 95% in 18-18.1 min, maintaining at 95% in 18.1-23 min. The samples were placed in an automatic sampler at 4°C throughout the analysis. A random sequence was used for the continuous analysis. QC samples were inserted into the sample queue for monitoring and evaluation of the system.

For Q-TOF MS analysis, electrospray ionized positive ions and negative ions were detected. The samples were separated by UHPLC and analysed by a Triple TOF 5600 mass spectrometer (AB SCIEX). The ESI source conditions after HILIC chromatographic separation were as follows: ion source gas 1 (Gas1): 60, ion source gas 2 (Gas2): 60, curtain gas (CUR): 30, source temperature: 600°C, ion spray voltage floating (ISVF) ±5500 V (positive and negative modes), TOF MS scan m/zrange: 60-1000 Da, product ion scan m/z range: 25-1000 Da, TOF MS scan accumulation time 0.20 s/spectra, and product ion scan accumulation time 0.05 s/spectra. The second-order mass spectrum was obtained by information-dependent acquisition (IDA) with a high sensitivity mode, a declustering potential (DP): of ± 60 V (positive and negative modes), collision energy of 35 ± 15 eV, and IDA excluded isotopes within 4 Da with 6 candidate ions to monitor per cycle.

The original data were converted into the mzXML format by ProteoWizard, and then, the XCMS program was used for

Table 1: Characteristics of the study cohorts.

	SLE	HC
Fecal samples	33	28
Female	26 (78.79%)	14 (50%)
Age, years, mean ± SD	12.39 ± 2.40	10.61 ± 3.67
BMI, kg/m^2, mean ± SD	18.79 ± 2.30	18.57 ± 4.51
DISEASE ACTIVITY PARAMETERS IN SERUM		
Elevated ESR, mm/h	11 (33.33%)	
Elevated CRP, mg/L	1 (0.30%)	
Reduced complement C3	18 (54.55%)	
Reduced complement C4	17 (51.52%)	
Positive ANA	21 (63.64%)	
Positive ANCA	2 (6.06%)	
Positive C1q	16 (48.48%)	
SLEDAI SCORE		
No or mild activity (0-4)	22 (66.67%)	
Moderate activity (5-9)	2 (6.06%)	
High activity (10-14)	9 (27.27%)	
Very high activity (≥15)	0	
TREATMENT		
GCs + HCQ	3 (9.09%)	
GCs + HCQ + CTX	12 (36.36%)	
GCs + HCQ + CsA + CTX	2 (6.06%)	
GCs + HCQ + MMF	15 (45.45%)	
GCs + HCQ + CTX + MMF	1 (3.03%)	

SD: standard deviation, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, ANA: antinuclear antibody, ANCA: antineutrophil cytoplasmic antibody, C1q: anti-C1q antibody. GCs: glucocorticoids, HCQ: hydroxychloroquine, CTX: cyclophosphamide, CsA: cyclosporin A, MMF: mycophenolate mofetil.

peak alignment, retention time correction, and peak area extraction. Accurate mass number matching (<25 ppm) and secondary spectrogram matching were used to identify the metabolite structure, and the laboratory database was retrieved. After pretreatment by Pareto scaling, multidimensional statistical analysis was performed, including principal component analysis (PCA), supervised partial least square discriminant analysis (PLS-DA), and orthogonal partial least square discriminant analysis (OPLS-DA). One-dimensional statistical analysis included Student's t-test and multiple analysis of variation.

2.5. Association Analysis. The correlation analysis is mainly based on statistical algorithms to find the correlation between significantly different flora ($p < 0.05$) and the significantly different metabolites obtained through nontarget metabolomics analysis. Principal component analysis (PCA) and multivariate statistical analysis were performed with SIMCA version 14.1. KEGG pathway analysis was based on the online Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/). Differentially abundant proteins/modified peptides/genes/and metabolites/lipids were $\log_2$ scaled (TMT/iTRAQ) or Z-score scaled (label free) and concatenated into one matrix. Then, the correlation coefficients among all the molecules in the matrix were calculated with the Pearson algorithm in R version 3.5.1. Cytoscape version 3.5.1 was used to calculate the correlation network.

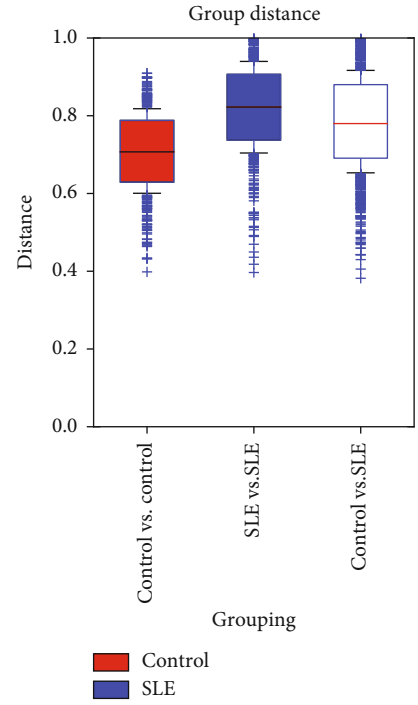

Figure 1: Bray-Curtis statistical algorithm was used to calculate the distance between each sample and obtain the distance matrix. The distance between the SLE group and the HC group with quartiles was calculated, and a box diagram of the distance between the two groups was drawn to compare the distance distribution differences. Student's two-sample t-test method was used to compare the distance between each group.

3. Results

3.1. Clinical Features of SLE Patients. The basic characteristics of the participants at the time point of sample collection are listed in Table 1, including their sex, age, disease activity parameters, autoantibody status, and treatment. According to two-tailed unpaired Student's t-test, BMI was not significantly different between the SLE group and HC group ($p = 0.234$). All the children in the SLE group were also diagnosed with LN.

3.2. Intestinal Flora Is Different between SLE Patients and Healthy Groups. After data optimization, the average length of 99.87% of the sequence bars was within the range of 401-450 bp, and a total of 2,159 operational taxonomic units (OTUs) were retained for subsequent analysis.

Using a t-test, we compared the Chao1 index ($p = 0.327$) of the two groups and found no significant difference, suggesting that the species abundance does not change in SLE patients. Additionally, Shannon ($p = 0.214$), coverage ($p = 0.988$), and Simpson ($p = 0.414$) indexes that estimate community diversity also showed similar results. Taken

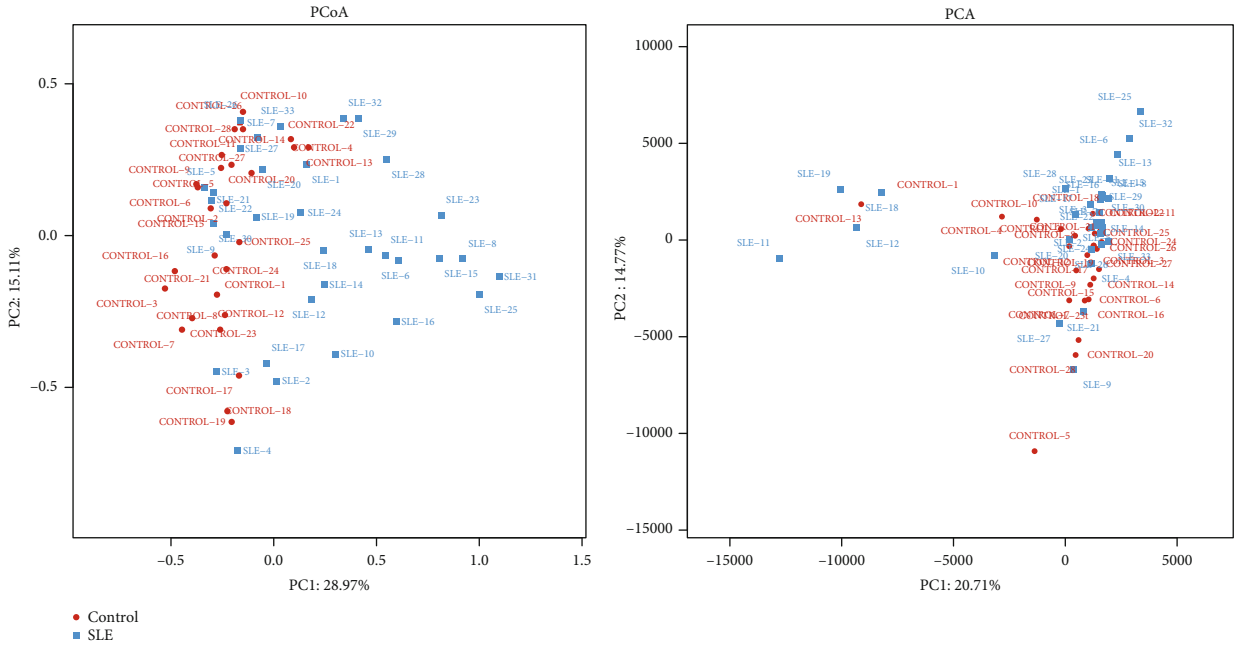

Figure 2: PCoA analysis and PCA analysis were conducted by R version 3.5.1. The red dots represent health controls, and the blue dots represent systemic lupus erythematosus patient samples. PCoA analysis sorts a series of eigenvalues and eigenvectors. It selects the most important eigenvalues ranked in the first few and shows them in the coordinate system. The result can be regarded as a rotation of the distance matrix. The red dots and the blue dots distribute in different clusters on the coordinate axis, revealing a clear separation between the SLE and HC groups. PCA analysis uses variance decomposition to reflect the differences of multiple groups of data on the two-dimensional coordinate graph. The PCA analysis of our study did not show clear separation.

together, these results show that the richness of the intestinal flora did not change among SLE patients.

As we can see in Figure 1, the sample distance of the control vs. control was significantly shorter than SLE vs. SLE group and control vs. SLE group ($p < 0.05$). Interestingly, although the alpha diversity of SLE patients remained unchanged compared with that of healthy controls, the sample distance of the former was obviously longer, suggesting healthy children possess microbiota with more similar species composition and the heterogeneity of the microbiota among SLE patients.

A scatter plot based on principal coordinates analysis (PCoA) revealed a clear separation between the SLE and HC groups (Figure 2). It should be highlighted that a principal component analysis (PCA) of SLE and HC subjects, based on 16 rRNA microbiota profiles, did not show distinct clustering patterns. We witnessed the association between gastrointestinal microbiota and immune factors at the level of bacterial composition but not at the level of the metabolite landscape of gut microbiota, which suggests that SLE can influence the heterogeneous species inhabiting the gut but not the metabolic pattern [20].

Twenty phyla were detected, among which Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Verrucomicrobia, Fusobacteria, and Synergistetes were the most abundant, with Firmicutes being the most dominant. At the genus level, a total of 380 genera were detected in all the samples, and the top 10 genera with the largest abundance were *Faecalibacterium* (average abundance was 17.26%), *Agathobacter* (6.23%), *Roseburia* (5.45%), *Megamonas* (5.37%), *Lachnoclostridium* (3.45%), *Subdoligranululum* (2.58%), *Bacteroides* of Bacteroidetes (7.14%), *Prevotella 9* (5.46%), *Escherichia-Shigella* (Proteobacteria, average abundance was 3.97%), and *Bifidobacterium* (Actinomycetes, average abundance was 2.79%).

Differently abundant species in both groups are listed in Table 2. There were a total of 54 bacteria, including 1 phylum, 3 classes, 5 orders, 8 families, and 37 genera. Proteobacteria was the only different bacterium at the phylum level, increasing in SLE patients. *Gammaproteobacteria*, *Bacilli*, *Enterobacteriaceae*, *Escherichia_Shigella*, *Ruminococcus_gnavus_group*, *Lachnoclostridium*, and *Kluyvera* were also more abundant in SLE patients, while Ruminococcaceae, *Agathobacter*, *Ruminococcus_2*, *Coprococcus_2*, *Eubacterium_coprostanoligenes_group*, *Dialister*, *Faecalibacterium*, and *Subdoligranulum* were distinctly richer among the healthy controls.

Using LEfSe software, a nonparametric factorial Kruskal-Wallis (KW) sum-rank test was conducted to identify species with distinct abundance differences before undertaking linear discriminant analysis (LDA) to estimate the influence of each component (species) on the difference. The difference in microflora from phylum to genus was determined by an LDA value greater than 2 and a p value less than 0.05 (Table 2). We can see that for SLE patients, Proteobacteria at the phylum level, Gammaproteobacteria at the class level, Enterobacteriales at the order level, and Enterobacteriaceae at the family level are the most relevant components, and they possess a subordinate relationship. On the other hand, Ruminococcaceae, Christensenellaceae, and Family_XIII at

Table 2: Different species.

Flora category	Log value of abundance	Group	LDA value	p value	Level
Proteobacteria	4.967	SLE	4.49	0.007	Phylum
Alphaproteobacteria	2.883	SLE	3.255	0	Class
Gammaproteobacteria	4.95061	SLE	4.486	0.007	Class
Bacilli	4.154298	SLE	3.656	0.006	Class
Enterobacteriales	4.92	SLE	4.495	0.006	Order
Xanthomonadales	2.178	SLE	3.563	0	Order
Caulobacterales	1.81	SLE	3.61	0.001	Order
Sphingomonadales	2.497641	SLE	3.013	0	Order
Lactobacillales	4.152046	SLE	3.655	0.008	Order
Ruminococcaceae	5.556	HC	4.821	0.005	Family
Streptococcaceae	3.838	SLE	3.284	0.017	Family
Christensenellaceae	3.909	HC	3.377	0.04	Family
Caulobacteraceae	1.788	SLE	3.589	0.001	Family
Enterobacteriaceae	4.920289	SLE	4.495	0.006	Family
Family_XIII	2.853446	HC	2.797	0.036	Family
Sphingomonadaceae	2.497641	SLE	3.021	0	Family
Rhodanobacteraceae	1.816683	SLE	3.679	0	Family
Erysipelotrichaceae_UCG_003	3.358	HC	2.768	0.003	Genus
Lachnospiraceae_UCG_008	1.772055	HC	3.586	0.043	Genus
Lachnospiraceae_UCG_004	3.600504	HC	2.964	0.025	Genus
Ruminococcaceae_NK4A214_group	3.618	HC	3.006	0.006	Genus
Lachnospiraceae_FCS020_group	2.815	HC	2.738	0.033	Genus
Family_XIII.Family_XIII_UCG_001	2.302	HC	3.209	0.01	Genus
Ruminococcaceae_UCG_013	3.423	HC	2.885	0.002	Genus
Ruminococcaceae_UCG_010	3.021	HC	2.775	0.03	Genus
Lachnospiraceae_NC2004_group	2.246211	HC	3.241	0.034	Genus
Christensenellaceae_R_7_group	3.89737	HC	3.376	0.021	Genus
Family_XIII_AD3011_group	2.580169	HC	3.234	0.037	Genus
Lachnospiraceae_NK4A136_group	3.869956	HC	3.367	0.001	Genus
Escherichia_Shigella	4.809	SLE	4.393	0.019	Genus
Agathobacter	4.932	HC	4.313	0.004	Genus
Akkermansiaceae	3.328	HC	2.979	0.007	Genus
Ruminococcus_2	4.314	HC	3.767	0.049	Genus
Clostridium_innocuum_group	2.778	SLE	2.938	0.037	Genus
Fusicatenibacter	3.892	HC	3.342	0.019	Genus
Streptococcus	3.829	SLE	3.271	0.032	Genus
Dorea	3.792	HC	3.122	0	Genus
Coprococcus_2	4.047	HC	3.686	0	Genus
Hungatella	3.387	SLE	3.087	0.033	Genus
Erysipelatoclostridium	3.226	SLE	2.98	0.017	Genus
Eubacterium_coprostanoligenes_group	4.435	HC	3.809	0.008	Genus
Ruminococcus_gnavus_group	4.308	SLE	3.958	0.003	Genus
Dialister	4.603	HC	4.101	0.001	Genus
Klebsiella	3.669467	SLE	3.274	0	Genus
Lachnoclostridium	4.698468	SLE	4.244	0.027	Genus
Faecalibacterium	5.329871	HC	4.598	0.015	Genus
Ruminiclostridium_5	3.464955	HC	2.995	0.014	Genus
Rudaea	1.816683	SLE	3.68	0	Genus

Table 2: Continued.

Flora category	Log value of abundance	Group	LDA value	p value	Level
Sphingomonas	2.425476	SLE	3.099	0	Genus
Kluyvera	4.006708	SLE	3.48	0.001	Genus
Subdoligranulum	4.57793	HC	4.024	0.012	Genus
Odoribacter	2.434041	HC	3.256	0.048	Genus
Akkermansia	3.327618	HC	2.973	0.007	Genus
Eubacterium_eligens_group	3.929	HC	3.478	0.022	Genus

SLE: systemic lupus erythematosus group; HC: health control group; LDA: linear discriminant analysis.

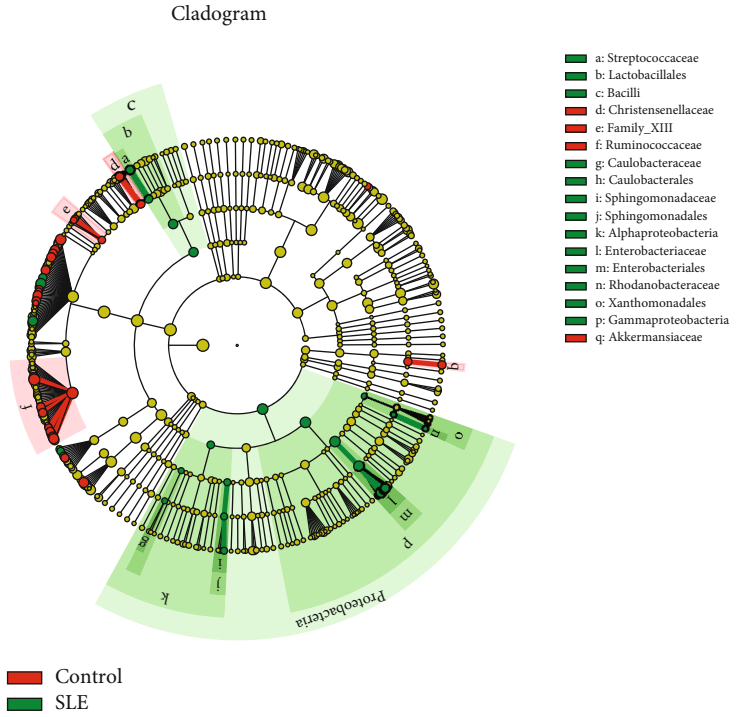

Figure 3: Differential analysis using LEfSe software. Red nodes represent the microbial group that plays an important role in the HC group, green nodes represent the microbial group that plays an important role in the SLE group, and yellow nodes represent the microbial group that does not play an important role in both groups.

the family level and *Akkermansiaceae* at the genus level were abundant in the healthy controls (Figure 3).

The nonparametric factorial Kruskal-Wallis sum-rank test was used for calculating the difference of abundance. Log value of abundance reflects the abundance of the different species. LDA value estimates the influence of the abundance of each component (species) on the overall abundance differences. $p < 0.05$ and LDA > 2 represent significantly different species.

3.3. Different Metabolites' Analysis by GC-MS. Variable importance for the projection (VIP) obtained from the OPLS-DA model was used to measure the influence intensity and explanatory ability of the expression pattern of each metabolite on the classification and discrimination of samples of each group, and the different metabolites with biological significance were excavated.

This study defined metabolites with VIP > 1 in the multidimensional statistical analysis and $p < 0.05$ in univariate statistical analysis as significantly different metabolites, including 24 in positive ion mode and 37 in anion mode. Ruling out 6 overlapping data, there were a total of 55 significantly different metabolites (Table 3), including mainly long-chain fatty acids and amino acids. Among them, cyclohexylsulfamate had the highest VIP value, indicating that its decline had a greater impact on the occurrence of SLE diseases.

Of the 55 SLE-altered metabolites submitted to the KEGG (Kyoto Encyclopedia of Genes and Genomes) website (http://www.kegg.jp/), we were able to match 27 of them with 66 pathways. The p value represents the significance of enrichment of the KEGG pathway, and the number of differentially expressed metabolites contained in the KEGG pathway reflects the degree of influence of SLE on each pathway to some extent (Figure 4). Taking two factors into consideration, protein digestion and absorption ($p = 1.75E - 9$) were the most significant among all these pathways, involving *L*-tryptophan, tyramine, *L*-phenylalanine, *L*-leucine, *L*-methionine, *L*-alanine, *L*-glutamine, *L*-valine, *L*-isoleucine, and *L*-tyrosine.

Table 3: Significantly different metabolites.

Description	VIP	Fold change (SLE/HC)	p value	m/z	rt (s)
(S)-2-Aminobutyric acid	1.134457	7.300207	0.027901	102.0559	714.909
(Z)-6-Octadecenoic acid	1.460343	0.604532	0.00674	282.2502	91.72
16-Hydroxypalmitic acid	1.874055	0.499351	0.009079	314.2681	92.142
1-Oleoyl-sn-glycero-3-phosphocholine	1.904087	0.331859	0.032946	522.3537	354.483
1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine	2.796585	0.225656	$7.95E-06$	454.2913	372.123
2-Hydroxy-3-methylbutyric acid	4.457651	4.059644	0.024633	117.0556	283.2115
2-Hydroxy-butanoic acid	1.365271	2.252586	0.000967	103.0398	364.888
3-(3-Hydroxyphenyl)propanoic acid	7.504876	3.292347	0.042029	165.0556	213.446
3-Phenylpropanoic acid	8.186158	0.482206	0.018599	149.0607	197.716
5,10-Methylene-THF	2.02514	5.177854	0.038319	456.1695	81.174
9,10-DiHOME	1.759624	0.622241	0.018751	313.2384	197.908
Acetyl-DL-leucine	2.436625	3.258067	0.020322	172.0978	372.417
all *cis*-(6,9,12)-Linolenic acid	1.996995	0.552529	0.003271	279.2309	91.4225
Alpha-linolenic acid	1.125754	0.495219	0.027657	296.2578	85.836
Alpha-N-phenylacetyl-L-glutamine	1.032981	6.993584	0.03739	263.1035	434.981
Bisindolylmaleimide I	1.251916	0.533825	0.034091	411.1837	60.485
cis-9-Palmitoleic acid	2.112812	0.530253	0.010487	315.2523	157.6505
Cyclohexylsulfamate	18.63932	0.399938	0.01068	178.0541	134.362
Daidzein	1.860198	0.37232	0.049911	253.0506	90.079
DL-Indole-3-lactic acid	1.389089	1.868281	$2.75E-05$	188.0698	482.9055
DL-Methionine sulfoxide	1.188165	1.570971	0.013233	164.0387	705.022
Enalapril	2.770035	3.292851	0.02267	375.1845	335.5975
Formononetin	1.159497	12.16737	0.031198	269.08	75.902
Formylanthranilic acid	1.108129	1.780982	0.030492	164.035	124.961
Gamma-L-glutamyl-L-valine	1.260303	1.995892	0.001099	245.1144	738.78
Glycyl-L-leucine	1.714816	1.468498	0.009308	187.1089	549.388
Hydrocortisone (cortisol)	2.126813	6.47518	0.003054	421.2224	94.857
Hydroxyisocaproic acid	10.89455	4.366298	0.014686	131.0711	244.439
L-Alanine	1.917025	1.859043	0.000362	88.04036	663.2525
L-Glutamine	1.81976	2.137778	0.00051	145.0619	715.39
Linoleic acid	3.644522	0.377719	$4.77E-06$	298.2736	84.196
L-Isoleucine	1.163985	4.459251	0.006748	261.182	497.738
L-Leucine	7.61484	1.835031	$9.53E-05$	130.0874	497.981
L-Methionine	2.024436	2.05734	0.005488	148.0437	536.5665
L-Norleucine	1.193737	5.606042	0.047861	263.1959	493.5235
L-Phenylalanine	6.291687	1.785839	0.000231	164.072	482.6735
L-Tryptophan	2.780279	1.785305	$2.45E-05$	203.0828	482.5715
L-Tyrosine	3.001494	1.658265	0.006866	180.0666	569.619
L-Valine	3.438313	3.006204	0.000931	116.0716	574.471
Methoprene (S)	1.236084	0.334761	0.004683	328.2844	64.9405
MG(18 : 2(9Z,12Z)/0 : 0/0 : 0)[rac]	3.617466	0.313071	$7.55E-05$	355.2834	69.542
N-Acetyl-L-aspartic acid	1.020191	0.48076	0.016099	174.0408	773.394
N-Acetylneuraminic acid	1.083761	0.539489	0.027489	310.1126	716.366
Nicotinate	1.873086	0.543751	0.001525	124.0383	412.64
Oleic acid	1.514581	0.359758	0.001028	265.2517	120.4355
Oxindole	1.01039	0.407327	0.033063	134.0594	80.262
Palmitic acid	4.166731	0.485449	0.035091	255.2329	88.202
p-Hydroxyphenylacetic acid	1.625958	2.672582	0.032862	151.0399	386.525

Table 3: Continued.

