

Intestinal flora alterations in patients with early chronic kidney disease: a case-control study among the Han population in southwestern China

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Abstract

Objective: In this case–control study, we retrospectively analyzed the intestinal flora compositions of patients with early-stage chronic kidney disease (CKD).

Methods: Forty-seven patients with early CKD who were treated at the Traditional Chinese Medicine Hospital between March and October 2018 were enrolled, and 150 healthy volunteers were enrolled in the healthy control group. Fresh stool samples were collected. The V3–V4 region of the bacterial 16S rRNA was amplified via PCR. Biterminal sequencing was performed using the Illumina MiSeq platform. The flora compositions were compared between the two groups.

Results: The Chao1 and Shannon indices showed significantly lower intestinal flora diversity and abundances in the CKD group than in the healthy controls. Beta diversity analysis revealed notable differences in the intestinal flora compositions between the groups. At the phylum level, Actinobacteria and Proteobacteria abundances were significantly higher in the CKD group. Thirty-one species differed significantly between both groups, among which, differences in *Ruminococcus* and *Roseburia* displayed the highest diagnostic values for distinguishing CKD patients from healthy controls.

Conclusions: Intestinal flora compositions are altered in early-stage CKD patients among the Han population in southwestern China.

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Keywords

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Introduction

Chronic kidney disease (CKD) is a syndrome characterized by symptoms/metabolic disorders caused by progressive renal dysfunction in patients with primary or secondary renal disease. CKD has an approximate incidence of 8% to 16%; it is one of the most common diseases affecting human health and thus has become a global public health issue.¹ Diabetes, hypertension, obesity, metabolic syndrome and autoimmune diseases are common causes of CKD.² Although the causes and risk factors for CKD can be much better controlled now than in the past, CKD-induced damage to the kidney structure and function is irreversible. CKD can ultimately progress to end-stage renal disease (ESRD) and affect multiple systems throughout the body, thus endangering the lives of CKD patients.

Human intestines contain over 10^{14} microorganisms, more than 10 times the total number of human cells. Types of intestinal microorganisms number in the thousands, constituting the largest symbiotic ecosystem in the human body.³ More than 400 species of intestinal microorganisms can be cultured and classified into dominant or secondary microflora according to their abundance. They can also be categorized as beneficial, harmful or neutral. Intestinal flora interact with the host organism, playing important roles in host metabolism, digestion, immunity and barrier protection.⁴ Studies have shown that alterations in the intestinal flora play important roles in the development of inflammatory bowel disease,⁵ hypertension,⁶ obesity,⁷ diabetes,⁸ and nonalcoholic fatty liver disease.⁹

A recent study showed that CKD progression is closely associated with alterations in the intestinal flora.¹⁰ The variety and amounts of probiotic bacteria, such as *Lactobacillus*, *Prevotella* and *Bacillus bifidus*, are significantly reduced in the intestines of CKD patients, whereas those of conditional pathogenic bacteria, such as *Enterobacteria* and *Pseudomonas*, are significantly increased.¹¹ Research on the association between intestinal flora alterations and CKD development remains in its infancy, and systemic studies remain rare. In addition, many factors, such as age, dietary habits and geographical environment, influence the intestinal flora composition, which can vary greatly according to host ethnicity.¹²

In southwestern China, the CKD incidence is 10.8%, and approximately 1.5% of CKD cases involving a progressive decrease in the glomerular filtration rate develop into ESRD annually.¹³ Therefore, kidney diseases must urgently be prevented and treated in this region. We conducted a case-control study in southwestern China to retrospectively explore the association between intestinal flora alterations and early CKD development. The results of this study may add to current knowledge regarding intestinal flora diversity in CKD patients and provide a reference for future research on preventing and treating kidney diseases.

Materials and methods

Clinical data collection

Forty-seven patients with early CKD who treated at the Traditional Chinese Medicine

Hospital Affiliated with Southwest Medical University between March and October 2018 were consecutively enrolled. Patients were diagnosed according to the Kidney Disease Outcomes Quality Initiative criteria for CKD, which defines CKD as abnormal kidney structure or function lasting more than 3 months (e.g., a urinary albumin excretion rate ≥ 30 mg/24 h).¹⁴ According to clinical staging,¹⁵ these patients were at stages II to III (glomerular filtration rate, 30–90 mL/minute/1.73 m²).

One hundred fifty healthy age- and sex-matched volunteers who received physical examinations at the same hospital and had no medical or family history of CKD were enrolled in the control group.