Description	VIP	Fold change (SLE/HC)	p value	m/z	rt (s)
Pregnenolone sulfate	1.984757	0.31761	0.008648	395.1888	55.708
Stearidonic acid	2.760427	23.20345	0.040155	337.2366	372.7625
Sunitinib	3.289336	0.283469	0.002701	397.2046	61.461
trans-Cinnamate	1.407338	1.766262	0.000204	147.045	482.794
trans-Vaccenic acid	3.069738	0.496621	0.012385	283.2626	93.176
Tridecanoic acid (tridecylic acid)	1.351245	0.338885	0.032456	213.186	91.512
Tyramine	2.011381	1.73279	0.001232	120.0798	479.944
Vanillin	1.308743	1.345909	0.016492	151.0398	75.058

VIP: variable importance for the projection; m/z: mass-to-charge ratio; rt (s): retention time. The VIP value was calculated based on orthogonal partial least squares discriminant analysis to measure the influence and explanatory ability of each metabolite expression pattern on the classification discrimination of each group of samples, and to excavate the different metabolites with biological significance. Fold change describes the ratio of metabolite content (SLE/HC). p value < 0.05 represents that metabolites have significant difference in one-way ANOVA or two-way ANOVA analysis.

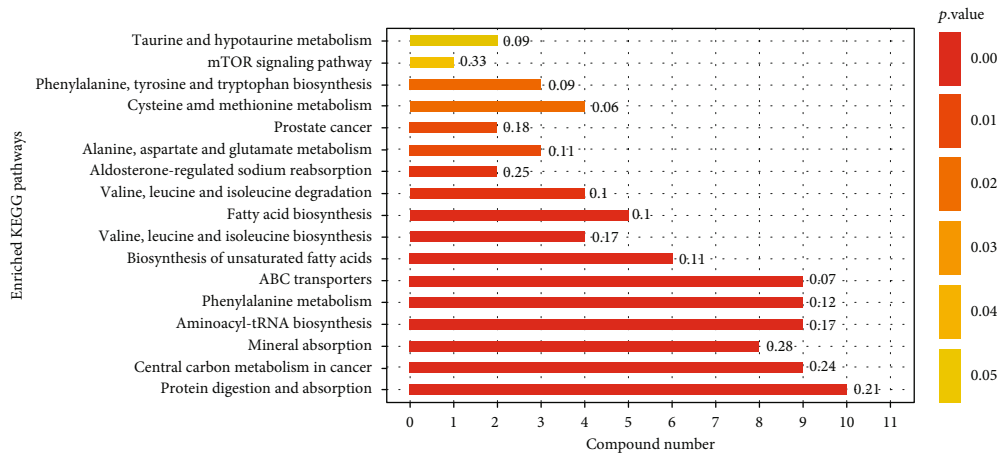

Figure 4: KEGG pathways. Numbers on the right of the bar are rich factor, reflecting the degree of enrichment (rich factor = the number of genes enriched in this pathway/the number of all genes in this pathway).

The network of metabolites and intestinal flora using Cytoscape version 3.5.1 is shown in Figure 5. We can see that the most central location belongs to 3-phenylpropanoic acid, which exhibits a strong positive correlation with *Family XIII UCG-001* and a negative correlation with all 5 differentially expressed genera of Proteobacteria. *Sphingomonas* of the Proteobacteria phylum plays the most important role as a bacterium, and it is obviously positively associated with all 10 metabolites in the protein digestion and absorption pathway mentioned above.

4. Discussion

The current study found that alpha diversity of the SLE group is not different when compared to healthy controls, and the beta diversity was partially altered, which was in accordance with a previous study among adult SLE patients [20]. The fact that the alpha diversity is not statistically different between the healthy control group and the group affected by the pathology could be related to age-dependent factors and also to the small number of children in the analysis. To be precise, PCoA successfully identified 2 separated clusters of SLE and HC subjects when PCA did not, indicating that the changes induced by SLE became marked at the functional level, i.e., the level of microbial population structure (or 16S rRNA), regardless of the heterogeneities that appeared above at the highest level of the functional hierarchy, i.e., the metabolite level (Figure 1). In contrast to our outcomes on the alpha and beta diversity, the outcomes of a study by Zhu revealed upregulated alpha diversity in the SLE group; in addition, Rojo et al. reported beta diversity in PCA but not PCoA [16, 21]. This inconsistency may be due to age and sex differences since previous papers contained only adult patients and fewer or no male patients.

Another important finding was the increase in Proteobacteria and Enterobacteriales at the phylum and family levels among SLE patients in our project in southern China, and this finding was also reported by Wei et al., He et al., and Hevia and Milani from northern China. However, results from studies conducted in Spain and southern China do not support our results [22–24]. In Spain, the intestinal flora of SLE patients was characterized by a significantly lower Firmicutes/Bacteroidetes ratio and showed a depletion of *Lachnospiraceae* and *Ruminococcaceae* and an enrichment of

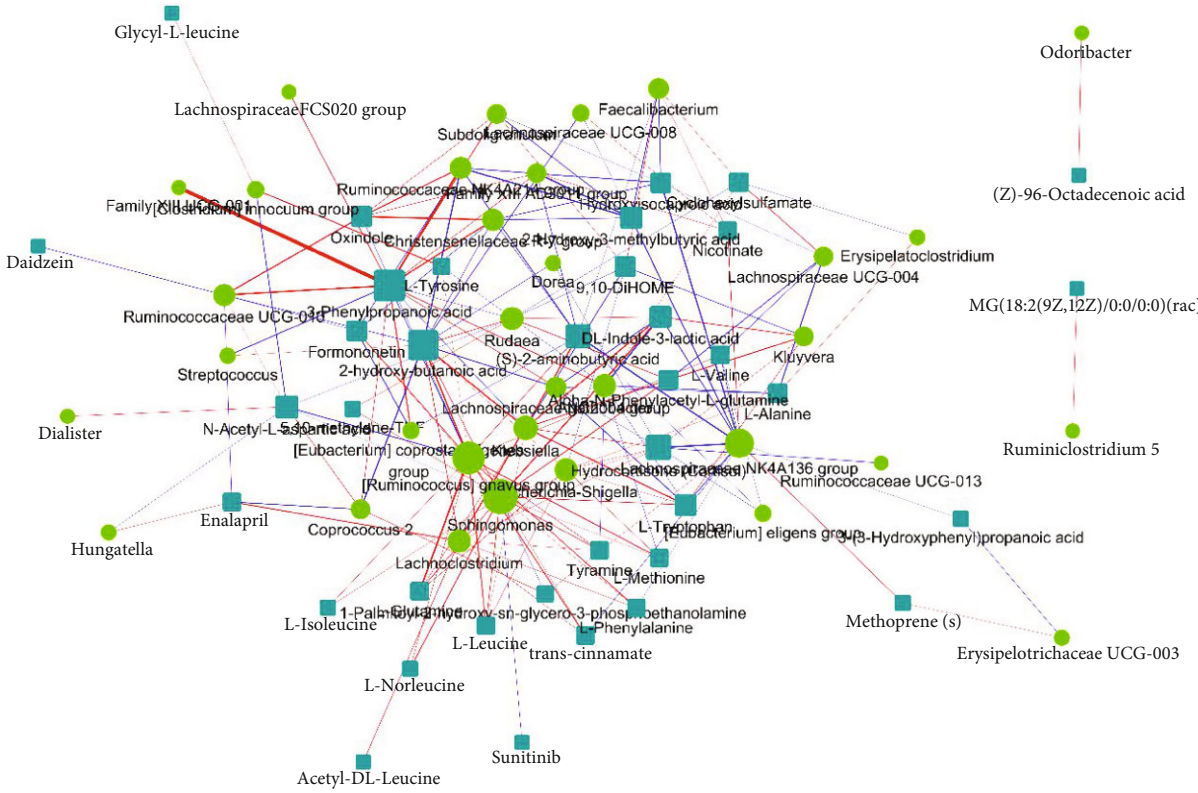

Figure 5: Circles represent significantly different genera, and rectangles represent significantly different metabolites. The color of the line represents the positive and negative value of correlation coefficient (blue represents a negative correlation and red represents a positive correlation), and the thickness of the line is proportional to the absolute value of the correlation coefficient. The node size is positively correlated with its degree (the greater the degree, the larger the node size).

Bacteroidaceae and *Prevotellaceae* [24]. In southern China, the intestinal flora of SLE patients was characterized by a significant increase in Actinobacteria [22]. It can therefore be assumed that the flora composition is irrelevant to the geographical location. On the other hand, consistent with the literature, this research found that Ruminococcaceae levels were higher in healthy controls [21, 23, 24]. Moreover, there was a list of other genus assigned to the Lachnospiraceae family that are widely descripted as beneficial bacteria in literature elevated in health controls, including Lachnospiraceae_UCG_008 and Lachnospiraceae_UCG_004 [25, 26]. Additionally, previous research has established that the increase in Proteobacteria and the decrease in Ruminococcaceae were associated with lupus nephritis with gastrointestinal damage [27]. Na et al. proposed that an increased prevalence of the bacterial phylum Proteobacteria is a marker for an unstable microbial community (dysbiosis) and a potential diagnostic criterion for disease [28]. Shang et al. identified Ruminococcaceae to be responsible for the degradation of diverse polysaccharides and fibres [29].

An obvious finding to emerge from the analysis is that amino acids were enriched in SLE faecal samples compared to HCs (Table 3), which was in accordance with previous research that found that their plasma concentration decreased [30–33]. Using gas chromatography-mass spectrometry, Yan et al. were able to detect the serum depletion of 12 amino acids, including tryptophan, alanine, proline, glycine, serine, threonine, aspartate, glutamine, asparagine, lysine, histidine, and tyrosine [30]. Similarly, Ouyang et al. found that *L*-alanine, *L*-isoleucine, *L*-lysine, *L*-phenylalanine, *L*-tyrosine, and *L*-valine were downregulated in the plasma of SLE patients [31]. Notably, four essential amino acids, *L*-valine, *L*-leucine, *L*-phenylalanine, and *L*-tryptophan, were listed among the metabolites depleted in plasma but enriched in faeces. These four amino acids have important physiological functions, such as the regulation of immunity, metabolism, and neural activity. Choi et al. reported that low dietary tryptophan prevented autoimmune pathology in lupus-prone mice, whereas high dietary tryptophan exacerbated disease [32]. Moreover, glucogenic amino acids, such as proline and *L*-methionine, and glucogenic and ketogenic amino acids, such as *L*-tyrosine, were increased in the faeces of SLE patients, indicating that there might be disorders in glucose metabolism and energy metabolism, as these amino acids could emerge as potential energy sources [33, 34]. Regarding the fatty acids, we found the abundance varied, as most long-chain fatty acids decreased while short-chain fatty acids increased in SLE children. This finding is contrary to a recent study that reported enriched fatty acids in the faeces of the SLE group [35]. Another study also established that long-chain fatty acids were less abundant in SLE patient serum [36]. At least half of SLE patients have gastrointestinal

symptoms, including nausea, vomiting, anorexia, abdominal pain, diarrhoea, and abdominal distension, which may lead to an imbalance of amino acids and fatty acids [37].

The results of this study show that SLE-enriched faecal amino acids were significantly located in the protein digestion and absorption, ABC transporters, and aminoacyl-tRNA biosynthesis pathways. Amino acids are mainly absorbed in human intestinal mucosal cells through carrier proteins and the γ-glutamine cycle. Through type I ABC transporters in the third subgroup, intestinal prokaryotes and archaea obtain amino acids, which can be used for the synthesis of microbial proteins, including the biosynthesis of aminoacyl-tRNA, microbial energy metabolism, and conversion into a variety of physiologically active substances, such as neurotransmitters and hormones [38]. As a result, the increase in amino acids in faeces can impact the physiological activities of microorganisms and their hosts. For example, SLE-rich faecal branched-chain amino acids, including *L*-leucine and *L*-valine, can be used as precursors of membrane fatty acids, as well as key synergistic regulators of pathogen growth and virulence. Low-proline or low-protein diets in germ-free mice parasitized by human intestinal flora led to reduced expansion of wild-type *C. difficile* after challenge, indicating that the effectiveness of amino acids may be important for *C. difficile* infection [39]. Tryptophan is essential for the growth of certain pathogens, and severe tryptophan deficiency may hinder normal *Chlamydia trachomatis* onset and reduce its activation [40].

Of association analysis, this study found that *Sphingomonas* plays the most important role in the network of metabolites and intestinal flora. We found higher levels of the bacterium in SLE children. It is encouraging to compare this rise with that found by Corrêa et al., who found that *Sphingomonas* was at lower relative levels in healthy periodontal sites of SLE patients [41]. Chen et al.'s research provided evidence for increased microbial transmission along the gastrointestinal tract of most certain species in SLE patients compared to HCs, implicating a damaged barrier in the upper gastrointestinal tract in SLE patients who possibly cause transmissible oral species to colonize the gut [42].

It is unfortunate that most studies concerning gut microbiota and metabolites in SLE and LN patients include a study cohort of only adults, so our literature search may not be comprehensive enough. There is also a potential confounding effect of medications, as most patients were receiving treatment at the time of sample collection, and a large list of medications is postulated to affect the balance of taxa within the gut microbiome community [43, 44]. Another limitation of this study is that the SLE group and the HC group were not perfectly matched.

5. Conclusion

This study set out to investigate intestinal flora and metabolomics in SLE children. This study has shown that Proteobacteria was increased in SLE patients. Of this phylum, the genus *Sphingomonas* plays a very important role, as it exerts a significant positive correlation with a series of proteins in the protein digestion and absorption pathway. *Sphingomonas* were reported to be less abundant in healthy periodontal sites of SLE patients than in those of HCs, indicating the transmission of oral species to the gut. Adjunct therapeutic modalities of adjusting microbiota can be innocuous and worth trying. Considerably, more work will need to be done to determine the pathogenesis and treatment of SLE in children.

Data Availability

All data are available in our manuscript.

Ethical Approval

Ethical approval for this study was obtained from Ethics Committee of the Second Xiangya Hospital of Central South University.

Conflicts of Interest

All authors declare that they have no conflict of interest.

Authors' Contributions

Min Wen, Taohua Liu, Mingyi Zhao, and Xiqiang Dang participated in the experiments, data analysis, and manuscript writing. Shipin Feng, Xuewei Ding, Zhiquan Xu, Xiaoyan Huang, Qiuyu Lin, Wei Xiang, and Xiaoyan Li contributed to the data collection, management, and analysis. Xiaojie He and Qingnan He designed the experiment. Min Wen and Taohua Liu contributed equally to this work and should be considered co-first authors.

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Review Article

A Role for Folate in Microbiome-Linked Control of Autoimmunity

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The microbiome exerts considerable control over immune homeostasis and influences susceptibility to autoimmune and autoinflammatory disease (AD/AID) such as inflammatory bowel disease (IBD), multiple sclerosis (MS), type 1 diabetes (T1D), psoriasis, and uveitis. In part, this is due to direct effects of the microbiome on gastrointestinal (GI) physiology and nutrient transport, but also to indirect effects on immunoregulatory controls, including induction and stabilization of T regulatory cells (T_{reg}). Secreted bacterial metabolites such as short-chain fatty acids (SCFA) are under intense investigation as mediators of these effects. In contrast, folate (vitamin B9), an essential micronutrient, has attracted less attention, possibly because it exerts global physiological effects which are difficult to differentiate from specific effects on the immune system. Here, we review the role of folate in AD/AID with some emphasis on sight-threatening autoimmune uveitis. Since folate is required for the generation and maintenance of $T_{reg,}$ we propose that one mechanism for microbiome-based control of AD/AID is via folate-dependent induction of GI tract $T_{reg,}$ particularly colonic T_{reg}, via anergic T cells (T_{an}). Hence, folate supplementation has potential prophylactic and/or therapeutic benefit in AID/AD.

1. Introduction

Autoimmune diseases (AD) develop when there is breakdown of immunological tolerance to self-antigen in the adaptive immune system while autoinflammatory diseases (AID) occur when there are defects or dysregulation in the innate immune system [1]. In both cases, a disordered microbiome has been implicated and, by inference, an altered bacterial flora including its secreted products [2]. Classical AD such as multiple sclerosis (MS) [3], type 1 diabetes (T1D) [4], and rheumatoid arthritis (RA) [5] is kept at bay by a healthy microbiome, while probable AID such as inflammatory bowel disease (IBD), Behçet's uveitis, and ankylosing spondylitis (AS) are negatively affected by a disordered microbiome (reviewed in [6]). Psoriasis, a debilitating skin inflammation, and uveitis, a major sight-threatening disease in which infection may be a direct or indirect cause, are considered in many cases to be either an AD or an AID [7, 8].

In both AD and AID, there is failure of immune regulation (tolerance) and a disturbed microbiome. Identifying possible causal links between these two biological domains is a major focus. In adaptive immunity, tolerance (homeostasis) is maintained by autoreactive T cell deletion/anergy or suppression by T regulatory cells (T_{reg}). T_{reg} are also effective in controlling innate immunity by regulating the activity of myeloid and NK cells [9] and so contribute to preventing AID. Circumstantial evidence for their role in AD and AID is the decline in T_{reg} numbers in many of these conditions such as AS [10] as well as the effectiveness of adoptive T_{reg} therapy in experimental models of AD and AID.

2. The Colonic Microbiota Shapes the Host's Health

The prenatal GI tract is sterile due to the protective immunological placental barrier preventing bacterial translocation into the fetal organism. Microbial colonialization develops gradually when environmental contact first occurs upon delivery. This has significant implications for overall health

in later life [11, 12]. For instance, the expanding gut microbiome exerts its effects on brain- (CNS-) related immune privilege (IP) in that the blood-CNS barriers only reach maturity in the neonatal period [13–15]. A key colonic metabolite that can modulate the immune system is folate. Naturally, occurring folate/vitamin B9 (pteroyl-glutamic acids and oligo-glutamic acid conjugates) and its synthetic form folic acid (FA) are water-soluble B vitamins that must be ingested through the diet (e.g., legumes and leafy greens [16, 17]) or supplements [18]. Commensal bacteria [19] are also capable of synthesizing folate and other B vitamins. Glutamic compounds [20] occur in the body as different metabolites with variable bioavailability [21] and the terms folate and FA are often used interchangeably. The role of folate in hematopoiesis, reproductive health and foetal development are well known, and an extended role for the vitamin particularly in later life is recognized in preventing a decline in cognitive and neurological functioning [16, 22, 23]. Indeed, most likely due to inadequate intake, folate deficiency is more prevalent in the older population [24] contemporaneously with a higher incidence of chronic disease.

Hence, a balanced microbiome with adequate folate and micronutrient production maintains homeostasis. Recently, however, the microbiome has come under scrutiny as a source of pathogenic antigens capable of inducing or promoting AD [25–28]. This is particularly linked to dysbiosis [29] and may be the result of infection with pathogenic bacteria, loss of commensal bacteria, or reduction in microbial diversity [30]. The human intestinal epithelium covers as much as 400 m^2 of surface area [31] with more than ten times as many resident microbes as the total number of cells in the body [32]. Overall, the gut microbiota comprises five phyla and about 160 species in the large intestine [33], and the number of genes of the intestinal microbiota is 150 times greater than the human genome [34]. Qualitative and quantitative changes in the microbial flora, their metabolic activity, and their local distribution [35] are a typical feature of IBD [36] that is otherwise characterized by the infiltration of the lamina propria with a mixed leukocyte population expressing proinflammatory cytokines [37]. Whether dysbiosis represents the cause or result of IBD (reviewed in [38]), it is a correlated biomarker of extraintestinal inflammatory disease (reviewed in [25, 39]). While mechanistic evidence is still limited, dysbiosis has long been linked to AD [40], including noninfectious uveitis [41–43], (reviewed in [25]), often occurring simultaneously with acute flare-ups of colitis [44]. Thus, it can be seen that dysbiosis and similar microbiota-related environmental factors impact up to 70% of all AD [45, 46], and while the etiology of IBD itself is not fully understood, it is considered to be the result of an interplay between environment/nutrition, microbiota, gastrointestinal immunity, and epigenetics.

3. The Microbiome Promotes Immunological Tolerance via an Immune Privilege-Like Mechanism

Immune privilege is a relative property of all tissues reflecting various degrees of tissue-based immunological tolerance [47] and has particular relevance for the large intestine, now considered a secondary immune organ [48, 49]. "Unconventional" IP of the gut [50] tolerates trillions of commensals and has two components, a physicochemical barrier and an immunological barrier [51]. The physical barrier is provided by the two cellular barriers which prevent translocation of pathobionts from the intestine to the general circulation (reviewed in [47]). These include a monolayer of enterocyte epithelium (i.e., an intestinal epithelial barrier, IEB) covering the entire mucosa and the subjacent lamina propria and a stringent gut-vascular barrier (GVB). The physical barrier to the passage of small molecules is provided by immunologically responsive [50] intraepithelial tight junctions [52] while a chemical barrier derives from specialized enterocytes (mucus-producing goblet cells and Paneth cells) which secrete antimicrobial peptides (reviewed in [53, 54]). A further physical barrier to hematogenous passage of any pathogens which may have penetrated the epithelium is provided by the GVB [55] with its closely associated pericytes and enteric glial cells which serve a vital function in retaining barrier properties [56–60].

The immunological component of the gut barrier is provided by a wealth of immune cells in the gut, including tolerogenic DC, several types of classical T cells including T_{reg}, three sets of innate lymphoid cells (ILC), myeloid suppressor cells, and mucosa-associated invariant T (MAIT) cells. These cells regulate aspects of both the adaptive and innate immune systems and are under the control of secreted factors both by host cells and the microbiome. For instance, flagellin associated mostly with gram-negative bacteria (e.g., *E.coli* and *Salmonella*) binds TLR5 on $CD103^+$ mucosal DC which secrete IL23 to act on ILC which in turn release IL22 to then induce the gut epithelium to release antimicrobial peptides [30], and colonic *Clostridia* through their metabolic activity have been found to induce and impact the colonic distribution of T_{reg} in mice [61–63]. T_{reg} are known to be stabilized by folate [64–66], that in turn is synthesized by some commensals including *Clostridia*, *Lactobacilli*, and *Bifidobacteria* [67]. These findings point to a tolerizing immunological role for the commensal microbiome that has the potential to exert effects on disease induction and progression. How gut-derived leukocytes might cross distant barriers at target sites and induce AD/AID remains to be clarified. Several mechanisms have been proposed [25] viewed from both the perspective of an adaptive immune response (TCR activation) and dysregulation of the microbiome.

4. The Microbiome Mediates Immunological Tolerance via the Products of Microbial Fermentation

Microbial fermentation in the gut generates secreted products which directly modify immune activity (Figure 1). These include products of tryptophan metabolism, short-chain fatty acids (SCFA), and folate.

4.1. Tryptophan. Tryptophan is an essential amino acid (AA) that is delivered through the diet, particularly dairy products

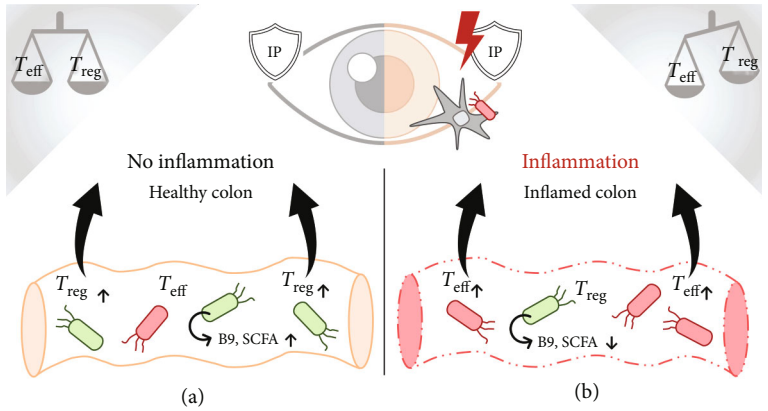

Figure 1: Bioavailable folate (B9) produced by certain phyla of the human microbiome (*Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Verrucomicrobia* [84]; green microbes) and short-chain fatty acids (SCFA) locally stabilize T regulatory cells (T_{reg}) in the colon, thereby increasing their abundance (a). T cells traffic from the colon to distant sites [85, 86] where they accumulate and exert their respective functional properties. The inflamed colon ((b); characterized by structural damage—"leaky gut") is frequently accompanied by dysbiosis (red microbes). This qualitative and quantitative shift in bacterial colonization is associated with decreased microbial folate and SCFA production and a consequential relative increase in autoreactive immunogenic T effector cells (T_{eff}) [87]. T_{eff} have been shown to traffic from the colon to target sites of autoimmunity [85, 86] (e.g., intraocular tissue in the case of autoimmune uveitis), skewing the ratio of immunogenic (T_{eff}) to regulatory cells (T_{reg}) at target sites [88–90], ultimately breaching ocular immune privilege (IP) through unknown mechanisms, and thereby triggering autoimmune disease. Due to impaired intestinal barrier integrity in dysbiosis, pathogenic bacterial/viral/fungal/environmental antigens have facilitated access to the circulation, possibly triggering inflammation through adjuvant effects at affected sites (following antigen presentation; APC) (b). In the case of uveitis, we propose that when a sufficiently high T_{eff} precursor frequency is generated [91], activated T cells access retinal tissue where they adopt a pathogenic phenotype upon further activation by retinal self-antigen and/or microbial antigen [92].

and fish. Host metabolic pathways of tryptophan include the serotonin and kynurenine routes, the latter of which via indoleamine 2,3 dioxygenase (IDO) is a major tolerizing pathway in DC and macrophages. Its downstream products such as kynurenic acid (KA), 3-hydroxy-anthranilic acid (HAA), quinolinic acid (QA), and niacin (vitamin B3) suppress both innate and adaptive immunity and promote immunological tolerance and gut homeostasis (reviewed by [68]). Tryptophan can also be metabolized by microbiota-generating metabolites that interact with the aryl hydrocarbon receptor [69]. The IDO pathway and the AhR system are active in many cell types and important in homeostasis, e.g., in epithelial health. In immune cells, it mediates tolerance and suppresses inflammation via DC-mediated induction of T_{reg}.

4.2. Retinoic Acid. Induction of T_{reg} in the gut may also require supplementation of dietary vitamin A (retinol) which is directly converted to bioavailable all *trans*-retinoic acid (atRA) by gut-associated lymphoid tissue DC [70] and in both mice and humans promotes conversion of naïve T cells into tissue-specific (mucosa homing) FoxP3$^+$$T_{reg}$ through FoxP3 promoter histone acetylation [71]. Moreover, atRA prevents the IL6-induced conversion of T_{reg} into Th17 cells and boosts the generation of TGFβ-induced T_{reg} in vitro that were effective in suppressing inflammation in a colitis model [72]. Similarly, atRA stabilized T_{reg} in an experimental autoimmune encephalitis model (EAE) through a TGFβ-dependent pathway [73], and in an experimental autoimmune uveitis (EAU) model, atRA acted as an adjuvant to induce antigen-specific type 1 T_{reg} (Tr1) attenuating autoimmunity [74].

4.3. SCFA. SCFA produced by the microbiome are major effectors of immunomodulation. Three main SCFA are recognized: acetate, butyrate, and propionate. Acetate accounts for ~50-70%, propionate ~20%, and butyrate, which is selectively restricted to certain *Clostridia* species, makes up the remainder [75]. SCFA regulates intestinal T_{reg} and macrophages, and the majority of SCFA produced remain in the gut generating beneficial effects locally, while only negligible quantities escape into the general circulation [76]. Dysbiosis in IBD (colitis) patients is typically associated with a reduced number of bacteria that produce SCFA particularly butyrate and propionate (Figure 1(b)). These include *Firmicutes* such as cluster IV *Clostridia* next to *Bifidobacteria*. Propionate and butyrate suppress inflammation by promoting the generation of tolDC and T_{reg} (reviewed in [77]). In germ-free mice treated with antibiotics, stool butyrate concentrations were decreased relative to littermate mice [78]. Oral acetate supplementation in NOD mice reduced autoreactive T cells in lymphoid tissues in a B cell-mediated fashion. A butyrate-rich diet increased T_{reg}, while a combination of both SCFA improved gut barrier function and decreased diabetogenic IL21 in serum of NOD mice [79]. Colonic concentrations of SCFA, including butyrate, correlated with the number of FoxP3$^+$ T_{reg} in the caecum of mice [62]. Furthermore, oral administration of butyrate to mice increased the FoxP3 expression in T_{reg}, higher numbers of T_{reg} in mucosal tissues, and an enhanced ability of DC to induce T_{reg} differentiation.

These data suggest that butyrate (and to a lesser extent propionate) promotes extrathymic differentiation of T_{reg} [78]. Recent findings from human trials investigating AD (MS and neuromyelitis optica) support this hypothesis [80, 81].

Vitamin B3 (niacin) also exerts immune-modulatory functions by increasing T_{reg} cell numbers and functioning [82, 83] together with butyrate through activation of its receptor Gpr109a, thereby protecting against colon inflammation [83].

Mechanistically, SCFA act by inhibiting histone deacetylases (HDAC) [78], in antigen-presenting cells affecting atRA and IL10 production [93]. HDAC also induce apoptosis in T_{eff} cells [94] and engage in loosening of chromatin, thus enabling transcription factor accessibility to the DNA backbone. These findings suggest a balancing effect of SCFA on mucosal and systemic immunity, possibly affecting inflammation in secondary organs, since mucosal inflammation is typically associated with epithelial damage ("leaky gut"). Fukuda et al. [95] demonstrated that acetate (produced by *Bifidobacteria* and *Clostridia*) may improve leaky gut by restoring gut epithelial integrity through activation of the inflammasome and IL18 [96].

4.4. Folate. Folate is synthesized de novo from phosphoenolpyruvate and guanosine triphosphate (GTP) and secreted by commensals of the phylum *Bacteroidetes* (*Prevotella*, *Bacteroides*, *Porphyromonas* [97]) (reviewed in [98]) (Figure 2). In contrast to dietary vitamins that are mostly absorbed in the small intestine, microbial folate metabolites are mainly absorbed in the colon where they are produced [99, 100] and assimilated into host tissues [100–102]. Various glutamylation profiles for commensal gut microbes (i.e., species-specific patterns of folate derivatives) may affect folate bioavailability in the intestine [84] and the general circulation [67], and the colon is recognized as a significant folate depot [19]. Folic acid deficiency is associated with disruptions of intestinal integrity and persistent diarrhea (reviewed in [103]). A folate-producing microbiome likely influences the T cell methylome, but the mechanism is unclear [94]. Rats fed a probiotic formulation of folate-producing *Bifidobacteria* exhibited increased plasma folate levels, confirming in vivo production and absorption of the vitamin. The same supplement when administered to humans raised folate concentration in feces (reviewed in [67]). A sufficient folate status therefore is likely to reduce the risk of AD/AID including uveitis.

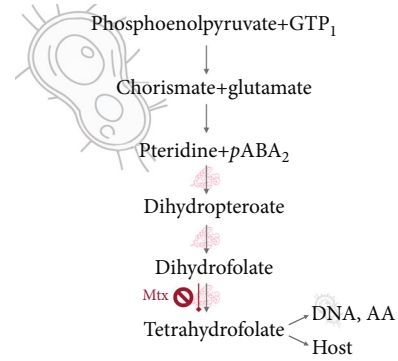

Figure 2: Bacterial route of de novo folic acid synthesis. Certain commensals of the human colonic flora produce folate de novo through the chorismate pathway (from phosphoenolpyruvate + guanosine triphosphate, GTP)$_1$. Further, 77% of the bacterial genome is capable of synthesizing folate using freely available *p*-aminobenzoic acid (*p*ABA) and dihydropteroate diphosphate (pteridine)$_2$ [84]. The pathway engages a series of enzymatic reactions (red protein symbols) including dihydropteroate synthetase and dihydrofolate synthetase. The resulting dihydrofolate (via the dihydropteroate intermediate) must be enzymatically reduced (through dihydrofolate reductase) to generate biologically active tetrahydrofolate. This process can be blocked by the folate antagonist methotrexate (Mtx), used for controlling some forms of autoimmune disease including anterior uveitis. Folate metabolites synthesized by commensals are used by the bacteria themselves (e.g., for DNA-synthesis or anabolic pathways such as generation of amino acids, AA). The remaining unused folates are released into the gut lumen and absorbed in a receptor-mediated fashion absorption into the circulation.

5. Microbiome-Mediated Immune Tolerance Is Maintained through Regulatory and Anergic T Cells under the Influence of Folate

Deletion, anergy, and induction of T_{reg} are the tenets of immune tolerance. T_{reg} are generated centrally in the thymus de novo (natural T_{reg}, nT_{reg}) and in the periphery (pT_{reg}) from conventional T cells (T_{conv}). Most autoreactive T_{conv} are deleted in the thymus or periphery but a proportion may enter a state of anergy (T_{an}) as they become tolerized [104–107]. pT_{reg} in the gut are recognized as major contributors to immune homeostasis and generated in response to tryptophan metabolites or SCFA secreted by the microbiome.

Less is known about the role of microbiome-generated folate in colonic T_{reg} formation. However, an important property of pT_{reg} is the high expression of the folate receptor, FR4 [65]. In addition, a developmental relationship between T_{reg} and T_{an} has been suggested [105, 106, 108, 109]. Both cell types have some overlapping features such as expression of the folate receptor FR4 [108]. Moreover, on adoptive transfer, anergic FoxP3$^-$ CD44^{hi} CD73^{hi} FR4^{hi} Nrp1$^+$ cells gave rise to FoxP3$^+$$T_{reg}$ in an autoimmune arthritis model and reduced the susceptibility of mice to IBD [110], through acting as progenitors for T_{reg} cell differentiation. Both T_{reg} and T_{an} rely on similar tightly regulated epigenetic programs to retain function [105, 109, 110]. nT_{reg} contain highly methylated CpG-rich regions in the conserved noncoding sequence 2 (CNS2) of the FoxP3 locus ([111, 112]; reviewed in [113]) since pT_{reg} are induced in the periphery, this level of methylation is lost allowing stable FoxP3 expression (summarized in [114]). Thus, systemic folate may be more important in the generation of nT_{reg} rather than stable pT_{reg} as exist in the colon. However, the increased number of total methylation sites in T_{an} in the periphery [115] probably allows a necessary degree of instability to permit interconversion of T_{an} and pT_{reg}. This points towards a potential indirect T_{reg} replenishing effect of folate through epigenetic modifications in T_{an}. Microbiome-derived folate might thus

generate a pool of T_{an} from T_{conv}, which have the option of losing their methylation sites and becoming stable T_{reg}. This degree of flexibility underpins the properties of immunological tolerance.

The mechanism whereby folate modifies T_{reg} appears to be through inhibiting cell death specifically by induction of Bcl-2. Adoptive transfer of T_{reg}-depleted cell suspensions induced autoimmune gastritis in susceptible nude mice [65] while adoptive transfer of folate-supplemented T_{reg} prolonged the cells' survival and protected the mice from the disease. Mice treated with the folate antagonist methotrexate (Mtx) show impaired survival of T_{reg} and decreased expression of Bcl-2, while in vivo depletion of dietary folate resulted in a reduction in T_{reg} cell numbers in the small intestine. In this study, folate was required for the survival of differentiated nT_{reg}, but not for the conversion of naïve T cells into pT_{reg} [66]. Remarkably, this effect is different from that of atRA, and less so vitamin D3, which both enhance the differentiation of naïve T cells into pT_{reg} [72, 116–119], emphasizing a unique role for folate in the generation of nT_{reg} [113]. This selective effect of folate on maintenance of FoxP3$^+$ T_{reg} has been further demonstrated [64], while a diet deficient in folate resulted in a marked reduction of FoxP3$^+$ T_{reg}, but not other T cell populations, in the colon. In the same study, blockade of FR4 and treatment with Mtx, led to decreased colonic FoxP3$^+$ T_{reg} and increased autoimmune bowel inflammation. These data have implications for human biology but remain to be verified in man, particularly as T_{reg} exhibit some degree of phenotypical variation between mice and humans [120, 121].

6. FoxP3 in T Regulatory Cells Controls the Expression of the Folate Receptor

At a molecular level, folate stabilizes overall cell proliferation, controls DNA modification (histone methylation), and metabolically detoxifies the prooxidative AA intermediate homocysteine, by recycling it to the essential sulphur-rich AA methionine (Figure 3) or alternatively, the semiessential AA cysteine (not depicted in Figure 3).

Folate is delivered to cells through three known routes: (1) via folate receptors (FR)/folate-binding protein (Folbps) [122], (2) the reduced folate carrier, and (3) through the proton-coupled folate transporter [123]. In humans, there are four FR isoforms, namely, α, β, γ, and δ, with tissue-specific expression patterns [122, 124]. Initially, three FR isoforms with greater than 70% homology were identified in humans (i.e., α, β, and γ) and two in mice (i.e., α and β) [125]. The human receptor homologue for murine FRδ (also known as FR4 or folate binding protein 3) is expressed on splenic and thymic lymphocytes [126] and is particularly abundant on both nT_{reg} and pT_{reg}, in both mice and humans [65, 127, 128]. Since FR4 is a glycosyl phosphatidylinositol–anchored protein, adapter molecules may assist the receptor in the maintenance of T_{reg} cell survival [129, 130] but little is known about its precise role. Importantly, based on its T_{reg}-specific expression, FR4 can be used to discriminate T_{reg} from T_{conv} following antigen stimulation [65].

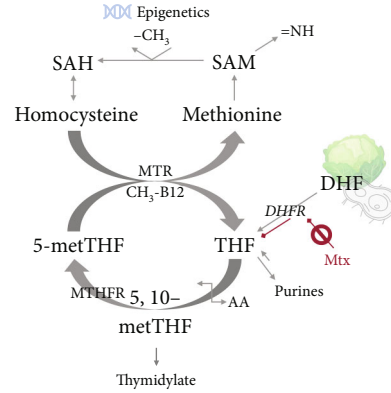

Figure 3: Pathways of folate metabolism and the interrelationships of folate-dependent reactions. Dihydrofolate (DHF) from nutritional sources and the gut microflora is enzymatically reduced engaging dihydrofolate reductase (DHFR) to biologically active tetrahydrofolate (THF), a process that is competitively blocked by the folate analogue methotrexate (Mtx). This has implications for cell proliferation, division, and survival. Folate metabolism branches out into anabolic pathways including synthesis of amino acids (AA) and amines (=NH) as well as purines and thymidylate for DNA production. Importantly, folate in the form of 5-methyl tetrahydrofolate (5-metTHF) serves as a methyl-group (CH_3) donor in the detoxification of proatherogenic homocysteine to the AA methionine. SAM is the universal CH_3 donor in histone- and DNA-methylation. This function gives folate powerful mediating properties at an epigenetic level with a potential role in thymic CD4$^+$ nT_{reg} expansion. Abbreviations: MTR: methionine synthase requiring the co-factor vitamin B12 (cobalamin) as a methyl transfer vehicle (methyl cobalamin, CH_3-B12); MTHFR: 5,10-methylenetetrahydrofolate reductase (requiring the co-factor NADPH, not shown); THF: tetrahydrofolate. Methionine cycle metabolites: SAH: S-adenosylhomocysteine; SAM: S-adenosylmethionine; =NH: amines.

The high folate requirement of murine nT_{reg} is met via upregulation of the FR4 surface expression, under the control of FoxP3 [65], suggesting a tight crosstalk between the transcription factor and receptor expression. Folate may also influence other T_{reg} molecular pathways (summarised in [114]).

7. The Microbiome in Uveitis

Uveitis (intraocular inflammation) is an AD/AID which causes significant blindness and visual handicap worldwide (10–15% in the developed world) [131]. A failure of T_{reg} as an underlying pathogenesis is suggested by the reduced numbers of circulating T_{reg} in patients with uveitis, and since the number of circulating T_{reg} correlates with certain taxa in the colonic microbiome and become stabilized in vivo by bacterial metabolites ([61, 63] see above), this supports a role for a dysregulated microbiome in uveitis. Uveitis occurs in two broad forms, anterior uveitis involving the iris and ciliary body and is closely linked to ankylosing spondylitis (AS) in many cases, and posterior uveitis involving the retina which is protected by the blood retinal barrier (BRB). Both forms of uveitis are subject to changes in the microbiome,

particularly anterior uveitis, in conjunction with AS and IBD [132]. Specific autoantigens for human uveitis have been intensively sought but not identified (reviewed in [7]).

Recently, an experimental model of spontaneous uveitis (experimental autoimmune uveoretinitis, EAU) in a transgenic TCR mouse with specificity for a retinal protein (potential autoantigen: interphotoreceptor retinol binding protein, IRBP), in which the mice next to uveitis also develop dysbiosis, has been described. It was suggested that the pathogenic antigen was an unidentified commensal protein which was crossreactive with the IRBP-TCR and, due to the loss of colonic IP (leaky gut), bacterial forms translocated across the gut wall and activated T cells in the gut draining lymph node. Included in this T cell population were autoreactive IRBP-specific T cells which in this mouse model are in increased frequency (~20%) [133]. Once activated, circulating T cells crossed the BRB and were further activated on contact with cognate antigen in the retina causing uveitis and retinal damage. The definitive proof-of-principle experiment was that no uveitis occurred in germ free IRBP-TCR specific mice, i.e., animals lacking a microbiome. Whether the commensal antigen translocated freely in lymphatics or was carried as cargo by trafficking antigen presenting cells is not clear, but trafficking of leukocytes to and from the gut occurs in both health and disease [85, 86], emphasizing a tight immunological crosstalk between the intestine and extraintestinal tissues. While an interesting hypothesis, a similar prevention of EAU was shown in germ free mice [26, 134] in which EAU was induced using a standard procedure of IRBP peptide emulsified in Complete Freund's Adjuvant (CFA) [135]. In this model, disease is induced by a specific antigen in IRBP-specific T cells, in which the precursor frequency of antigen-specific T cells is vanishingly low. In this case, the effect of the microbiome on the induction of uveitis is more likely to be indirect. In another model, in which EAU develops spontaneously due to lymphopenia and imbalance in [T_{eff}:T_{reg}] ratio, we have shown that disease can be prevented by adoptive transfer of antigen-experienced T_{reg}, but not by naïve T_{reg}. Furthermore, there was evidence of T_{an} to T_{reg} conversion [90].

It is therefore relatively unexplained how the microbiome influences susceptibility to uveitis and in the context of this review, what might be the role of folate? Recent studies (reviewed in [136]) proposed that EAU in mice might be mediated through epigenetic changes possibly involving Tbx21 and Rorc—two transcription factors important for the differentiation of T_{reg} and Th17 cells [137]. Interestingly, hypomethylation of these factors (along with FoxP3) was found in the retinas and RPE-choroidal tissues of B10.RIII mice developing CFA-induced EAU after IRBP immunization, together with an increase in proinflammatory IFNγ and IL17 and reduced DNA-methyltransferase 1 (DNMT1) expression in these tissues corresponding to the genes' methylation status ([138]; reviewed in [136]). These findings highlight a requirement for folate to modulate inflammation at an epigenetic level in the prevention of AD and although not stated in that paper, may be linked to the interconversion of T_{an} to generate stable T_{reg}, all under the control of FoxP3. In a separate study, upregulation of miRNA-223 was detected in IRBP-specific Th17 cells from an induced EAU mouse model [139] as well as in uveitis patients' sera [140]. The latter study revealed a pattern of six miRNAs that were linked to inflammatory signalling cascades, such as MAPK, FOXO, and VEGF. Of those miRNAs highlighted, miRNA-223 stood out, as it not only promoted an inflammatory response through activation of DC and T cells but also hinted at a dysbiotic microbiome [141–143] with reduced colonic folate synthesis/bioavailability. Interestingly, hyperhomocysteinaemia, and its underlying polymorphisms in folate metabolism-associated genes [144], occurs in autoinflammatory (Behçet's) uveitis patients [145] indicating a link for FA in noninfectious uveitis [146].

It is clear thus that in the model of autoimmune uveitis, both experimental and clinical there is a strong association with dysbiosis and dysregulated folate metabolism. There is also a clear deficiency in the T_{reg} function and/or numbers. Since folate is required for T_{reg} physiology [65] the link between folate, T_{reg} and autoimmune uveitis speaks for itself [64, 66, 77]. We propose that folate deficiency as part of a dysfunctional microbiome is part of the backdrop to autoimmune uveitis and probably other AD/AID.

8. The Microbiome and Its Metabolites as Therapeutic Intervention

There is much interest in potential therapeutic modulation of AD using microbiome-based metabolites including fecal microbial transplantation (FMT), SCFA, folate, and probiotics.

8.1. FMT. FMT has been proposed for treatment of AD but it is as yet unclear whether this approach may have a beneficial or deleterious effect [147]. FMT from patients with autoimmune Vogt-Kayanagi-Harada disease (VKH) exacerbated EAU in mice [148]. To date, there are no studies of FMT in uveitis patients.

8.2. SCFA. SCFA have been shown to be reduced in patients with RA and in mice with experimental arthritis and interestingly, treatment of such mice with SCFA induced upregulation of the AhR in regulatory B cells [149]. SCFA such as butyrate and propionate have also been effective in reducing inflammation in experimental models including in EAU [86] and endotoxin-induced uveitis [150] but to date have not been translated to clinical use in uveitis. However, the SCFA propionate has been trialed in patients with MS, and a significant shift in the balance towards T_{reg} vs Th1/Th17 cells was observed [151].

8.3. Folate. Folate and folate supplementation have also been proposed for therapy of AD. In a focal model of EAE, a novel folate-aminopterin construct (EC2319) was found to be tolerated and provided anti-inflammatory benefit by suppressing $CD68^+$ macrophage activity [152]. Similarly, a novel FR-targeted drug EC0746 was found to be effective in the treatment of EAE and EAU [153]. The folate receptor FRβ is expressed on activated macrophages and has been

suggested as a target in AD including RA [154]. However, we suggest here that the preferential expression of FR4 on T_{reg} promotes their expansion, particularly of colonic T_{reg}, which then have the ability to suppress macrophage activity.

8.4. Probiotics. Delivery of dietary folate to supplement microbiome-generated folate is also a promising approach and may be incorporated in probiotics [155] in combination with prebiotics [156]. Folate-producing lactic acid bacilli, *Streptococcus (Strep.) thermophilus CRL 808 and Strep. thermophilus CRL 415*, have been shown to prevent intestinal inflammation in experimental models and proposed for the treatment of dysbiosis [157]. Probiotics have been proven to prevent EAU in mice, and delivery of folate-producing probiotics offers a safe and tolerable supplement in the treatment of AD [158].