In each group, participants meeting one or more of the following criteria were excluded: 1) concomitant diarrhea or other intestinal diseases; 2) use of antibiotics or probiotics/prebiotics within 4 weeks before stool sampling; and 3) use of preparations containing diuretic constituents within 4 weeks before sampling. All participants were from the Han population.

The Ethics Committee of the Traditional Chinese Medicine Hospital Affiliated with Southwest Medical University (approval no. KY2019053) approved the study, which was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent.

Sampling and DNA extraction

Fresh stool was collected on the examination day or from the first stool after hospitalization. Samples (1–2 g) were stored in collection tubes containing a nucleic acid protection solution (LongseeMed, Guangzhou, China). Microbial DNA was extracted using a DNA extraction kit (LongseeMed) and stored at -80°C .

16S rDNA sequencing

Extracted DNA quality was assessed using 1% agarose gel electrophoresis, and the quantity was determined with a NanoDrop spectrophotometer (Thermo Fisher, Waltham, MA, USA). Primers targeting the V3–V4 region of the bacterial 16S rRNA genes V3F (ACTCCTACGGGAGGCAGCA) and V4R (GGACTACHVGGGTWTCTAAT) were used for amplification. The 25- μL reaction system consisted of 12.5 μL of KAPA HiFi HotStart ReadyMix (2X), 0.5 μL of Amplicon PCR Forward Primer (10 μM), 0.5 μL of Amplicon PCR Reverse Primer (10 μM), and 11.5 μL (20 ng) of Fast Pfu DNA. The amplification conditions were 95°C for 3 minutes; 25 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s; and a final step at 72°C for 5 minutes. The PCR products were purified with KAPA Pure Beads, then subjected to a second round of amplification. The 50- μL PCR system consisted of 5 μL of Nextera XT Index Primer 1 (N7xx: 10 μM), 5 μL of Nextera XT Index Primer 2 (S5xx: 10 μM), 5 μL of KAPA HiFi HotStart ReadyMix (2X), 5 μL of Adaptor-ligated DNA, and 10 μL of sterilized ddH₂O. The reactions were performed at 95°C for 3 minutes, followed by 6 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s and a final step at 72°C for 5 minutes. Libraries of the sequenced samples were constructed. Concentrations were determined with a Qubit[®] 2.0 Fluorometer (Invitrogen; Carlsbad, CA, USA), and the library sizes were determined using an Agilent 2100 (Agilent; Chandler, AZ, USA). The V3–V4 hypervariable region of the amplified 16S rRNA was sequenced on the Illumina MiSeq PE250 platform (Illumina, San Diego, CA, USA).¹⁶

Bioinformatic analysis

The raw sequencing data (FASTQ) were trimmed and filtered using Trimmomatic

v.0.32 software (<http://www.usadellab.org/cms/index.php?page=trimmomatic>). High-quality sequences with at least 97% similarity were clustered to obtain operational taxonomic units. Alpha and beta diversities were determined using QIIME2 V.2017.12 (<http://qiime.org/>) and R software, respectively. Chao1 and Shannon indices were used to assess α -diversity of the intestinal flora, and principal coordinate analysis (PCoA) and principal component analysis (PCA) were performed to assess β -diversity. Microbial taxa showing significant differences were analyzed using the linear discriminant analysis effect size (LEfSe) method. Intestinal flora functions were predicted via phylogenetic investigation of communities by reconstruction of unobserved states (PICRUST). A receiver operating characteristics (ROC) curve was plotted to evaluate the diagnostic value of the flora markers, and canonical correspondence analysis (CCA) was performed to evaluate the correlation between the flora and clinical indices.

Statistical analysis

Data were analyzed using IBM SPSS Statistics for Windows version 20.0 (IBM Corp., Armonk, NY, USA). Wilcoxon rank-sum and Kruskal–Wallis tests were performed. High-flux detection outcomes were subjected to descriptive analysis. $P < 0.05$ was considered statistically significant.

Results

Participant characteristics

The average age of the 47 participants with CKD was 43.2 ± 12.6 years; the average age of the 150 healthy age- and sex-matched volunteers was 38.5 ± 15.4 years.

Intestinal flora diversity

A total of 7,006,107 valid sequences were obtained, with an average of 35,564

sequences per sample. Chao1 and Shannon indices were used to evaluate the diversity and abundance of the microorganisms in the sample; higher values typically indicate greater diversity. Alpha-diversity analysis showed significantly lower Chao1 and Shannon values for the CKD group than for the healthy controls ($P < 0.001$, Figure 1a and b).