The mechanism of action of folate is distinctly different from other known vitamin-based immunomodulators such as vitamin A/atRA and D, as well as SCFA. While atRA, cholecalciferol (vitamin D3) and SCFA had been found to enhance the peripheral differentiation of naïve T cells into pT_{reg} [72, 116–118], and folate is required during clonal expansion of nT_{reg} [66]. Hence, folate exerts its modulatory effects at two levels in vivo, namely, (1) as a mediator of epigenetic control in thymic nT_{reg}; and (2) as an antiapoptotic signal in induced pT_{reg} supporting their survival in the circulation. As adoptive transfer of antigen-experienced T_{reg} prevents development of EAU [88, 90], it would be interesting to see whether folate-treated antigen-experienced (or even naïve) T_{reg} were more effective in control of AD. This could be combined with SCFA to maximize the differentiation of naïve T cells to T_{reg}. Complementary effects of the two metabolites are likely based on their different modes of action.

9. Folate Deficiency and Current Therapy for AD

Folate deficiency has strong implications for overall health and may also complicate the management of AD. Methotrexate (Mtx; amethopterin) is routinely used to manage a range of AD including certain types of uveitis. As indicated above (see Figure 2), the drug's effectiveness and toxicity vary among individuals and are likely determined by polymorphisms in folate, pyrimidine, and purine metabolic enzymes [159]. The mechanism of action is presumed to be inhibition of T cell proliferation but overall, the efficacy of Mtx in uveitis is limited [160–163]. This may be due to the drug's competitive antifolate effects. Macrophages are major agents of tissue destruction in AD including uveitis [164, 165] and require high amounts of folate to remain active via high surface expression of the folate receptor FRβ [166, 167]. Mtx is structurally similar to folate with 1000-fold higher affinity for the enzyme dihydrofolate reductase (DHFR) [168, 169] (Figure 3). It thus starves cells with high folate requirement such as activated macrophages, thereby abrogating the cells' survival and halting disease progression. While this might be of benefit to control tissue-damaging macrophages in noninfectious uveitis [170], there may be a negative side-effect on the T_{reg} function. Thus, the action of Mtx is likely to be rather complex having both antiproliferative and anti-inflammatory roles and targeting activated macrophages as well as T_{reg}. In the event, Mtx seems to be more effective in acute AU (with significant myeloid involvement) than in sight-threatening chronic PU (with Th1 and Th17 T_{eff} cells being the main drivers of disease).

An alternative mechanism for the immunosuppressive effect of Mtx and other immunosuppressants has been proposed: in humans, hypomethylation of the TSDR (T_{reg}-specific demethylated region) is required for the functional stability of peripherally expanding FoxP3$^+$ pT_{reg} [171] and correlates with the duration of oral immunosuppressive therapy. This indicates that in patients, conventional immunosuppression can induce pT_{reg} leading to remission of the disease (reviewed in [172]). This finding is particularly interesting with regard to folate being a mediator of epigenetic programming (Figure 3) and emphasizes the importance of coordinating appropriate treatment regimens with the dynamics and kinetics of disease progression.

10. Conclusions

Folate as one of the colonic bacterial fermentation products is a powerful micronutrient with a broad spectrum of well-known functions at various levels. Its importance in ocular health is well established [144–146, 173–179], but its immunomodulatory properties represent an emerging concept for functional T_{reg} stabilization. The colon is a significant folate depot [19] that participates in metabolism and contributes to bioavailable folate levels. It is an important immunological organ with "unconventional IP" properties [50] with a core function in oral tolerance [180] and prevention of microbial antigen escape into the general circulation. Pathological structural ("leaky gut") and/or environment-induced proinflammatory changes (dysbiosis) in the gut therefore jeopardize this role, allowing for the transmigration of commensal antigen into the blood stream. The use of folate- and SCFA-producing microbes has the potential to form the basis for a novel approach to prophylactic control of AD. While emerging data suggests oral probiotics [158, 181] or alternatively FMT [182] might help eliminate dysbiosis, knock-on effects on colonic folate synthesis/bioavailability and its implications for immune cell functioning remain to be explored. As dysbiosis has been found to occur in a range of AD, including uveitis [36, 183, 184], targeted administration of certain beneficial folate-producing bacteria or even direct oral folate treatment in AD merits clinical evaluation as a low-risk effective adjunctive treatment option. A daily oral folate supplementation of 5,000–10,000 μg (i.e., 25–50 times the daily recommendation) is generally well tolerated by healthy, nonpregnant individuals. Neurological side-effects have been reported in cases of pernicious anemia (B12 hypovitaminosis), and interference with intestinal zinc absorption has been demonstrated in animals which is likely irrelevant in humans (reviewed in [185]). Some evidence suggests that long-term folic acid supplementation can promote the progression of preexisting malignant lesions in advanced

age [186]. Importantly, as T_{reg} phenotypes between mice and humans vary to some degree [120, 121], research is needed to clarify those differences and assess whether those AD-attenuating folate effects observed in mice are equally valid in humans. Regardless, folate is an important and undervalued micronutrient with powerful direct and indirect effects in the organism and a potential regulatory role in autoimmunity and chronic inflammation.

Data Availability

Original data will be made available upon request.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

CM researched, collated, and summarized available literature, wrote the main body of the paper, created the figures, and edited/formatted the manuscript. JVF and HMW assisted with the conceptualization, writing and revising the text. LK and JVF delivered clinical insight. HMW, LK, and JVF provided expert opinion, edited and critically revised the article.

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Review Article

The Immunomodulatory Effect of the Gut Microbiota in Kidney Disease

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The human gut microbiota is a complex cluster composed of 100 trillion microorganisms, which holds a symbiotic relationship with the host under normal circumstances. Intestinal flora can facilitate the treatment of human metabolic dysfunctions and interact with the intestinal tract, which could influence intestinal tolerance, immunity, and sensitivity to inflammation. In recent years, significant interests have evolved on the association of intestinal microbiota and kidney diseases within the academic circle. Abnormal changes in intestinal microbiota, known as dysbiosis, can affect the integrity of the intestinal barrier, resulting in the bacterial translocation, production, and accumulation of dysbiotic gut-derived metabolites, such as urea, indoxyl sulfate (IS), and p-cresyl sulfate (PCS). These processes lead to the abnormal activation of immune cells; overproduction of antibodies, immune complexes, and inflammatory factors; and inflammatory cell infiltration that can directly or indirectly cause damage to the renal parenchyma. The aim of this review is to summarize the role of intestinal flora in the development and progression of several renal diseases, such as lupus nephritis, chronic kidney disease, diabetic nephropathy, and renal ischemia-reperfusion injury. Further research on these mechanisms should provide insights into the therapeutic potential of regulating intestinal flora and intervening related molecular targets for the abovementioned nephropathy.

1. Introduction

Kidney disease is a general term for renal heterogeneous disorders affecting the kidney structure and function, which is a dominant contributor to global morbidity and mortality [1]. Although it has been increasingly identified as a significant public health problem worldwide with increasing prevalence and poor outcomes, clinical diagnosis and therapeutic interventions are lagging. Nowadays, most therapeutic methods are limited to lowering blood pressure, controlling blood glucose, and reducing proteinuria [2]. Current studies are aimed at developing more effective therapeutic strategies to prevent the progression of renal diseases. Over the past years, our understanding of the composition and function of the gut microbiota has been expanded, mainly on account of the evolution and advances of modern molecular techniques. Developing research studies on gut microbiota have shown that this formerly "neglected organ" plays a significant role in many diseases within and beyond the intestinal tract. Substantial differences of the gut microbiota composition, immunogenicity,

and metabolic activity have been observed by comparing healthy individuals to patients presenting with different types of kidney diseases [3–6] and other noncommunicable illnesses, such as diabetes mellitus, obesity, atherosclerotic cardiovascular disease, heart failure, and liver diseases [7–11]. These changes of the gut microbiota are causally correlated with disease phenotypes, complications, and outcomes according to experimental studies in animals and humans [7, 12, 13].

The human gut microbiota, also known as the intestinal flora, is composed of ~100 trillion microorganisms constituted by a broad spectrum of over 500 genera of bacteria from two main phyla, namely, *Bacteroidetes* and *Firmicutes*. Generally, the diversity and abundance of the intestinal microbiota differ along the intestinal tract and maintain a dynamic balance. Known as the body's "second brain," the intestinal microbiota plays a major role in the absorption and metabolism of nutrients, hormone secretion, and toxin degradation, which enable it to control the human intestinal homeostasis and even the whole internal environment. The SCFAs including acetate, propionate, and butyrate are predominant final products of the distal gut microbiome, fermented from a variety of plant polysaccharides produced under anaerobic conditions [14]. SCFAs can provide about 10% of the caloric needs for the human body [15] and enhance the barrier function by regulating the retinol production and mediating the secretion of mucin and IgA [16]. In addition, SCFAs come into the blood circulation and then exert their systemic effects such as increasing anti-inflammatory factors, downregulating autoimmunity-related factors, and developing regulatory T (Treg) cells [17–20] via the G protein-coupled receptors (e.g., GPR41, GPR43, and GPR109A). Furthermore, accumulating pieces of evidence have reported the positive effects of SCFAs in treating kidney problems caused by several diseases [13, 21–24].

In addition to the metabolic function, the gut microbiota performs some basic roles to promote the maturation of intestinal immunity [25–28] and maintain the integrity of the intestinal epithelial barrier to prevent the invasion and colonization of pathogenic microorganisms [29]. The intestinal epithelial barrier is essential for intestinal homeostasis, which enables the bilateral passage of vast metabolites and immune signals and simultaneously obstructs the passage of the pathogenic bacteria, toxic metabolites, and microbial byproducts [30–32]. To counterpoise these apparently contradictory roles [33, 34], the epithelial cells and immune cells closely interact with each other, establishing the first line of protection against invading pathogens. Its mechanism of action is the recruitment of phagocytes or direct bacterial prevention and killing by releasing chemokines, cytokines, AMPs, and other soluble molecules [35–38]. The intestinal immune system is built up and matures with the participation of the gut microbiota. Microfold cells (M cells) in the epithelium capture lumen contents and deliver them to the underlying antigen-presenting cells, such as macrophages and dendritic cells (DCs) [39]. Once the pattern recognition receptors of the DCs bind to the pathogenic microorganisms, the stimulated DCs that process and present the antigens express costimulatory molecules and cellular factors. These processes contribute to the regulating helper T (Th) cells, such as Th1, Th2, and Th17, and immunosuppressive Treg cells differentiated from naive $CD4^+$ T cells, maintaining the Treg/Th17 balance and the immune homeostasis [14, 40–42] (Figure 1). Intestinal flora imbalance induces the activation of immune cells through this pathway, secreting a large amount of proinflammatory factors (e.g., IL-4, IL-5, IL-6, and interferon-γ (IFN-γ)), which results in immune dysregulation and inflammation. The intestinal microbiome promotes the differentiation of IgA-secreting plasma cells by activating a proliferation-inducing ligand (APRIL) receptor and B cell-activating factor (BAFF) in DCs. sIgA has a regulatory effect on intestinal microorganisms. Bacterial metabolites, such as SCFAs, histamine, spermine, and taurine, can also influence the host's immune homeostasis [43].

As a contributing factor and indicator of human health, the gut microbiota plays an important role in the prevention, diagnosis, and treatment of many human diseases. Although a dynamic balance is established between intestinal flora, host, and external environment, it is susceptible to changes caused by age, diet, antibacterial drugs, psychological pressure, and other factors, resulting in an imbalance of intestinal flora [44, 45]. Once this microecological balance of intestinal flora is destroyed, known as dysbiosis, it will lead to a variety of gastrointestinal and systemic diseases (Figure 1). Microbial dysbiosis promotes the production of bacteria-produced uremic toxins, such as IS, p-cresyl sulfate (PCS), and trimethylamine N-oxide (TMAO). These metabolites translocate into the circulation through the impaired intestinal barrier, and most of which are excreted by the kidneys, where their retention would lead to kidney dysfunction [46]. In addition to dysbiosis, the increased permeability and structural damage of the intestinal barrier result in the translocation of pathogenic bacteria and their byproducts which is a vital step leading to local or systemic inflammation [47–50], affecting various organs, including the kidneys [51, 52].

Taken together, the colonization of intestinal microorganisms is a double-edged sword for the host. The healthy microbial community plays an indispensable role in the host's nutrient absorption and metabolism, the maturation of intestinal immunity, the maintenance of the integrity of the intestinal epithelial barrier, and the prevention of colonization by pathogenic microorganisms. These are what the microbiome has contributed to the overall health of the host. However, changes in the intestinal flora can cause diseases of different organs and exacerbate existing diseases. This review summarizes the current understanding of the role of intestinal flora in the occurrence and development of kidney disease, focusing on select components of the immune system that have been shown to drive the pathogenesis of each kidney disease. Further research on the association between the immune system and the gut microbiota may contribute to the understanding of the intricate pathogenesis of kidney disease. Likewise, the regulation of intestinal flora and the intervention of related molecular targets may have a potential therapeutic utility in the treatment of kidney diseases.

2. Gut Microbiota in Lupus Nephritis

Systemic lupus erythematosus (SLE) is a multisystemic autoimmune disease characterized by lymphocyte overactivation and the production of antinuclear autoantibodies that drive

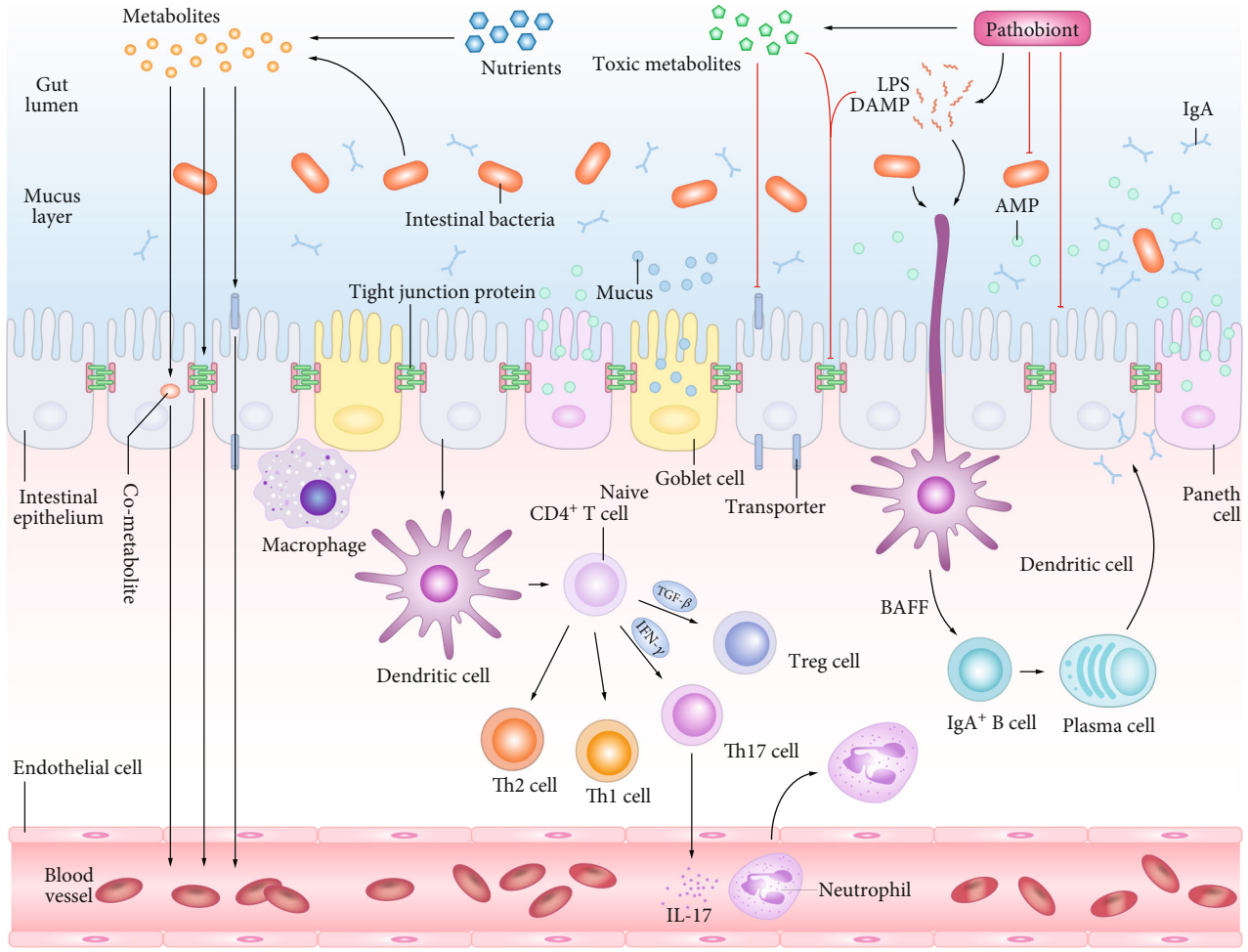

FIGURE 1: The intestinal epithelial barrier allows a large number of metabolites and immune signals to pass in both directions while blocking the pathways of pathogenic bacteria, toxic metabolites, and microbial byproducts. The outermost layer of the intestinal barrier is the mucus layer which is composed of mucin glycoprotein, AMPs, and sIgA, produced by goblet cells, Paneth cells, and plasma cells, respectively, and excludes the microbiome from the epithelial surface. Adjacent cells are linked together by the tight junction protein families that can determine the permeability and prevent mechanical disruption of the epithelial sheet. The intestinal immune system is established and mature with the participation of the gut microbiota. Once bound to luminal antigens, DC pattern recognition receptors express costimulatory molecules and cellular factors involved in regulating Th1, Th2, Th17, and Treg cells differentiated from naive $CD4^+$ T cells, maintaining Treg/Th17 balance and forming immune homeostasis. The intestinal microbiome promotes the differentiation of IgA-secreting plasma cells by activating APRIL (a proliferation-inducing ligand) receptor and B cell-activating factor in DC.

arthritis, glomerulonephritis, and other different inflammatory tissue damage [53]. Approximately 60% of SLE patients are suffering from lupus nephritis (LN), which is one of the leading causes of morbidity and mortality in SLE, resulting in acute or chronic kidney damage through inflammation, deposition of immune complexes, and glomerular or interstitial scarring [54]. Nowadays, the etiological understanding of LN is limited in the genes and environment [55], but the specific causes still remain unclear. In recent years, the alterations of the gut microbiota have been associated with multitudinous autoimmune disorders, and present data has reported the distinctive microbiota composition in the gastrointestinal tract of LN patients [3, 56–61]. Thus, the role of intestinal flora in LN has increasingly attracted the attention of researchers [62].

In the symbiotic condition, intestinal microorganisms can affect gut tolerance, immunity, and sensitivity to inflammation through B cell maturation, Treg/Th17 ratio balance, and anti-inflammatory cytokine secretion. However, the intestinal inflammatory microenvironment in SLE patients may influence intestinal tolerance, exceeding the immunologic reactions, autoimmunity, and damage of tissues/organs in SLE patients. In the pathological circumstances of SLE, chronic inflammation disrupts the intestinal barrier, which is termed as leaky gut [63], and bacterial pathogens are directly exposed to various organs and immune systems of the body. Through the toll-like receptor (TLRs) [64, 65], antigen-presenting cells (APCs, e.g., macrophages and DCs) secrete cytokines to activate the differentiation and proliferation of T cells [14, 40]. Proinflammatory factors, such as IL-6 and IFN-α, are released, which play an important role in inducing B cells to release autoantibodies and cause an imbalance in the Treg/Th17 ratio [66]. A large number of autoantibodies, most of which are anticellular antibodies

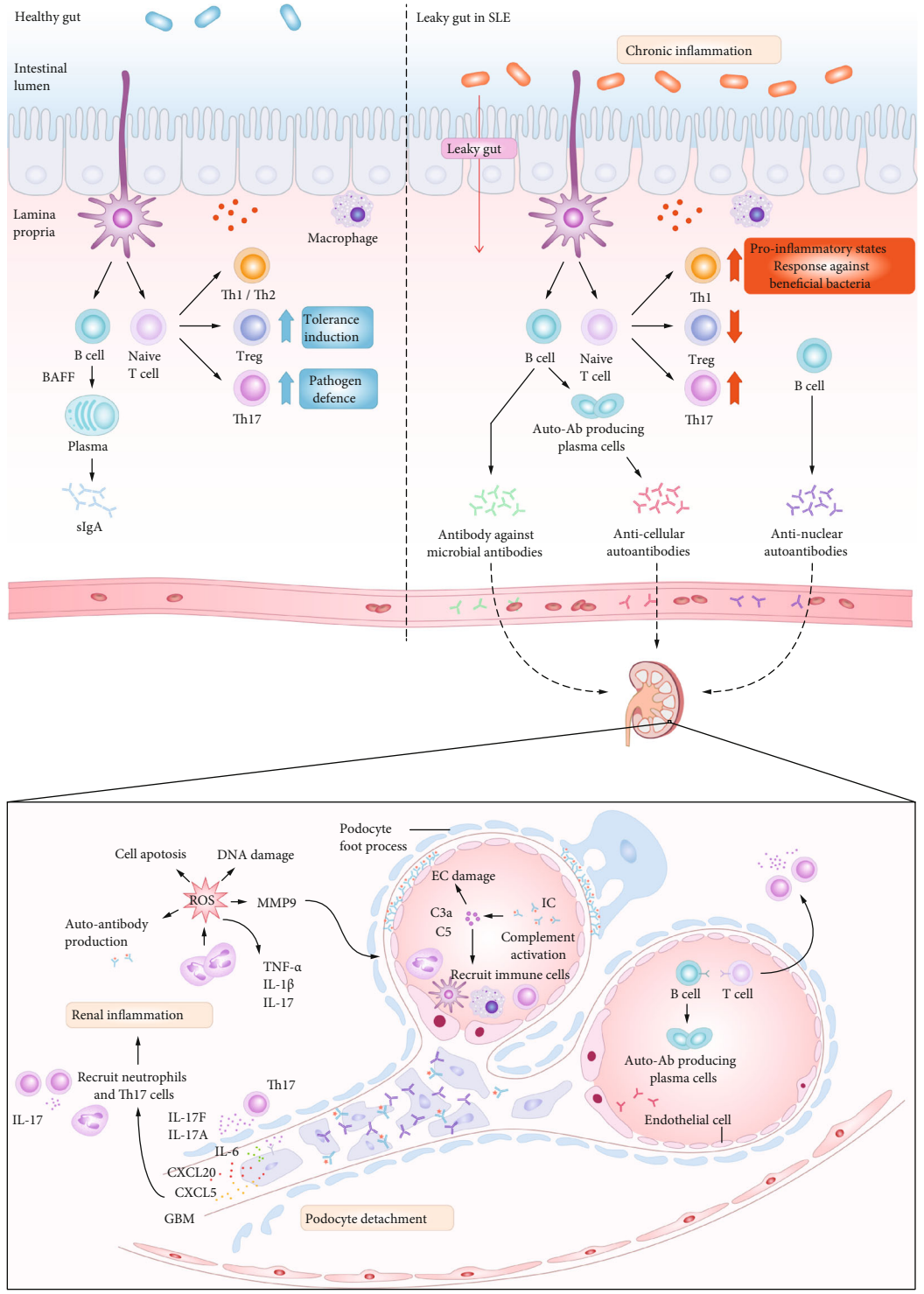

Figure 2: Correlation between intestinal microbiota and the incidence of lupus nephritis. Under the symbiotic condition, intestinal microorganisms can affect gut tolerance, immunity, and sensitivity to inflammation through B cell maturation, Treg/Th17 ratio balance, and anti-inflammatory cytokine secretion. In SLE patients, the intestinal inflammatory atmosphere can induce B cells to release autoantibodies, resulting in an imbalance of the Treg/Th17 ratio, leading to intestinal tolerance disorders, beyond immune response and autoimmunity, and tissue/organ damage (such as lupus arthritis (LN)). A large number of autoantibodies and immune complexes are produced and enter the circulation. The deposition of autoantibodies and immune complexes in the glomeruli leads to the activation of complement components (e.g., C3a and C5), resulting in the endothelial cell or podocyte injury and recruitment of immune cells. Infiltrated Th17 cells in the kidney secrete cytokines IL-17A and IL-17F, which activate mesangial cells and tubular epithelial cells to produce CXCL5 and CCL20, then recruit more Th17 cells and neutrophils through CCR6 and CXCR2, respectively. At the same time, ROS produced by infiltration of immune cells can lead to further renal inflammation and tissue destruction.

and antinuclear antibodies [67–69], combine with ligands to form immune complexes. Moreover, molecular mimicry may be an important link between intestinal microbiota and SLE. Bacteria can express orthologs of human Ro60 autoantigens in the SLE patients' gut. This characteristic would lead to T cell cross-reaction and the production of human anti-Ro60 autoantibodies in SLE patients [70, 71]. Particularly, it was found that *Ruminococcus gnavus* cross-reacts with human DNA, whose relative abundance in the intestinal tract is positively correlated with SLE activity and LN [72].

These changes initiated by the gut microbiota can lead to an acceleration of the process of kidney injury. The deposition of autoantibodies and immune complexes in the glomeruli leads to the activation of complement components, resulting in the injury of endothelial cells or podocytes and the recruitment of immune cells. Some pieces of evidence have reported the essential role of renal resident cells (e.g., podocytes, renal tubular epithelial cells, and glomerular mesangial cells) in the development of LN [73]. Infiltrated Th17 cells in the kidney secrete cytokines IL-17A and IL-17F, which activate the mesangial cells and tubular epithelial cells to produce C-X-C motif chemokine 5 (CXCL5) and chemokine (C-C motif) ligand 20 (CCL20) and then recruit more Th17 and neutrophils through chemokine receptor 6 (CCR6) and CXCR2, respectively. At the same time, reactive oxygen species (ROS) produced by the infiltration of immune cells can lead to further renal inflammation and tissue destruction (Figure 2).

The current treatment for LN includes the administration of high doses of corticosteroids and broad-spectrum immunosuppressants, but treatments are not ideal at present [74]. The study of the interaction between intestinal microflora and LN provides a new idea for the treatment of LN. In experimental studies, the restoration of the composition of intestinal flora through the administration of acidic water [67], vitamin A [61], probiotics [3, 75, 76], or prebiotics can moderate the inflammatory status and possibly favor renal protection in SLE models. Rodgers et al. demonstrated the renal protective potential of drug-like analogs of ES-62, which is a type of phosphorylated cholinergic glycoprotein secreted by *Acanthocheilonema viteae* and involved in maintaining the balance of regulatory/effector B cells and desensitized renal effector function [77]. Since limited reports have investigated the impact of the abovementioned treatments in SLE and LN patients, further research is needed to confirm their efficacy in clinical application.

3. Gut Microbiota in Chronic Kidney Disease

Chronic kidney disease (CKD) is a global health issue and is increasingly considered a social burden. More than 10% of the population has been diagnosed with CKD, in which 50% are classified as high-risk subgroups [78]. As a result of progressive renal parenchymal injury, clinical symptoms, such as the reduced glomerular filtration rate, increased urinary protein excretion, reduced synthesis of erythropoietin, and hypertension, can be noticed in patients with CKD.

CKD induces numerous alterations in internal and external factors that potentially alter the microbiota composition. Furthermore, intestinal dysbiosis is closely associated with gut inflammation and intestinal barrier disruption [79, 80]. For instance, dietary changes in CKD patients might contribute to intestinal dysbiosis and the generation of excessive uremic toxins. Urea is produced from amino acids in the urea cycle and is excreted by the kidneys (80%) and the digestive tract (20%). As renal function is impaired in patients with CKD, the digestive tract becomes the main route for urea excretion. Urea in the intestinal lumen could be converted by bacteria to NH_3 or NH_4OH, wherein the formation of which and increased intestinal lumen pH can promote the proliferation of pathogenic microorganisms and destroy the intestinal barrier. Other causes that probably contribute to intestinal barrier disruption in CKD include the use of numerous medications and hypervolemia [81, 82] which lead to uremia, azotemia sympathetic overactivity [83, 84], and intestinal congestion [5]. These processes lead to systemic inflammatory responses through increasing the production of proinflammatory cytokines, activating the nuclear factor-kappa B (NF-κB) pathway, and dysregulating the immune response, thus exacerbating the ecological imbalance [79, 85]. The destruction of the intestinal barrier facilitates the bacterial endotoxin to enter the circulatory system, which is known as endotoxin translocation. Endotoxemia has various effects on systemic inflammation, oxidative stress, cardiac injury, and atherosclerosis [52]. More importantly, endotoxemia is positively correlated with the reduced survival of CKD and hemodialysis patients [52]. The inflammation in CKD involves the endotoxin-induced overactivation of APCs and lymphocytes [86]. However, evidence suggested that host defense against infectious microorganisms is impaired in end-stage renal disease (ESRD) patients [87, 88]. This seemingly paradoxical immune response can be explained by the endotoxin tolerance; that is, persistent innate immune activation induces immune paralysis [89], which contributes to the presence of acquired immunosuppression and systemic inflammation.

In the liver and colon, dysbiotic gut-derived uremic toxins, such as indoles and phenols, are further metabolized into TMAO, IS, and PCS [80, 90]. They enter the circulation through the impaired intestinal barrier and then exert harmful effects on the kidney. IS has the ability to promote the production of ROS in renal tubular epithelial cells; activate NF-κB, p53, and other regulatory factors; and upregulate the expression of chemokines, leading to the aggregation of renal interstitial monocytes/macrophages and finally causing renal fibrosis [91]. IS can also promote the expression of transforming growth factor-β (TGF-β) and accelerate renal function deterioration by activating the renin-angiotensin-aldosterone system (RAAS) [92]. PCS has a proinflammatory effect, which can promote renal interstitial monocyte/macrophage infiltration and upregulate the expression of inflammatory factors, such as IL-6 and TGF-β, thus promoting renal fibrosis [93]. Both IS and PCS can lead to the hypermethylation of the Klotho gene, inhibit Klotho gene expression, weaken the protective effect of its products on the kidneys, and ultimately accelerate renal function deterioration [94]. In the circulation, 100% of the PCS and IS are bound to proteins, which limits their clearance; they could

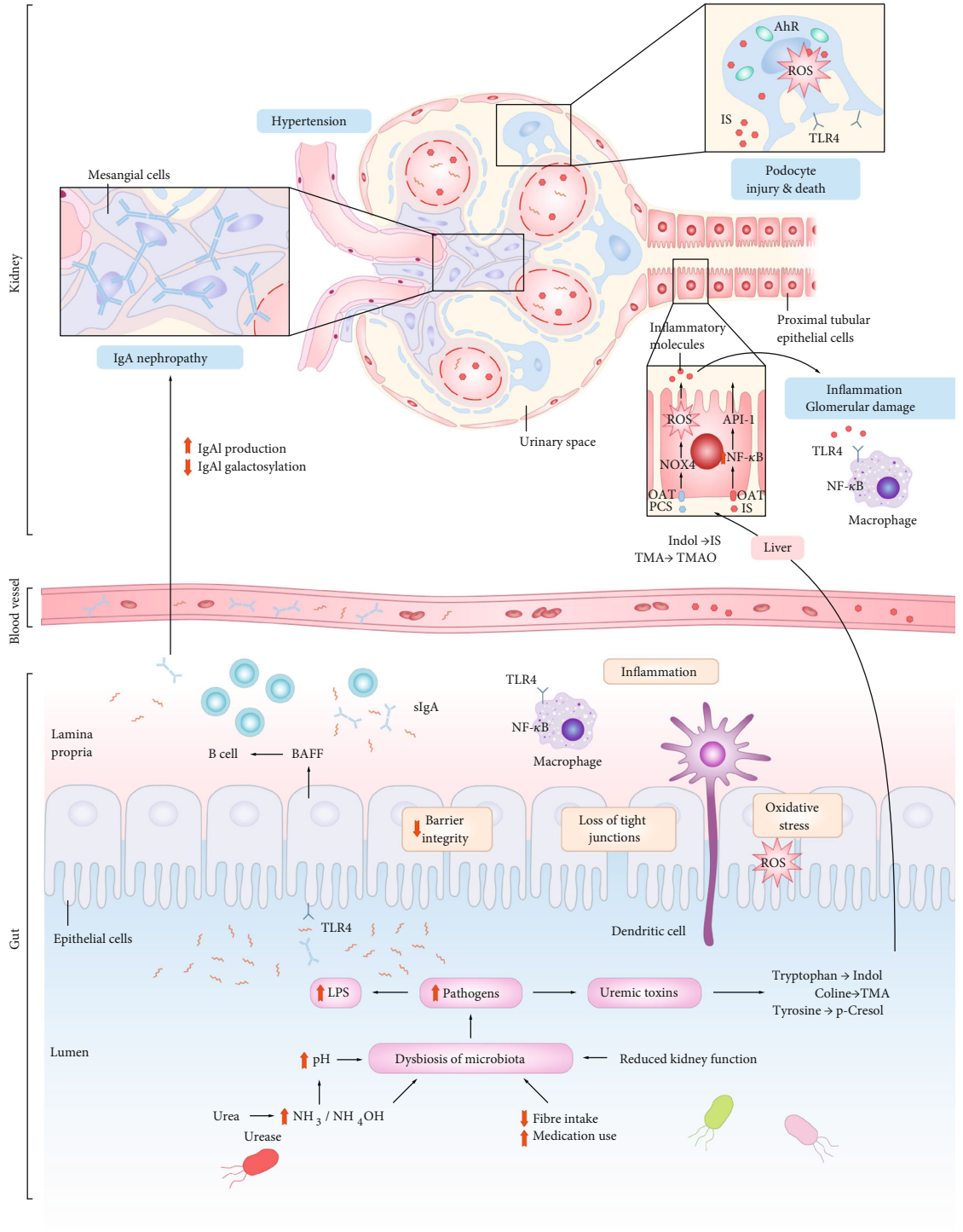

Figure 3: Changes in diet, numerous medication use, and decreased kidney function in CKD patients may lead to intestinal dysbiosis. The digestive tract becomes the main route for urea excretion in CKD patients with impaired renal function. A large amount of NH3 or NH4OH which are produced by bacteria can increase the intestinal pH value, promote the intestinal dysbiosis and destroy intestinal barrier. Thus, the transport of endotoxins (e.g., lipopolysaccharides (LPSs)) into the bloodstream has different effects on systemic inflammation, oxidative stress, cardiac injury, and atherosclerosis. Dysbiotic gut-derived uremic toxins, such as indoles and phenols, are further metabolized into trimethylamine N-oxide (TMAO), indoxyl sulfate (IS), and p-cresyl sulfate (PCS) in the liver and colon. The introduction of uremic toxins into the circulation causes inflammation and tubulointerstitial damage and promotes ROS production, tubulointerstitial damage, and nephrotoxicity of proximal tubuloepithelial cells. Intestinal microbial metabolites SCFAs are associated with hypertension and are important risk factors for CKD. SCFAs trigger hypertension through Olfr78, leading to renin secretion and regulation of peripheral resistance. In addition, bacteria and their components are involved in the hyperproduction and hypogalactosylation of IgA.

not be eliminated by dialysis. However, the binding capacity of proteins as a whole is decreased in patients with CKD [95], augmenting the circulating levels of unbound metabolites. The increased levels of PCS and IS in serum were positively correlated with renal degeneration, nephropathy progression, cardiovascular diseases, and mortality in patients with CKD [51]. Collectively, uremic toxins will trigger inflammation and tubulointerstitial damage and promote ROS production, tubular injury, and renal toxicity in the proximal renal tubular epithelial cells [96]. In addition to the tubules and mesenchymal damage, IS also injures the glomeruli where the podocytes play an important role. Podocytes are highly differentiated cells that are involved in the formation of glomerular filtration membranes and have a limited regenerative capacity [22]. Once the podocytes are damaged, proteinuria and other clinical manifestations of renal disease would occur. The abnormal increase of IS induces AhR activation, which contributes to the progressive impairment of the podocytes and glomeruli [97].

Renal disease is inextricably linked to cardiovascular diseases. Overall, 85%–90% of patients with CKD have hypertension [98], which is an important risk factor for CKD. Emerging studies have demonstrated a strong link between gut microbiota and hypertension in animals and patients [84, 99–103]. Yang et al. compared the fecal microbiome of spontaneously hypertensive rats and angiotensin-induced hypertensive rats. They noticed a prominent dysbiosis characterized by decreasing microbial abundance, variety, and evenness and the increased *Firmicutes*/*Bacteroidetes* ratio in hypertensive rats [103]. Moreover, treatments with antibiotics can lower the blood pressure of patients with treatment-resistant hypertension [102], indicating that the intestinal microbiome plays a role in hypertension pathogenesis and one of the possible causes is increased gut permeability and translocation of bacterial products [84]. Accumulating pieces of evidence suggest that gut-derived SCFAs contribute to the regulation of blood pressure via olfactory receptor 78 (Olfr78) and GPR41 [23, 84, 101, 104, 105]. Specifically, SCFAs trigger hypertension through Olfr78 in the peripheral blood vessels and renal afferent arterioles, leading to the secretion of renin and modulation of peripheral resistance when an intestinal microbial imbalance occurs. By contrast, SCFAs are able to lower blood pressure by binding with GPR41 and GPR43. Moreover, further research on the relationship between the intestinal microflora and the renal-cardiovascular system is helpful in the development of effective treatment methods for hypertension and CKD (Figure 3).

In view of the role of hypertension and other pathogenesis of CKD, the most useful management in the early stage of CKD is the control of blood pressure, along with reducing protein and salt intake to prevent acute renal injury and control blood glucose levels. With the exception of dialysis and kidney transplantation, no effective strategy to cure or prevent ESRD is currently available. Considering this, it could be hypothesized that regulating the intestinal microbiota can lower blood pressure, ameliorate kidney disease, and prevent complications in patients with CKD. Intervening measures (e.g., increasing fiber intake, rational use of antibiotics [106, 107], and therapeutic use of probiotics, prebiotics, and synbiotics) can restore the composition of intestinal flora and inhibit the accumulation of urotoxins in the blood [108, 109]. According to the research of Lakshmanan et al. [110], prebiotic gum acacia (GA) treatment restored the intestinal balance of CKD rats and relieves the inflammation of kidney tissue by increased production of butyrate, as well as its anti-inflammatory and antioxidant capacity. Future studies are needed to improve dialysis techniques to isolate protein-bound uremic toxins and to discuss the feasibility of fecal microflora transplantation.

4. Gut Microbiota in Diabetic Nephropathy

The worldwide prevalence of diabetes is rising rapidly, and it is estimated to increase to 578 million in 2030 [111]. Diabetes increases the risk of multiple complications, such as decreased kidney function and cardiovascular disease [112]. Although only 30%–40% of diabetic patients develop diabetic nephropathy (DN), it is a leading cause of ESRD in most developed countries and a key determinant of survival in people with diabetes [113]. Pathologically, the major changes in the kidney are the deposition of the extracellular matrix (ECM), thickening of the glomerular basement membrane, tubular atrophy, and cellular proliferation that results in interstitial fibrosis and glomerulosclerosis [113]. Accumulating pieces of evidence have revealed that increased ROS and low-grade inflammation, due in part to hyperglycemia, are strongly associated with diabetic complications [114, 115]. These changes lead to kidney damage, such as glomerular hyperfiltration, glomerular hypertension, altered glomerular composition, and hypernephrotrophy. Although the relationship is not clear, most studies believe that dysbiosis is involved in the occurrence and development of diabetes and DN, which may be related to the induction of insulin resistance and long-term chronic inflammation in diabetes.

Studies have investigated intestinal dysbiosis among diabetic patients and nondiabetic individuals [10, 11, 116], and it was found that intestinal dysbiosis is associated with insulin resistance and lipid metabolic disorders [117]. Intestinal dysbiosis itself and abnormal lipid metabolism in diabetes can decrease the expression of connective proteins, resulting in increased intestinal permeability and bacterial translocation. LPSs translocate into the circulation through the dysfunctional barrier and mediate host inflammatory responses through TLR2- and TLR4-related pathways, which is associated with the occurrence and development of many metabolic diseases. Chronic inflammation may also lead to the apoptosis of islet cells and eventually diabetes [7]. He et al. found that probiotics could delay the occurrence and development of diabetes by improving insulin resistance and stabilizing fasting blood glucose (FBG) levels [118]. The abovementioned studies have shown the correlation between intestinal flora and diabetes and found that restoring the gut microbiota is considered to be an effective strategy in preventing and treating diabetes.

In addition to the effect on insulin resistance, the intestinal microbiota may also be closely related to the occurrence and development of renal disease in diabetes through some

other ways. Although magnesium lithospermate B is unable to decrease the FBG levels in STZ mice, the study of Zhao et al. showed that it can improve renal function (decreasing 24 h urinary protein) in diabetic patients by restoring the intestinal microbial composition and regulating the bile acid metabolism [119]. In 2019, one study first discovered a direct association between intestinal flora and DN. Through analyzing the fecal flora composition among diabetic biopsy-proven DN patients and healthy controls, the researchers found that the composition of the gut microbiota of DN patients is different from diabetic patients and healthy controls with several strains, such as *Escherichia-Shigella* [4]. The increased abundance of *Escherichia-Shigella* could penetrate the intestinal barrier and then exacerbate the intestinal leakage [120], which could contribute to the chronic low-grade inflammatory status in diabetic patients [121]. The interaction of bacterial LPS with TLR2 and TLR4 has been shown to be involved in the ongoing inflammatory process of DN by activating NF-κB and inducing the release of proinflammatory cytokines (TNF, IL-1, IL-6, etc.) in an inflammatory cascade that exacerbates renal damage [118]. Moreover, the accumulation of toxic metabolites produced by intestinal microorganisms stimulates the production of ROS through the NADPH pathway, which triggers the NF-κB pathway and induces an inflammatory response, and contributes to proteinuria and podocyte damage. Kikuchi et al. suggested that phenyl sulfate, a type of bacterial toxin, could potentially be an early diagnostic marker and a therapeutic target of DN in the future [122]. The NF-κB pathway is a key point for the progression of inflammation and fibrosis in DN, whose activation can reduce the expression of inflammatory cytokines and fibrosis degree [123].

Recent studies have focused on the relationship between enterogenic products such as SCFAs and DN, which is a hot research field recently. Lu et al. speculated that intestinal microorganisms produce excessive SCFAs, especially acetate, which could bind to the renal Olfr78 receptor, and activate the intrarenal renin-angiotensin system (RAS) [124]. The activation of the RAS has long been regarded as one of the initiators of DN. The kidney is sensitive to angiotensin II (Ang II) which leads to renal vasoconstriction, increased blood pressure, and glomerular hypertension [125]. Moreover, Ang II promotes the morphological changes of podocytes and glomerular endothelial cells, the deposition of the extracellular matrix, and the secretion of inflammatory factors and profibrotic chemokines, accelerating the progress of DN. Furthermore, Hu et al. demonstrated that the acetate produced by the intestinal flora mediates the dysregulation of cholesterol homeostasis by activating GPR43, which leads to the tubulointerstitial injury of DN [126].

Clinical studies have demonstrated that traditional treatments that control glucose levels and inhibit RAS and inflammation could not absolutely prevent the progression of renal damage in DN. Considering this, the gut microbial factors could be involved in the pathogenesis of DN besides the traditional risk factors [127–129]. Modulating the gut microbiota may lead to better glycemic control and favorable outcomes in people with diabetes. Probiotics and prebiotics (e.g., fructooligosaccharides, lactulose, inulin, and resistant starches) are commonly used to regulate the gut flora, and the application of synbiotics and probiotics has been found to regulate the metabolic profile (e.g., glycemic, blood pressure, and lipid profile) of people with diabetes [130]. Studies have also reported the effect of probiotics and synbiotics in decreasing the biomarkers of inflammatory factors and oxidative stress [24, 131, 132], which could ameliorate kidney injury in diabetes. Chinese herbal medicine, QiDiTangShen granules, has been confirmed to modulate the gut microbiome composition and improved bile acid profiles in a mouse model of DN [133]. These benefits could be attributed to the ability of probiotics to restore epithelial barriers, producing SCFAs, modulating the immune response locally and systemically, and improving the gut barrier function. Moreover, in patients with type 2 diabetes, dietary fiber intake was strongly associated with glycemic control [11] and negatively associated with the prevalence of metabolic syndrome, both of which were associated with a lower risk of renal disease [134].

5. Gut Microbiota in Renal Ischemia-Reperfusion Injury

Renal ischemia-reperfusion injury (IRI) contributes to acute kidney injury (AKI) and delayed graft function after kidney transplantation [135]. Blood reperfusion of ischemic tissue increases the production of ROS that could attack the cells and tissue. Endogenous danger signals are released after cell stress and death, which could activate the tubules and endothelial cells to enhance the expression of adhesion molecules that can recruit innate and adaptive immune cells and promote ROS production [136]. Moreover, excessive ROS destroy the ratio of oxidant/antioxidant enzymes, leading to mitochondria-mediated cell apoptosis. Tubular epithelial cells and APCs secrete cytokines and chemokines that lead to the inflammatory response. APCs, such as macrophages and DCs, could activate $CD4^+$ and $CD8^+$ T lymphocytes by increasing the expression of total stimulus molecules, thereby leading to tissue damage [137].

According to the study of Emal et al., applying antibiotics leads to the diminution of the gut microbiome that can profoundly protect against kidney IRI by reducing the maturation status of the bone marrow monocytes and $F4/80^+$ renal resident macrophages [138], suggesting that intestinal microbes play a role in the progression of ischemia-reperfusion injury to AKI. Furthermore, the treatment with the SCFAs (acetate, propionate, and butyrate) that the gut bacteria produced in the distal colon can improve renal dysfunction in mice with IRI. This protection was associated with the functions of SCFAs, such as reducing inflammation, cellular oxidative stress, and immune cell infiltration and regulate DNA methylation status [21].

6. Gut Microbiota in IgA Nephropathy

IgA nephropathy (IgAN) is the most common type of glomerulonephritis globally and a dominant cause of CKD and renal failure [139]. A characteristic of IgAN patients is the circulating elevation and glomerular accumulation of

Table 1: The related mechanism in the relationship between gut microbiota and kidney diseases.

Kidney diseases	Related mechanism	Conclusions	References
LN	Molecular mimicry	In susceptible individuals, symbiotic bacterial antigens cross-react with human DNA to activate the immune system and destroy self-tolerance, which is positively correlated with SLE activity and LN.	[70, 71]
	Treg/Th17 imbalance	Treg/Th17 imbalance can trigger immune responses and promote the production of SLE autoantibodies.	[40, 42]
	↑TLR7 and TLR9	An increase of TLR7 and TLR9 can contribute to alterations of proinflammatory cytokines in lupus patients.	[64, 65]
	Antinuclear antibodies	Mice with reduced gut bacteria developed nephritis more slowly and had lower levels of circulating antinuclear antibodies (ANAs) compared to the control group.	[67, 69]
		Germ-free lymphotoxin-deficient animals monocolonized with SFB produced more ANAs than lymphotoxin-deficient controls monocolonized with *E. coli*.	[68]
CKD	Endoxin	Endotoxemia can lead to systemic inflammation, oxidative stress, cardiac injury, and atherosclerosis.	[52]
	Uremic toxins (TMAO, IS, and PCS)	Uremic toxins cause inflammation and tubulointerstitial damage and promote ROS production, tubulointerstitial damage, epithelial cytotoxicity of proximal renal tubules, and progressive podocyte and glomerular damage.	[51, 96, 97]
	SCFAs	Gut-derived SCFAs trigger hypertension through Olfr78 in the peripheral blood vessels and renal afferent arterioles, which in turn leads to renin secretion and regulation of peripheral resistance.	[23, 84, 101]
DN	Insulin resistance	Intestinal dysbiosis is involved in insulin resistance and apoptosis of islet cells in diabetes.	[7, 10, 11, 116, 118]
	Activation of the RAS	Ang II accelerates the progression of DN by inducing renal vasoconstriction, promoting renal cell morphology, extracellular matrix deposition, inflammatory cytokine secretion, and fibro-promoting chemokines.	[125, 126]
	Uremic toxin	Phenyl sulfate can cause proteinuria and podocyte injury in diabetic mice. Inhibition of phenyl sulfate can reduce proteinuria in diabetic mice.	[122]
IRI	Bone marrow monocytes and renal resident macrophages	Applying antibiotics can diminish the gut microbiome and protect against kidney IRI profoundly by reducing the maturation status of bone marrow monocytes and F4/80$^+$ renal resident macrophages.	[138]
IgAN	TGF-β, BAFF, and APRIL	Intestinal dysbiosis and chronic bacterial infections could stimulate epithelial cells to produce BAFF and APRIL which could promote excessive production of IgA.	[140, 144–147]
	Endoxin (LPS)	LPS is involved in the presence of important features of IgAN pathogenesis: hyperproduction and hypogalactosylation of IgA1.	[151]

immune complexes consisting of aberrantly glycosylated IgA1, IgG autoantibodies, and C3, which leads to glomerular inflammation [139]. The generation of IgA in the intestinal mucosa is a predominant immunological process that is critical for homeostasis between the intestinal microbiota and the local immunological environment [140, 141]. Considering this, it could be hypothesized that gut dysbiosis and the abnormalities of the IgA mucosal immune system could be a significant element in the pathogenesis of IgAN [142].