Bray–Curtis-based PCoA and PCA were performed to evaluate the β -diversity of the intestinal flora. The CKD patients and healthy controls showed a separating trend, suggesting that the intestinal flora composition in the CKD group differed from that in the healthy controls (Figure 1c and d).

Species variations of the intestinal flora

The intestinal flora of both the CKD patients and healthy controls contained 15 bacterial phyla, among which, Firmicutes, Bacteroidetes, Proteobacteria, and Fusobacteria were dominant (Figure 2). Compared with the healthy controls, the CKD group had significantly higher relative abundances of Proteobacteria and Actinobacteria ($P = 0.023$ and $P = 0.016$, respectively).

Bacterial species that differed significantly ($P < 0.05$) between the CKD patients and healthy controls were screened using LEfSe to construct a cladogram showing these species (Figure 3a). The histogram obtained via linear discriminant analysis showed that the abundances of 13 genera in the CKD group, i.e., *Bacteroides*, *Escherichia*, *Ruminococcus*, *Blautia*, *Enterococcus*, *Clostridium*, *Eubacterium*, *Klebsiella*, *Sarcina*, *Eggerthella*, *Turicibacter*, *Bilophila* and *Pseudoramibacter*, were significantly higher than those in the healthy controls. In the healthy controls, the abundances of 18 genera were significantly higher: *Roseburia*, *Prevotella*, *Faecalibacterium*, *Megamonas*, *Coprococcus*, *Burkholderia*, *Dialister*,