The mucosal IgA is mainly produced in mesenteric lymph nodes (MLNs), Peyer's patches (PPs), and isolated lymphoid follicles (ILF) [140, 143]. The microenvironmental signals and controlling factors that drive the mass production of IgA in the intestine include the transforming growth factor-β (TGF-β) [144], BAFF, and APRIL [140, 145–147], which can reveal the commensal dependence in the IgA switch and the IgA-driven pathology [148]. Intestinal dysbiosis and chronic bacterial infections could stimulate the epithelial cells to produce BAFF and APRIL that promote the excessive production of IgA. Furthermore, studies have reported the distinct differences of the gut microbiome and metabolome in IgAN patients and healthy controls [149, 150] (Figure 3).

Otherwise, the potential link between the gut microbiota and the pathogenesis of IgAN could be revealed in the inhibition of IgA1 glycosylation by bacterial LPS. Qin et al. suggest that LPS could significantly inhibit the chaperone Cosmc, which is essential for the activity of galactosyltransferase, via toll-like receptor 4 (TLR4) [151]. The low Cosmc mRNA expression restrains the galactosylation level of IgA1 in IgAN

patients. Combined with the fact that the bacterial LPS itself can stimulate a local and systemic inflammatory response, LPS is involved in the presence of the important features of IgAN pathogenesis including hyperproduction and hypogalactosylation of IgA1 [151].

7. Conclusion

The gut microbiome can be considered a giant bioreactor of the human body, which holds a bidirectional relationship with the host. Human-produced factors, such as sIgA and AMPs, can affect and control the intestinal microbiota potentially. The colonization of intestinal microorganisms is a double-edged sword for the host, and it can elicit a variety of effects on the host's health and diseases. A healthy microbial community plays an indispensable role in supporting symbiotic homeostasis by helping the body in resisting sudden changes from the internal and external environment, metabolizing nutrients, and secreting hormones, promoting the maturation of immune cells, maintaining the integrity of the intestinal epithelial barrier, and preventing the colonization of pathogenic microorganisms. A great deal of basic research can also confirm the role of microbiota in the treatment of a variety of renal disorders. The morbid state of the kidney leads to gut microbial dysbiosis, and in turn, gut microbial alteration induces renal injury. Imbalanced microbial composition leads to intestinal barrier permeability increase, accumulation of uremic toxins, and impaired autoimmune tolerance. Microbial dysbiosis and increased intestinal barrier permeability would be involved in the translocation of pathogenic bacteria, bacterial endotoxins, and toxic metabolites. Circulating microbial components may not lead to a clinical manifestation of infection but instead promote many pathological changes. Bacterial endotoxins and toxic metabolites cause chronic inflammation by activating the NF-κB pathway and promoting the production of proinflammatory chemokines; bacterial pathogens destroy autoimmune tolerance and induce autoimmunity by causing an imbalance in the Treg/Th17 ratio and abnormal activation of B cells. Moreover, the alternation of the gut microbiota can increase oxidative stress and induce hypertension (Table 1). These processes are thought to contribute to the further progression of kidney diseases. Clinical evidence that clarified the intricate pathogenesis of kidney diseases from a gut microbial perspective has opened the possibility for the development of innovative treatments in copious microbial pathways as both potential pharmacological targets and mediators for renal diseases. It may be helpful in the long term to modulate the composition of intestinal flora and restore the epithelial barriers through diet, probiotics, and antibiotics. Ranganathan et al. [152] found that BUN levels showed statistically apparent differences in outcomes ($P < 0.05$) between the placebo and probiotic treatment periods at all four sites (46 patients). Oral administration of *Lactobacillus casei* or *L. acidophilus*, both of which can be used as probiotics, reduced the production of phenolic and indole uremic toxins significantly in hemodialysis patients [153]. In another set of trials, CKD patients who took *L. acidophilus* orally had a significant decline in their levels of serum urea concentration (dimethylamine and nitrosodimethylamine) [154]. In addition, a randomized controlled clinical trial demonstrated that synbiotic therapy significantly alters uremic toxin, PCS, and a palpable shift in the stool microbiome (particularly with the increase of *Bifidobacterium* and the decrease of *Ruminococcaceae*) [155]. Parasite-derived glycoprotein is involved in maintaining the balance of regulatory/effector B cells and desensitized renal effector function. Moreover, gut-derived SCFAs were proven to reduce inflammation, cellular oxidative stress, and immune cell infiltration and contribute to the regulation of DNA methylation status. These significant findings can contribute to the future development of treatment methods for renal diseases. Although numerous studies have been conducted, major advances are still needed to expand our understanding of the interaction mechanism between bacterial molecules and peripheral organs. Further investigations will be needed to prove the research achievement in animal models successfully in patients.

Abbreviations

SCFAs: Short-chain fatty acids
GPR41: G protein-coupled receptor 41
GPR43: G protein-coupled receptor 43
GPR109A: G protein-coupled receptor 109A
Treg cells: Regulatory T cells
NLRP3: NLR family pyrin domain containing 3
IL-18: Interleukin-18
HDACs: Histone deacetylases
IS: Indoxyl sulfate
PCS: p-Cresyl sulfate
TMAO: Trimethylamine N-oxide
AhR: Aryl receptor
AMPs: Antimicrobial peptides
M cells: Microfold cells
DCs: Dendritic cells
Th1: Helper T cell 1
Th2: Helper T cell 2
Th17: Helper T cell 17
IL-4: Interleukin-4
IL-5: Interleukin-5
IL-6: Interleukin-6
IFN-γ: Interferon-γ
APRIL: A proliferation-inducing ligand
BAFF: B cell-activating factor
sIgA: Secretory IgA
LN: Lupus nephritis
SLE: Systemic lupus erythematosus
TLRs: Toll-like receptors
TLR7: Toll-like receptor 7
TLR9: Toll-like receptor 9
IFN-α: Interferon-α
APCs: Antigen-presenting cells
C3a: Complement component 3a
C5: Complement component 5
IL-17A: Interleukin-17A
IL-17F: Interleukin-17F
CXCL5: C-X-C motif chemokine 5

CCL20: Chemokine (C-C motif) ligand 20
CCR6: Chemokine receptor 6
ROS: Reactive oxygen species
CKD: Chronic kidney disease
NF-κB: Nuclear factor-kappa B
LPSs: Lipopolysaccharides
ESRD: End-stage renal disease
Olfr78: Olfactory receptor 78
DN: Diabetic nephropathy
ECM: Extracellular matrix
FBG: Fasting blood glucose
STZ: Streptozotocin
RAS: Renin-angiotensin system
Ang II: Angiotensin II
IRI: Ischemia-reperfusion injury
AKI: Acute kidney injury
IgAN: IgA nephropathy
MLNs: Mesenteric lymph nodes
PPs: Peyer's patches
ILF: Isolated lymphoid follicles
TGF-β: Transforming growth factor-β
TLR4: Toll-like receptor 4.

Ethical Approval

This article does not contain any studies with human participants/animals performed by any of the authors.

Conflicts of Interest

All the authors declare that they have no conflict of interest.

Authors' Contributions

Mingxuan Chi, Kuai Ma, and Linjiang Song were involved in the conception of the study. Mingxuan Chi and Kuai Ma were involved in writing the article. Jing Wang, Zhaolun Ding, Yunlong Li, Shaomi Zhu, Xin Liang, Qinxiu Zhang, and Chi Liu critically revised the manuscript. All authors read and approved the final manuscript. Mingxuan Chi and Kuai Ma contributed equally to this work.

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Research Article

Ursolic Acid Regulates Intestinal Microbiota and Inflammatory Cell Infiltration to Prevent Ulcerative Colitis

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Ulcerative colitis (UC) is a chronic and relapsing inflammatory bowel disorder in the colon and rectum leading to low life-quality and high societal costs. Ursolic acid (UA) is a natural product with pharmacological and biological activities. The studies are aimed at investigating the protective and treatment effects of UA against the dextran sulfate sodium- (DSS-) induced UC mouse model and its underlying mechanism. UA was orally administered at different time points before and after the DSS-induced model. Mice body weight, colon length, and histological analysis were used to evaluate colon tissue damage and therapeutic evaluation. Intestinal transcriptome and microbe 16 s sequencing was used to analyze the mechanisms of UA in the prevention and treatment of UC. The early prevention effect of UA could effectively delay mouse weight loss and colon length shorten. UA alleviated UC inflammation and lowered serum and colon IL-6 levels. Three classical inflammatory pathways: MAPKs, IL-6/STAT3, and PI3K were downregulated by UA treatment. The proportion of macrophages and neutrophils in inflammatory cell infiltration was reduced in UA treatment groups. UA could significantly reduce the richness of intestinal flora to avoid the inflammatory response due to the destruction of the intestinal epithelial barrier. The function of UA against UC was through reducing intestinal flora abundance and regulating inflammatory and fatty acid metabolism signaling pathways to affect immune cell infiltration and cytokine expression.

1. Introduction

Ulcerative colitis (UC) is a chronic and relapsing inflammatory bowel disorder in the colon and rectum, which can induce recurrent episodes of bloody diarrhea, abdominal pain, and even colorectal cancer [1]. The unhealthy lifestyle, gut microbiota, and genetic factors may be the main cause of the pathogenesis of UC [2]. At present, many drugs are used to treat UC including aminosalicylates, corticosteroids, immunosuppressants, and biological reagents; however, a large of patients are still ineffective or have more side effects [3]. Therefore, new therapeutic strategies for UC need to be developed.

Ursolic acid (UA), purified from medicinal plants and foods such as lavender and apple peel, is a natural pentacyclic triterpenoid carboxylic acid with pharmacological and biological activities [4]. The UA may possess broad-spectrum anticancer effects by promoting the apoptosis and autophagy of cancer cells to inhibit cell growth [5–9]. The anti-inflammation mechanisms of UA are reported to inhibit the production of proinflammatory cytokines such as IL6, IL1β, and TNF and reduced the high nuclear level of NFκB p65 [10–13]. UA can reduce transaminase (AST and ALT) levels and fat accumulation to protect against liver diseases [14–16]. Moreover, UA showed antibacterial activity to reduce bacterial biofilm mass of Streptococcus mutants, Pseudomonas aeruginosa, Actinomyces viscosus, etc. and has a synergistic effect against both Staphylococcus aureus and Bacillus cereus with ampicillin and tetracycline [17, 18]. The antibacterial activities of UA are realized by destroying

the integrity of the bacterial membrane and inhibiting the metabolic and protein synthesis pathway [19]. Meanwhile, UA can improve intestinal flora imbalance and play a protective role in the intestinal tract of liver fibrosis mice [20–22]. The character of UC is intestinal immune imbalance and intestinal microbial disorder [23, 24]. The infiltration of macrophage, dendritic cells, and T cells plays crucially important roles in the dextran sulfate sodium- (DSS-) induced mouse UC model [25–27]. The commensal bacteria diversity in UC patient pattern decreases, particularly in Firmicutes and Bacteroides, but some bacterial species are a relative increase like Enterobacteriaceae [28–30].

In our study, we hypothesized that UA may improve the microenvironment of intestinal flora and regulate the infiltration of immune cells to prevent ulcerative colitis. So, we used the DSS-induced mouse UC model to investigate the protective effects of UA against ulcerative colitis.

2. Materials and Methods

2.1. Reagents. Reagent-grade DSS salt (MW, 36-50 kDa, MP Biomedicals); UA (purity, 99.27%, MedChemExpress); Fast DNA Spin Kit for feces (6570200, MP Biomedicals); Qiagen RNeasy Kit (74104, Qiagen); BD™ Cytometric Beads Array (BD).

2.2. DSS Induced the Mouse UC Model and UA Treatment. The 6-week-old C57/BL6 (male, 18-20 g) were provided and fed a basal diet at 24°C and 55% humidity with 14 : 10 light-dark cycle in SPF laboratory animal facility according to the approval of the Animal Ethics Committee of Zhejiang Academy of Traditional Chinese Medicine (KTSC20200058). After a week of adaptation, mice were divided into 4 groups ($n = 8$) according to the experimental plan. In our experiment, all mice were randomly divided into four groups: control group (Con group), model group (DSS group), preventive treatment group (UA + DSS group), and treatment group (DSS + UA group) (Figure 1(a)). The DSS-induced UC model was given 3% (w/v) DSS in the drinking water for 5 days ad libitum. UA (200 mg/kg body weight) was administered by oral gavage once a day for 7 days, commencing 24 hours before changing 3% (w/v) DSS of the drinking water in the UA + DSS group. In the DSS + UA group, UA (200 mg/kg body weight) was administered by oral gavage once a day for 4 days, commencing 3 days after changing 3%(w/v) DSS of the drinking water. The vehicle was administered by oral gavage in the Con group and DSS group. The mice were weighed daily. The mice were anesthetized to collect whole blood from the hearts of mice and sacrificed to collect rectal feces and colonic tissues for follow-up experiments.

2.3. Histopathological Analysis and Serum Cytokine Measurement. The colonic tissue of mice was fixed with 4% formalin, embedded in paraffin, and then stained with hematoxylin and eosin (HE). The score of Nancy index was performed according to the Marchal-Bressenot method [31]. The Nancy index is defined by 5 level classification ranging from grade 0 (no significant disease activity) to grade 4 (severely active disease). The whole blood in the EP tube was left at room temperature for more than one hour and centrifuged at 1500 g for 20 minutes, and the supernatant was the serum. The 9 cytokine (IL-6, IL-10, MCP-1, TNF, IFN-γ, IL-17, IL-2, GM-CSF, and IL-4) levels were measured using the BD™ Cytometric Beads Array (CBA) according to manufacturers' protocol.

2.4. Transcriptome Analysis of Colonic Tissues. Total RNA was isolated from 3 colonic tissues of each group using the Qiagen RNeasy kit following the manufacturers' protocol. RNA samples with good quality control (RIN values > 8) were sequenced using Hiseq-2500 by Novogene. The raw data of RNA-seq was inspected using FastQC and mapped to the reference genome (GRCm38). The read count of genes was calculated by Hisat2 [32]. The expression of genes was analyzed by principal component analysis (PCA). According to a different group, colon length, and Nancy index, all genes were clustered by weighted gene coexpression network analysis (WGCNA) [33]. The clusters of genes were annotated by GO and KEGG by clusterProfiler [34]. Based on the RNA-seq data, the immune cell inflation was analyzed by seq-ImmuCC, which is a tool of tissue transcriptome measuring cellular compositions of the immune microenvironment from mouse RNA-seq data [35].

2.5. Intestinal Microbiota 16S rRNA Sequence. The total DNA of the rectal feces was isolated using the Fast DNA Spin Kit for feces. The V4 region of the 16S rRNA gene was amplified and sequenced with the 515F/806R primer set by Illumina MiSeq platforms at Novogene. The operational taxonomic units (OTUs) and representative sequences for each OTU were obtained at 97% similarity by FLASH, QIIME, and UPARSE software [36–38]. The species of OTUs sequence were annotated at setting a threshold of 0.8 ~ 1 by the Mothur method and SILVA132 SSUrRNA database (http://www.arb-silva.de/) [39]. And then, the abundance of species, Alpha diversity of each group, was calculated by *R* software.

2.6. Real-Time RT-PCR. Briefly, 1 μg of extracted RNA was reverse transcribed (Applied Biosystems) and amplified using the SYBR green PCR master mix (Roche 480). The relative quantification of the gene expression was calculated with the $2 - \Delta\Delta$Ct method referring to Gapdh.

2.7. Immunoblot Analysis. The colonic tissues were washed with ice-cold PBS and lysed with lysis buffer (20 mM Tris at pH 7.5, 1 mM PMSF, 0.1% Triton X-100, and 10 μg/ml aprotinin). The concentration of protein was determined using a BCA assay (Sangon Biotech), and 20 μg of protein per lane was added on an 8-12% SDS-polyacrylamide gel. The protein electrophoretically transferred to a nitrocellulose membrane (0.1-μM pore size). The proteins were detected using rabbit polyclonal antibodies against mouse Tgfb-1, Col1a1, Itga5, and Gapdh (Proteintech Group) as primary antibodies and peroxidase-conjugated anti-rabbit IgG (Proteintech Group) as a secondary antibody. Protein was detected by an enhanced chemiluminescence system (ECL) and exposure to X-ray film.

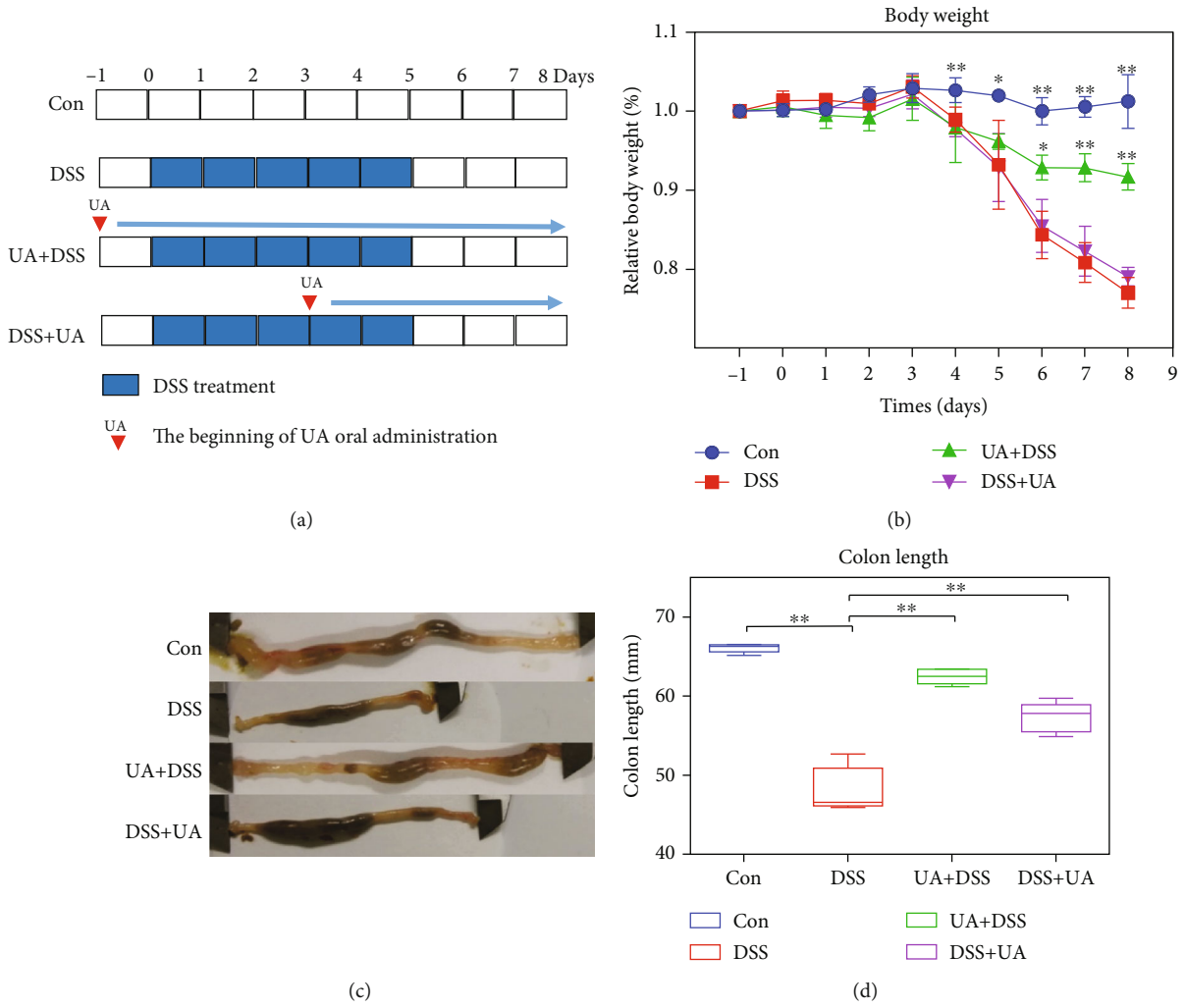


Figure 1: Effects of the UA on DSS-induced UC model. (a) Flow chart and time point of the DSS-induced UC model and UA treatment. The blue frame represented a 3% DSS drinking solution for one day. (b) The daily record of body weight in the DSS-induced UC model and UA treatment groups ($n = 8$). (c) The morphology and length of the colon in the DSS-induced UC model and UA treatment groups. (d) The box plot of colon length in the DSS-induced UC model and UA treatment groups ($n = 8$). Data represent the mean ± SD of values per group. Statistically significant results in different groups are marked by $^{*}P < 0.05$ and $^{**}P < 0.01$. There was no significant difference in the unmarked group.

2.8. Statistical Analysis. The Image-Pro Plus software was used to calculate the score of Nancy index. Image production and data analysis were performed using GraphPad Prism and *R* software. All data are presented as mean ± SD or SEM. The number of each experimental group was at least 3 samples to ensure confidence in the results. Student's *t*-test and Kruskal-Wallis *H* test were used to analyze the significant differences between groups. $^{*}P < 0.05$ was considered significant. $^{**}P < 0.01$ was considered extremely significant.

3. Results

3.1. Ursolic Acid Attenuated DSS-Induced Ulcerative Colitis of Mice. To investigate the effect of UA on prevention and treatment for UC, the DSS-induced mouse UC model was performed by oral administration of UA before and after DSS treatment. Early intervention with UA (UA + DSS group) was able to alleviate DSS-induced weight loss and shortening the colon in mice (Figure 1). However, the DSS + UA group only reduced DSS-induced shortening of the colon in mice for UA treatment five days (Figure 1). The histopathology of the colon was evaluated by the score of Nancy index and HE. The Nancy index of the UA + DSS group and DSS + UA was significantly lower than the DSS group (Figure 2(b)). The 3% DSS induced severe mucosal and epithelial cell structural damage and inflammation response (Figure 2). However, the mucosal epithelium was more intact and regularly arranged between the UA + DSS and DSS + UA groups than the DSS group (Figure 2(a)). The UA also reduced the level of proinflammatory factor IL6 in serum (Figure 2(c)). So, our results implied that UA had protective and therapeutic effects on colon damage and inflammation.

3.2. Core Gene Expression Characteristics of UA Attenuated DSS-Induced Mouse UC. To state the molecular mechanism of UA treatment on UC, we analyzed the transcriptome of

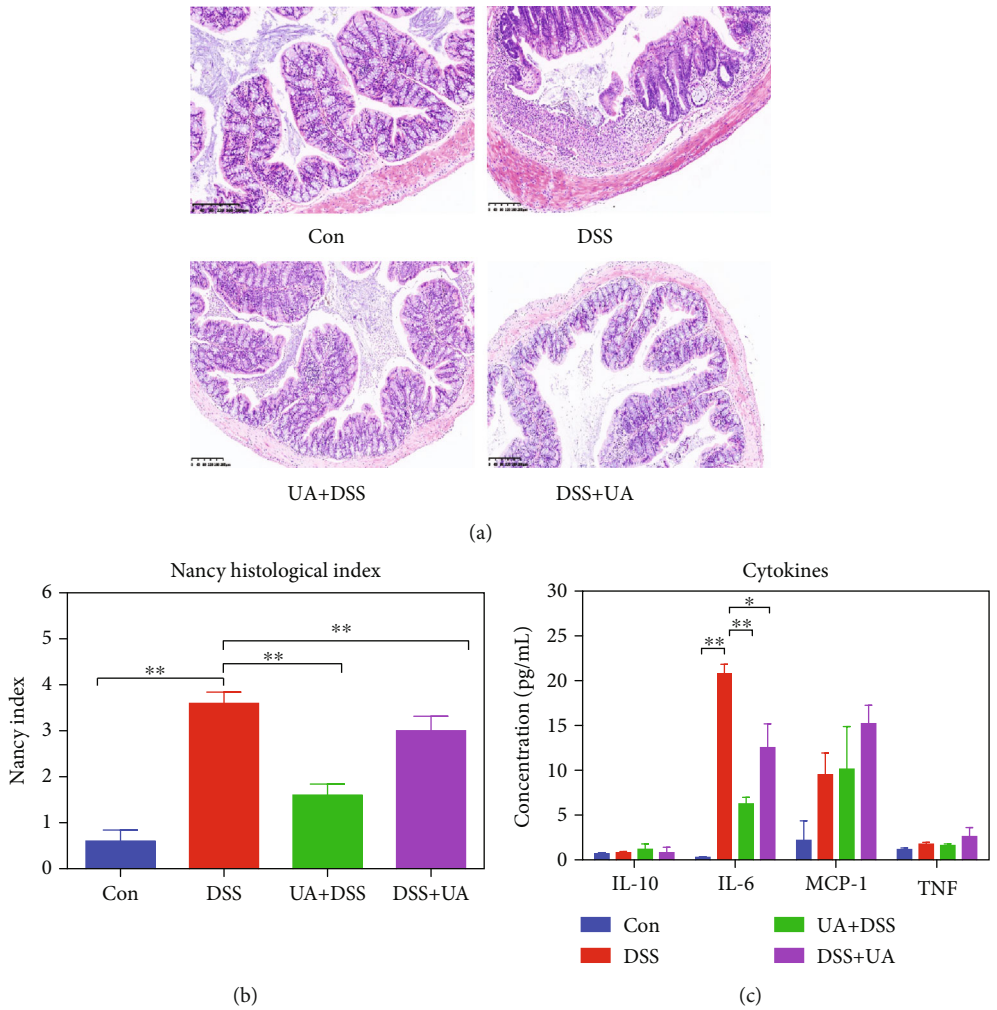
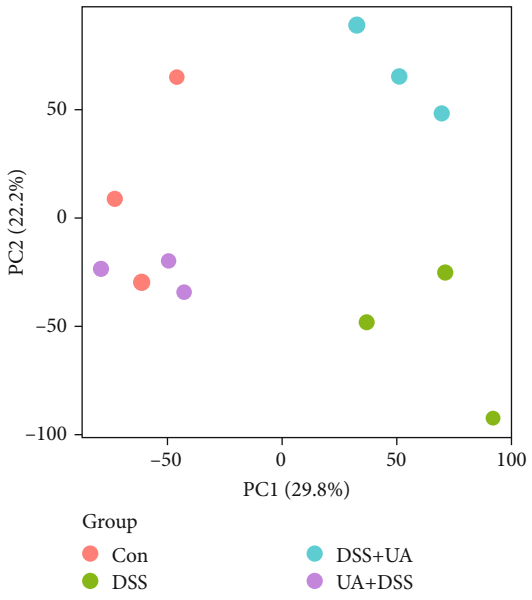


Figure 2: Effects of the UA against colon inflammatory and injure. (a) The histopathology of the colon by HE stain in the DSS-induced UC model and UA treatment groups. (b) Nancy index of each group according to the Marchal-Bressenot method in each group ($n = 8$). (c) Effects of UA on serum levels of four cytokines in the DSS-induced UC model and UA treatment groups ($n = 8$). Data represent the mean ± SEM of values per group. Statistically significant results in different groups are marked by $^{*}P < 0.05$ and $^{**}P < 0.01$. There was no significant difference in the unmarked group.

four groups (Con, DSS, UA + DSS, and DSS + UA) by high throughput sequencing (GSE150688). The Con and UA + DSS groups were clustered, while DSS and DSS + UA groups were individually separated according to transcriptomic data by PCA analysis (Figure 3(a)). The specific gene coexpression modules of four different treatment groups were analyzed by WGCNA. The 15 gene coexpression modules were found using calculating parameter (power = 14 and mergecutheight = 0.25) (Figure 1S and Table 1S). The gene expression profile of the black module was significantly positively correlated with the DSS group (correlation = 0.97, P value = $2e-07$). The genes of the black module were clustered into inflammation signaling pathways such as PI3K-Akt, MAPK, and cytokine interaction (Figure 3(b)). The ECM (extracellular matrix) and TGF-β signaling pathway were activated by DSS (Figure 3(c)). The blue module was significantly positively correlated with the UA + DSS group (correlation = 0.64, P value = 0.02) and negatively correlated with the DSS group (correlation = -0.61, P value = 0.04) (Figure 1S). The genes of the blue module were mapped into fatty acid metabolism, bile secretion, and virus infection pathways by KEGG analysis (Figures 4(a) and 4(c)–4(e)). The red module was significantly positively correlated with the DSS + UA group (correlation = 0.59, P value = 0.05) and negatively correlated with the DSS group (correlation = −0.65, P value = 0.02) (Figure 1S). The genes of the red module were clustered into neurological disease, oxidative phosphorylation, and fatty acid metabolism in (Figure 4(b)). Interestingly, We found gene modules (blue and red) associated with phenotypes that can be enriched to fatty acid metabolism in both the preventive treatment group (UA + DSS group) and treatment group (DSS + UA group) (Figures 4(a) and 4(b)). We also found that the gene expression of fatty acid metabolism and fatty acid degradation in the UA-treated group and the normal control group was higher than that in the DSS model group (Figure 4(c)). Meanwhile, AMPK and FOXO signaling pathways were downregulated in the



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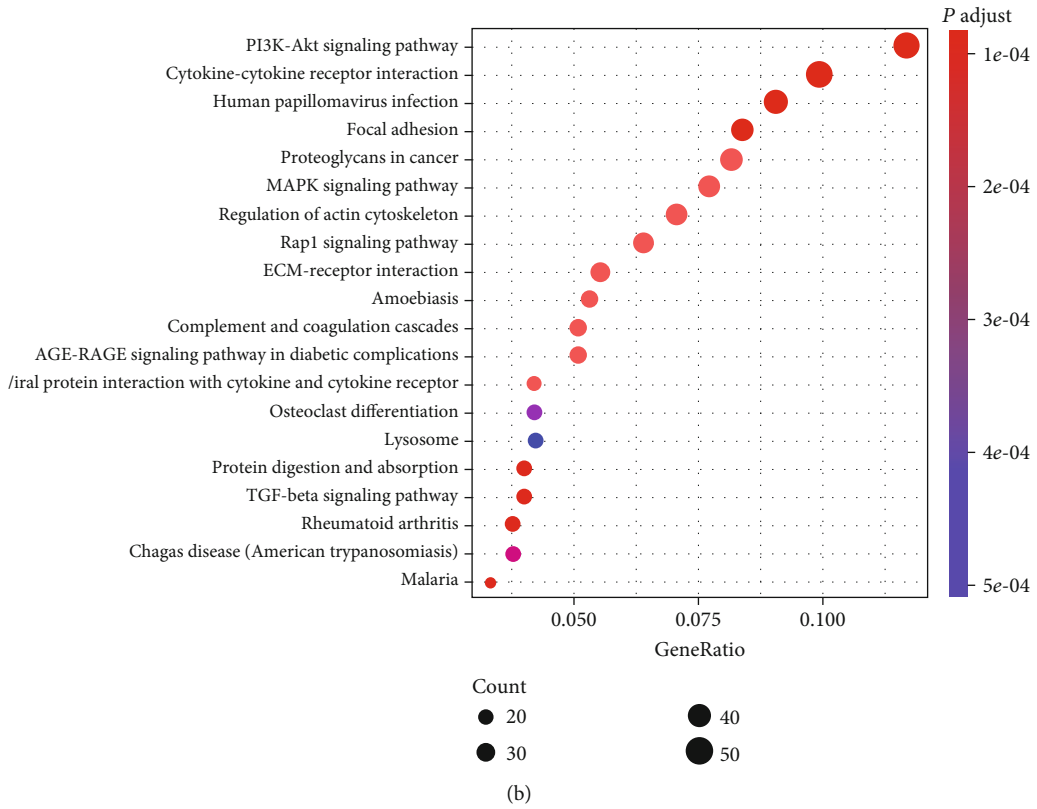

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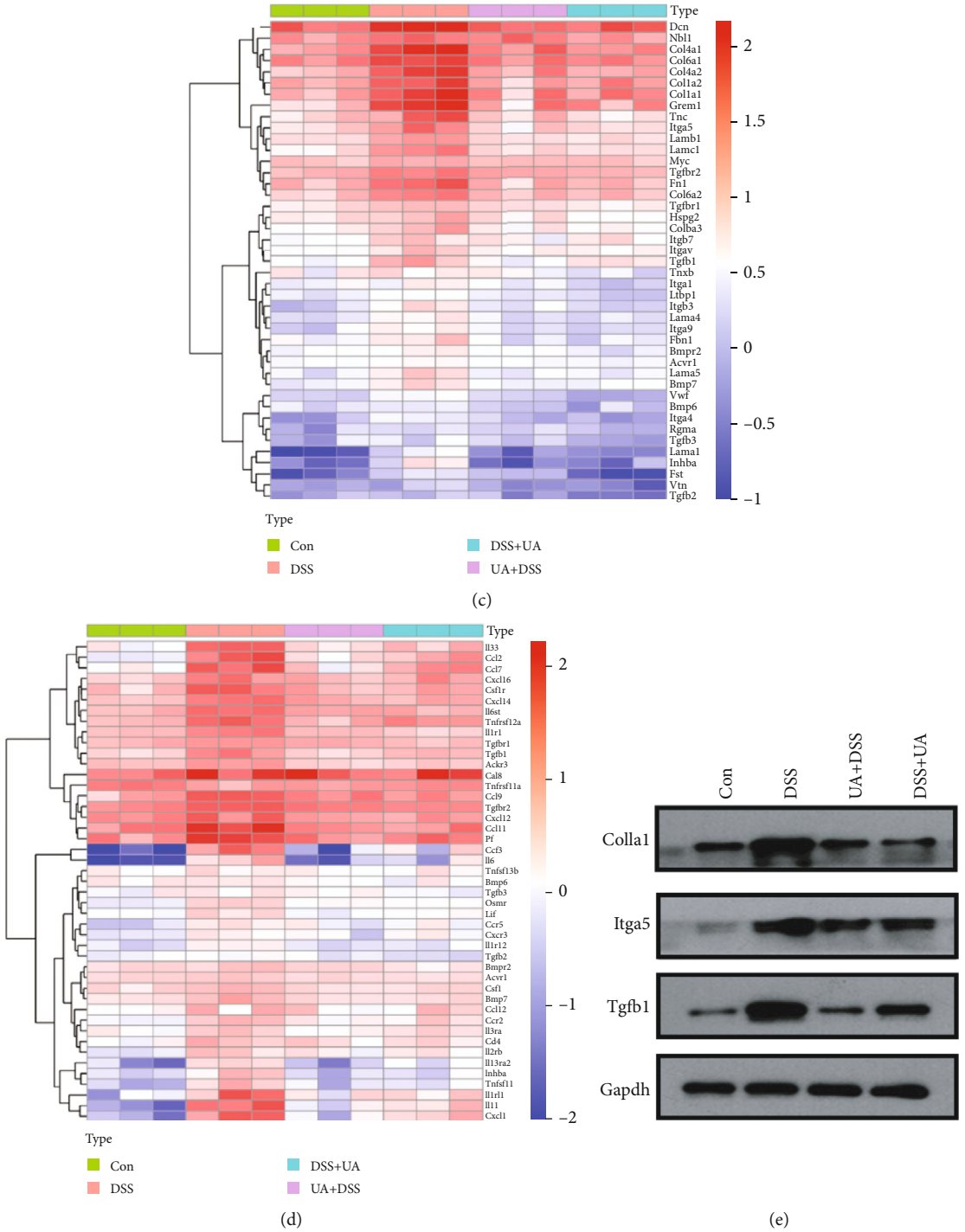

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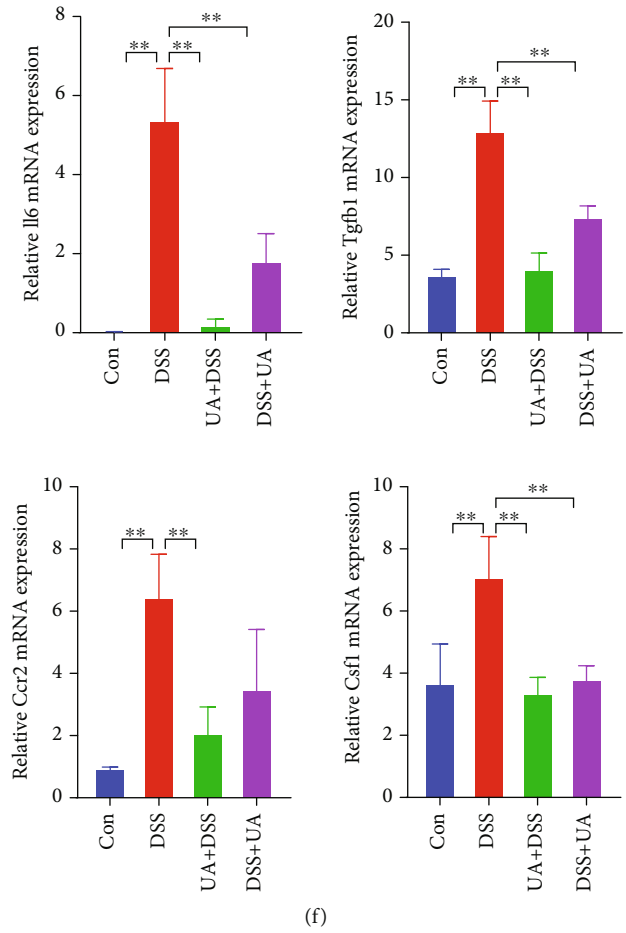


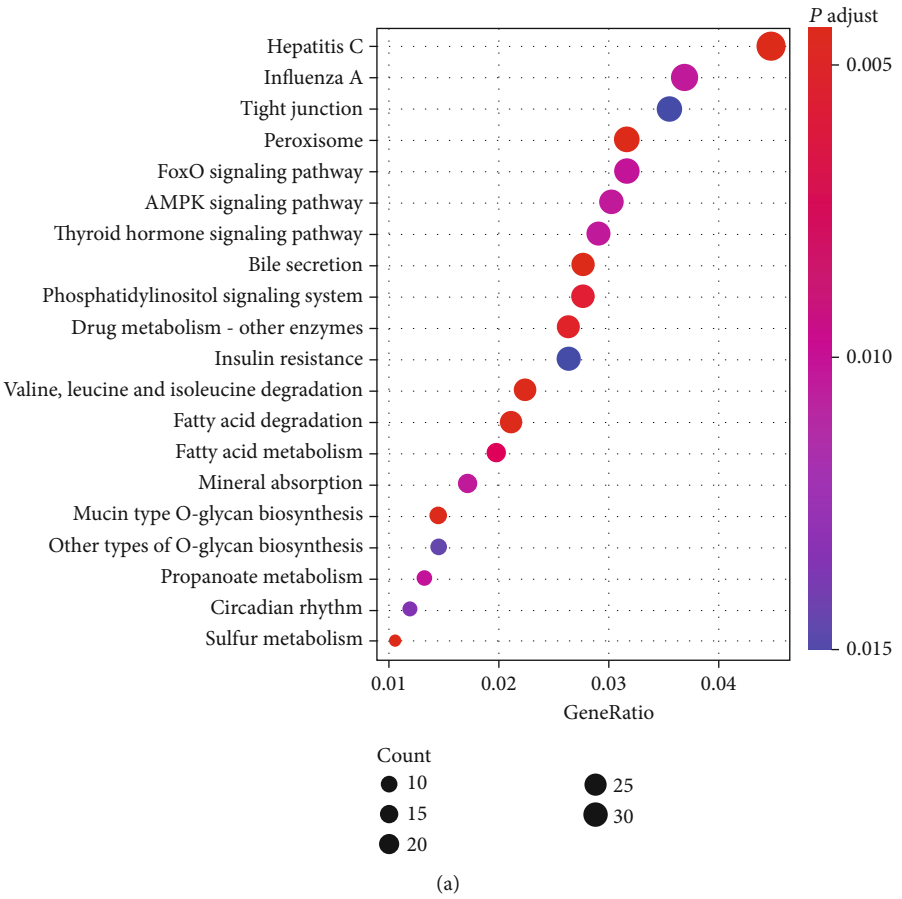
(f)

Figure 3: Transcriptome analysis and protein expression of the prevention and treatment of the DSS-induced UC model with UA treatment. (a) PCA analysis results of transcriptome under different treatments. (b) The KEGG signaling pathway enrichment results of black module genes by WGCNA. (c) Heatmap of ECM receptor interaction and TGF-β signaling pathway gene expression. (d) Heatmap of the cytokine-cytokine receptor signaling pathway gene expression. (e) Immunoblot analysis of Col1a1, Tgfb-1, and Itag5 in four groups of colonic tissues. (f) The relative mRNA expression of Il6, Tgfb-1, Ccr-2, and Csf-1 gene in four groups of colonic tissues.

DSS group compared with UA treatment and Con groups (Figure 4(d)). These results suggested that the protection and treatment effect of UA on UC is mainly through the regulation of fatty acid metabolism.

3.3. UA Regulates Immune Cell Infiltration of DSS-Induced Mouse UC. To understand the composition of infiltrated immune cells in inflammatory microenvironments Figure 5(h) of different treatment groups, the transcriptomic data were analyzed by seq-ImmuCC. Compared with the Con group, the proportion of macrophages and neutrophils in the DSS group was significantly increased, while the proportion of CD4 T and dendritic cells was decreased significantly (Figures 5(a)–5(c)). The proportion of macrophages and neutrophils was decreased, and the proportion of CD4 T and dendritic cells was increased in the UA treatment group including UA + DSS and DSS + UA (Figures 5(a), 5(b), and 5(k)). The proportion of NK and CD8 T cells was significantly increased in the UA + DSS group (Figures 5(d) and 5(g)). The proportion of mast cells was increased in the DSS + UA group (Figure 5(h)). These results suggested that UA could regulated immune cell infiltration in the UC mouse model.

3.4. UA Regulates the Microenvironment of the Intestinal Flora of DSS-Induced Mouse UC. To investigate the regulatory effect of UA on intestinal microorganisms, the observed species and diversity were analyzed by 16S rRNA amplicon sequencing. We found that UA could significantly reduce the community richness of bacteria and the Chao index (P value = 0.038) in the gut (Figure 6(a) and 6(b)). However, there was no significant difference in the Shannon index (P value = 0.319) (Figure 6(c)) and the Beta diversity index (data not shown). To determine the bacteria biomarkers of each treatment group, the LDA (Linear Discriminant Analysis) effect size of four groups was analyzed by LEfSe software. The Con and DSS groups owned more biomarkers, and the biomarkers were reduced by UA treatment (Figure 6(d)). The major biomarker of UA + DSS was Verrucomicrobia, while the major biomarker of the DSS + UA group was Gammaproteobacteria (Figure 6(d)).



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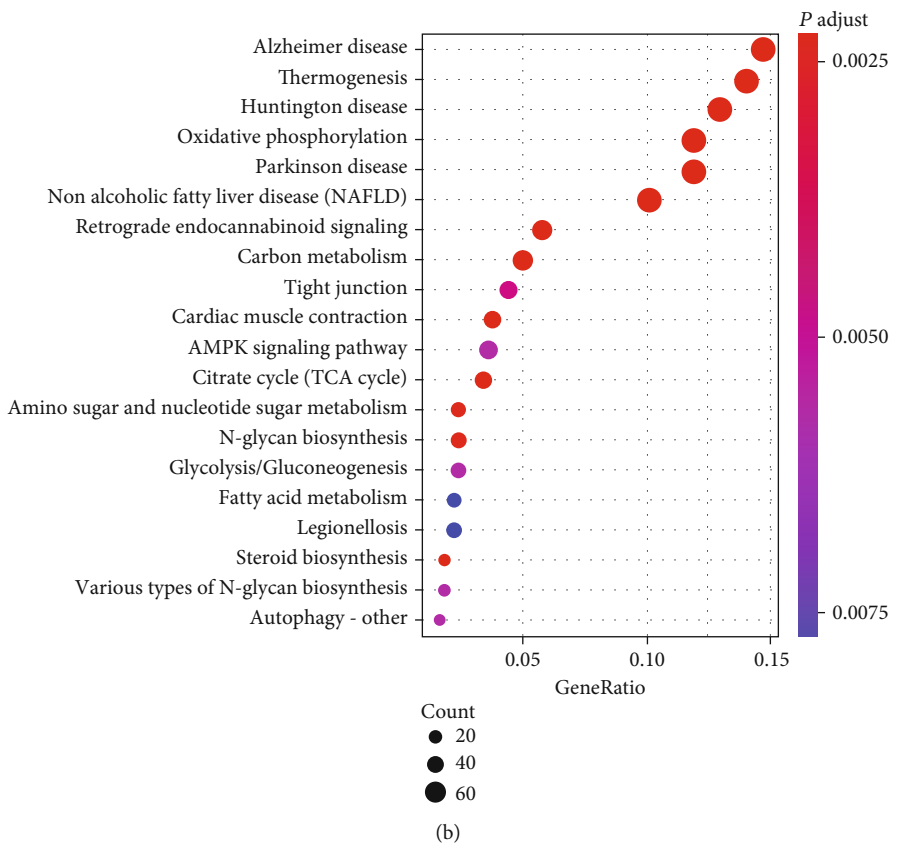

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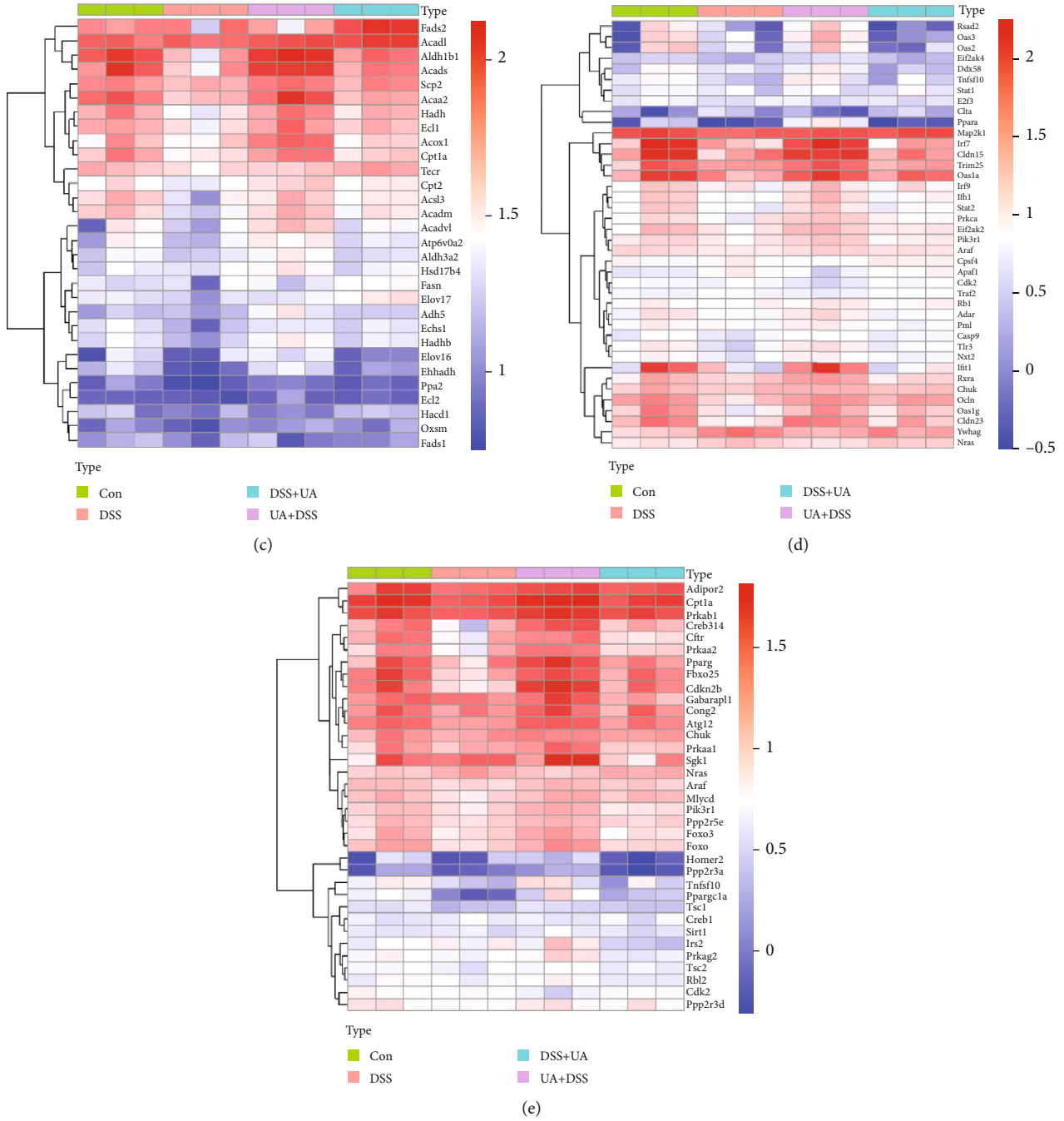
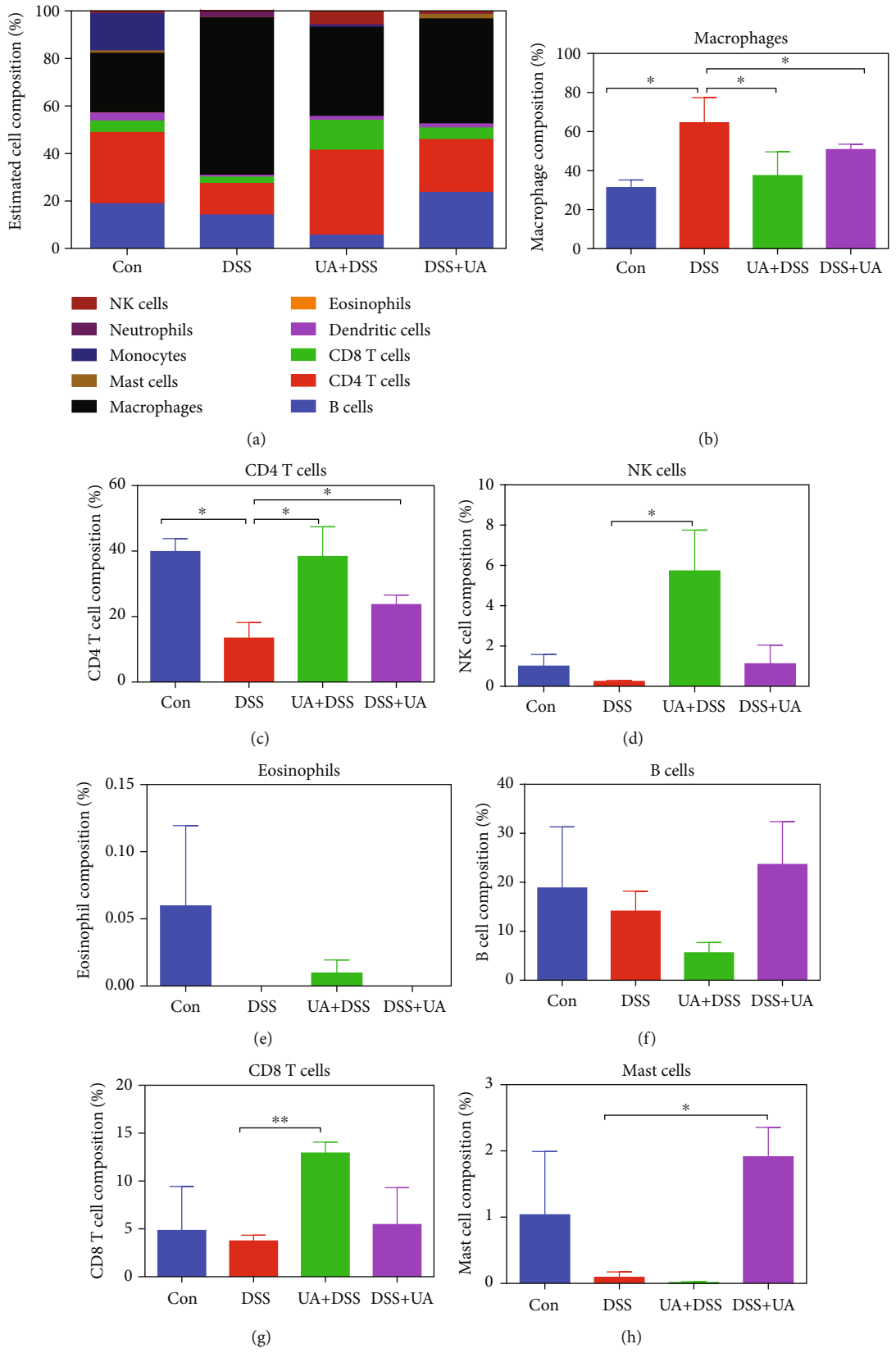


Figure 4: Gene expression modules associated with UA therapy and prevention. (a) The KEGG signaling pathway enrichment results of blue module genes associated with the phenotype of the UA + DSS group. (b) The KEGG signaling pathway enrichment results of red module genes associated with the phenotype of the DSS + UA group. (c) Heatmap of fatty acid metabolism signaling pathway gene expression. (d) Heatmap of the virus infection signaling pathway gene expression. (e) Heatmap of the AMPK and FoxO signaling pathway gene expression.

4. Discussion

Inflammatory bowel disease (IBD) has been a global disease leading to low life-quality and high societal costs [40]. UC and Crohn's disease (CD) are the two main IBD pathological subtypes. In contrast to CD, UC lesions occur mainly in the mucosa of the colon due to genetic immune, environmental factors, and intestinal microbes [2]. In this study, the early prevention (UA + DSS group) and late treatment (DSS + UA group) effects of UA were analyzed by the DSS-induced UC mouse model. The early prevention effect of UA could effectively delay mouse weight loss and colon length shorten (Figure 1). However, the late treatment effect of UA only delayed colon length shorten, and there was no significant difference in body weight between the DSS + UA group and the DSS group (Figure 1), but the no difference in body weight might be caused due to short UA administration time (only 5 days). However, in previous studies, UA treatment



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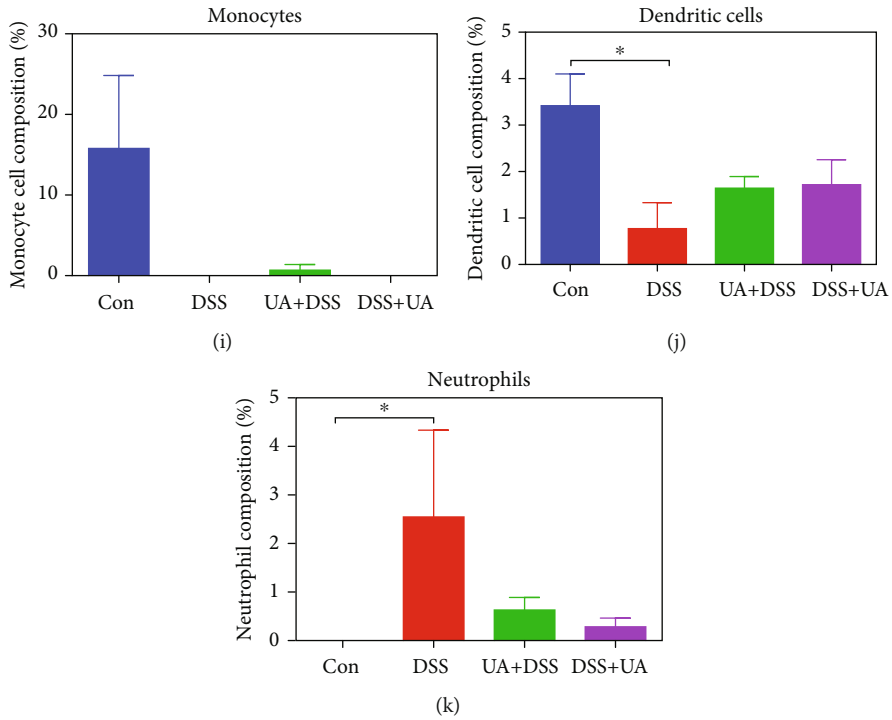
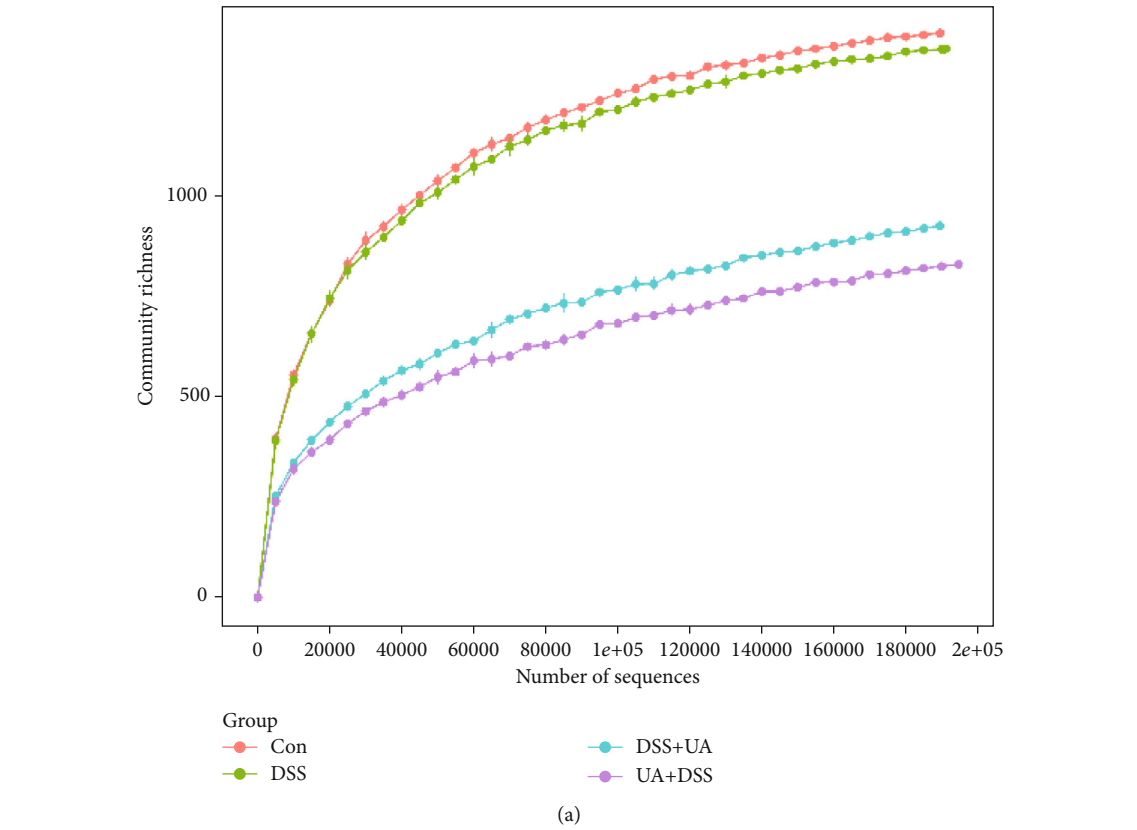


FIGURE 5: Composition of infiltrated immune cells among four UC experiment groups. (a) Fractions of 10 immune cells in Con, DSS, UA + DSS, and DSS + UA groups ($n = 3$ each group). (b) The proportion of macrophage change in four experimental groups. (c) The proportion of CD4 T cells change in four experimental groups. (d) The proportion of NK cell change in four experimental groups. (e) The proportion of eosinophil change in four experimental groups. (f) The proportion of B cell change in four experimental groups. (g) The proportion of CD8 T cell change in four experimental groups. (h) The proportion of mast cell change in four experimental groups. (i) The proportion of monocyte cell change in four experimental groups. (j) The proportion of dendritic cell change in four experimental groups. (k) The proportion of neutrophil change in four experimental groups. Data represent the mean ± SEM of values per group. Statistically significant results in different groups are marked by $^{*}P < 0.05$ and $^{**}P < 0.01$. There was no significant difference in the unmarked group.

for 7 days could improve SOD activity and reduce malondialdehyde (MDA) and myeloperoxidase (MPO) activity to relieve the reduction of body weight and stool blood [12]. Meanwhile, UA treatment for 9 days in the UA + DSS group showed significant weight recovery (Figure 1(b)). So, prolonged administration of UA may contribute to weight recovery in UC mice.

To further elaborate the mechanism of UA in the prevention and treatment of UC, the correlation between the transcriptome and the phenotypes of each treatment group was analyzed by WGCNA. Three classical inflammatory pathways: MAPKs, IL-6/STAT3, and PI3K were enriched into the black module which was significantly positively correlated with the DSS group (Figure 3(b) and 1S). Mitogen-activated protein kinases (MAPKs) involve in the regulation of the synthesis of inflammation mediators by transcription and translation [41]. PI3K isoforms (PI3Kα, β, γ, δ) play a particularly important role in chemokine-mediated recruitment and activation of innate immune cells at sites of inflammation and B and T cell development, differentiation, and function [42]. UA could also significantly reduce the IL-6 level in serum and downregulate the expression of inflammation-related genes (Figures 2(d), 3(c), and 3(d)). IL-6 regulates various cells including epithelial cells, macrophages, neutrophils, and T cells to activate early immune responses [43]. In IBD patients, multiple aberrancies in lipid metabolism have been found, and fatty acids may affect cytokine production and inflammation response [44–46]. AMP-activated protein kinase (AMPK) plays a key role as a master regulator of cellular energy homeostasis and is also thought to be important for regulating fatty acid metabolism [47]. The activation of the AMPK-FOXO3 pathway reduces the fatty acid-induced increase in intracellular reactive oxygen species [48]. In our results, the prevention and treatment effects of UA on UC are achieved by activating AMPK/-FOXO signaling pathways that upregulate fat acid metabolism (Figure 4).

Immune cell infiltration is an important biomarker of inflammation. DSS feeding resulted in an increased production of macrophage-derived cytokines in BALB/c mice [26]. We also found macrophages accounted for the highest proportion of immune infiltrating cells in the DSS-induced UC model (Figures 5(a) and 5(b)). The proportion of macrophages was significantly reduced in two UA treatment groups (Figure 5(b)). UA inhibits NF-κB activation in both intestinal epithelial cells and macrophages and attenuates experimental murine colitis [13]. However, the infiltration of neutrophils in IBD leads to loss of barrier function and apoptosis of epithelial cells [49]. UA treatment reduced the proportion of neutrophils in the colonic mucosa of DSS-induced UC models (Figure 5(k)). CD4 T cells, also known as T helper (Th) lymphocytes, differentiate into a variety of Th cell types



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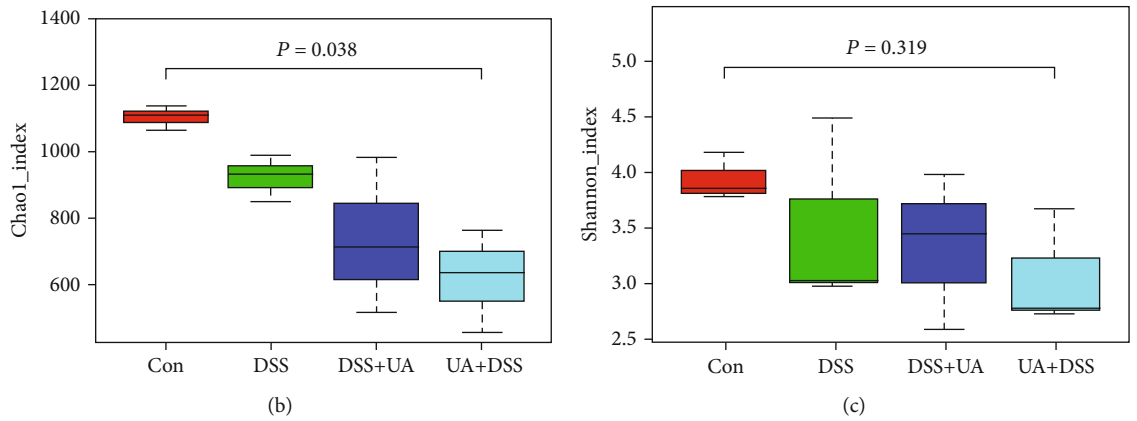

(b) (c)

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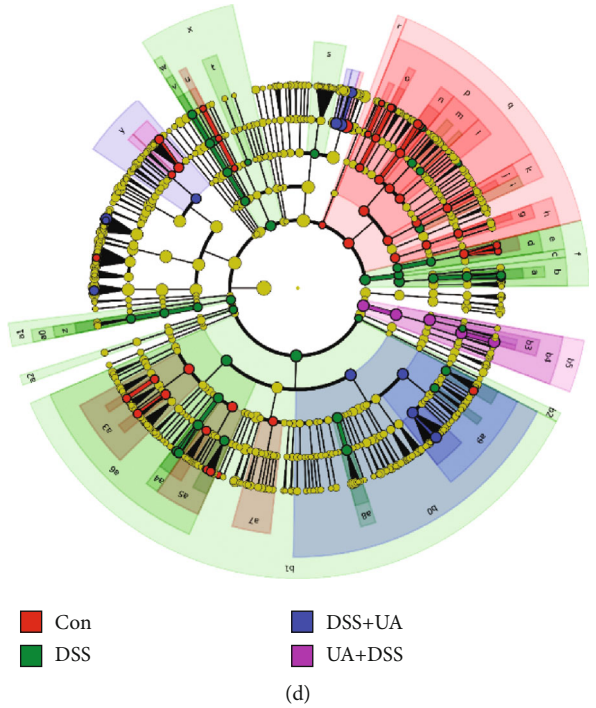

Figure 6: Analysis of the differential microbial community among the group. (a) Rarefaction curve of microbial community richness of Con, DSS, UA + DSS, and DSS + UA groups. (b) Alpha diversity index (Chao1_index) of intestinal within groups. (c) Alpha diversity index (Shannon_index) of intestinal within groups. (d) Cladogram of the LDA value from the Con, DSS, UA + DSS, and DSS + UA groups. The Kruskal-Wallis *H* test was used to analyze the significant differences between groups by *R*.

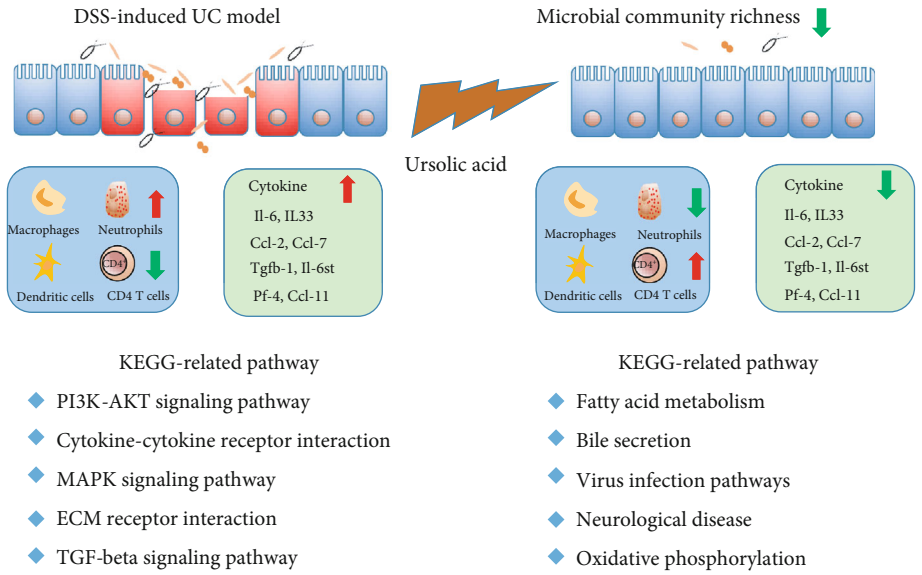

Figure 7: Schematic representation of UA function on the prevention and treatment of UC. DSS induced injury to the intestinal epithelial immune barrier, leading to the transfer of antigens into gut lamina propria. The red arrow means upregulation, and the green means downregulation.

and are key in mediating inflammation [50]. In our model, the proportion of CD4 T cells was downregulated in the DSS-induced UC model, while UA could mitigate this decline in the ratio (Figure 5(c)). However, the types of these CD4 T cells still need further analysis.

At present, there is no single agent that has been proven to cause IBD. The role of gut microbes has been suspected because of potential infectious, particularly when the intestinal epithelial barrier is destroyed [51]. UA has a potential antibacterial effect by inhibition of protein synthesis and the metabolic pathway [18]. In our studies, the community richness of bacteria, Chao index, and bacteria biomarkers were markedly decreased in two UA treatment groups (Figure 6). This reduction of community richness of bacteria

would reduce the innate immune response and inflammation due to the destruction of the epithelial barrier (Figure 7). In studies on liver fibrosis, it has been found that UA could prevent intestinal damage caused by carbon tetrachloride by improving intestinal flora disturbance [19–21]. In our research, the major biomarker of the UA + DSS group was Verrucomicrobia by LEfSe (Figure 6(d)). Meanwhile, Verrucomicrobia has been recently proposed as a hallmark of a healthy gut due to its anti-inflammatory and immunostimulant properties and its ability to improve gut barrier function, insulin sensitivity, and endotoxemia [52].

In conclusion, we demonstrated that UA could prevent and ameliorate the DSS-induced UC mouse model. The function of UA against UC was through reducing intestinal flora abundance, regulating inflammatory and fatty acid metabolism signaling pathways to affect immune cell infiltration and cytokine expression (Figure 7). These results suggested that IBD susceptible populations would eat some foods or drink herb tea rich in UA such as apple, berries, and mulberry leaf tea to prevent and treat IBD. Of course, how much UA content through diet and tea per day is still to be further studied to prevent UC.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request. The transcriptome data of this research have been submitted GEO database (Accession: GSE150688).

Conflicts of Interest

All of the authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

MQ Li, QS Sheng, and KQ Chai conceived and designed the experiments. F Li, GP Chen, J Li, YF Wang, YY Lu, and Q Li performed the experiments. J Li, YF Wang, and Q Li analyzed the data. MQ Li, F Li, and Q Li wrote the paper. QS Sheng and KQ Chai critically reviewed the manuscript. Qinsong Sheng and Fei Li contributed equally to this work.

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Supplementary Materials

Supplementary 1. Figure 1S. Relationship between gene expression modules and phenotype of experiment groups. The 15 gene expression modules were found by WGCNA. The upper number of each frame represented the correlation value. The lower number of each frame standard for significance *P* value. The abscissa represents different groups and phenotypes, and the ordinate represents related gene expression modules.

Supplementary 2. Table 1S. The table of the related gene in 15 gene expression modules by WGCNA.

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