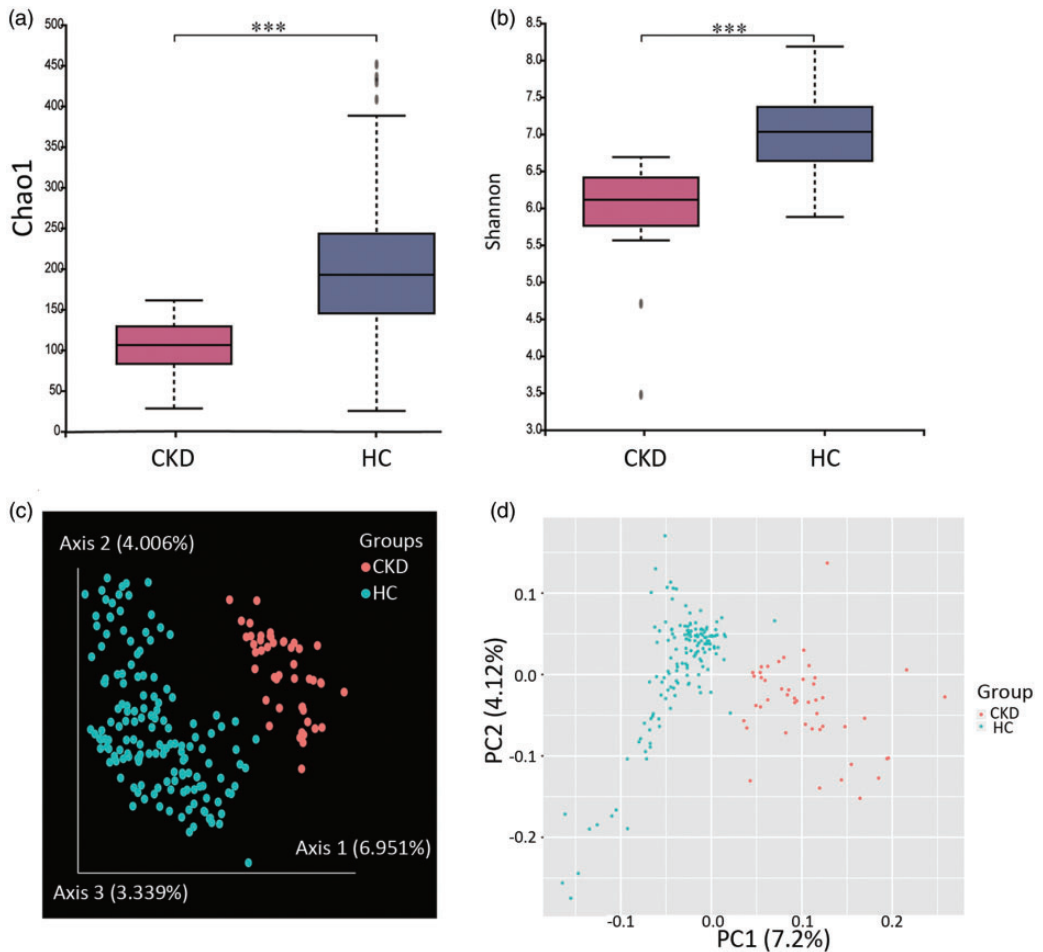


Figure 1. Comparisons of intestinal flora diversity between chronic kidney disease patients and age- and sex-matched healthy controls. (a) Chao1 index for assessing α -diversity. (b) Shannon index for assessing α -diversity. (c) Principal coordinate analysis outcomes based on Bray–Curtis distance for β -diversity. % denotes the interpretation percentage of the principal component. (d) Principal component analysis outcomes for β -diversity. *** $P < 0.001$, according to the Kruskal–Wallis test.

Lachnospira, *Streptococcus*, *Megasphaera*, *Sutterella*, *Collinsella*, *Stenotrophomonas*, *Haemophilus*, *Odoribacter*, *Butyrivimonas*, *Acidaminococcus* and *Granulicatella* (Figure 3b).

Functional annotations

PICRUSt functional prediction analysis was performed on the obtained 16S rRNA

sequences to determine the abundance and enrichment of functional genes at different levels in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. According to the second-level KEGG pathway entries, the average relative abundances of genes in 35 KEGG pathways, including the endocrine system ($P = 0.0001$), amino acid metabolism ($P = 0.0024$) and lipid metabolism ($P = 0.0048$), were

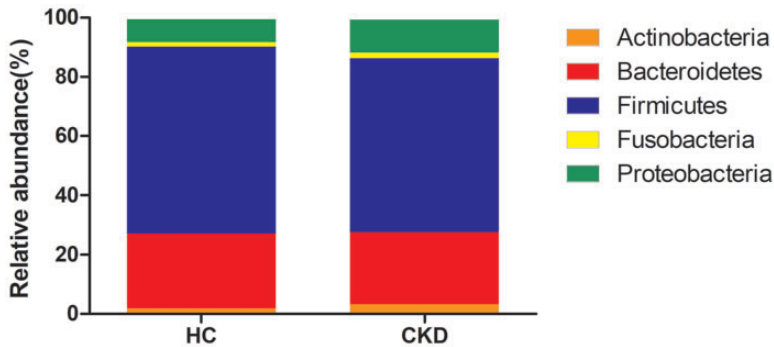


Figure 2. Intestinal flora composition at the phylum level.

significantly lower in the CKD group than in the healthy controls (Table 1).

Diagnostic values of the microbial markers

ROC curves were plotted using the top five genera of both groups according to linear discriminant analysis scoring, and the area under the curve (AUC) was calculated to assess whether the intestinal microflora could be used to distinguish between the CKD patients and healthy controls. The results showed that *Ruminococcus* had the highest diagnostic value for the CKD group (AUC = 0.771; 95% confidence interval [CI], 0.771–0.852) (Figure 4a), whereas *Roseburia* had the highest diagnostic value for the healthy controls (AUC = 0.803; 95% CI, 0.804–0.864; Figure 4b).

Canonical correspondence analysis (CCA)

To further assess the association between intestinal microflora and environmental factors, CCA was performed between cystatin C (Cys_c) and C-reactive protein (CRP) and the intestinal microflora. *Escherichia*, *Bilophila* and *Blautia* were positively correlated with Cys_c and CRP and were enriched in the CKD group; *Roseburia* was negatively correlated with Cys_c and

CRP and was enriched in the healthy controls (Figure 5).

Discussion

The intestinal microecosystem is continuously evolving, and its microbial diversity and abundance play important roles in maintaining a normal physiological state; however, the host also influences the intestinal microflora.¹⁷ In this study, 16S rRNA sequencing was performed on stool samples from patients with early-stage CKD and healthy volunteers in southwestern China using high-flux sequencing. The relative abundances of each intestinal microbial species and their diversities and compositions were compared between CKD patients and healthy age- and sex-matched controls. The results showed significantly decreased intestinal flora diversity and an altered intestinal flora composition in the CKD patients compared with those of the healthy controls.

CKD is closely associated with intestinal flora imbalance.^{18–20} Among the imbalanced intestinal flora in CKD patients, flora that induce carbohydrate fermentation are decreased, whereas those that induce protein fermentation are increased. Furthermore, endogenous uremic toxins, such as indoxyl sulfate, p-cresol sulfate and trimethylamine oxide, accumulate in

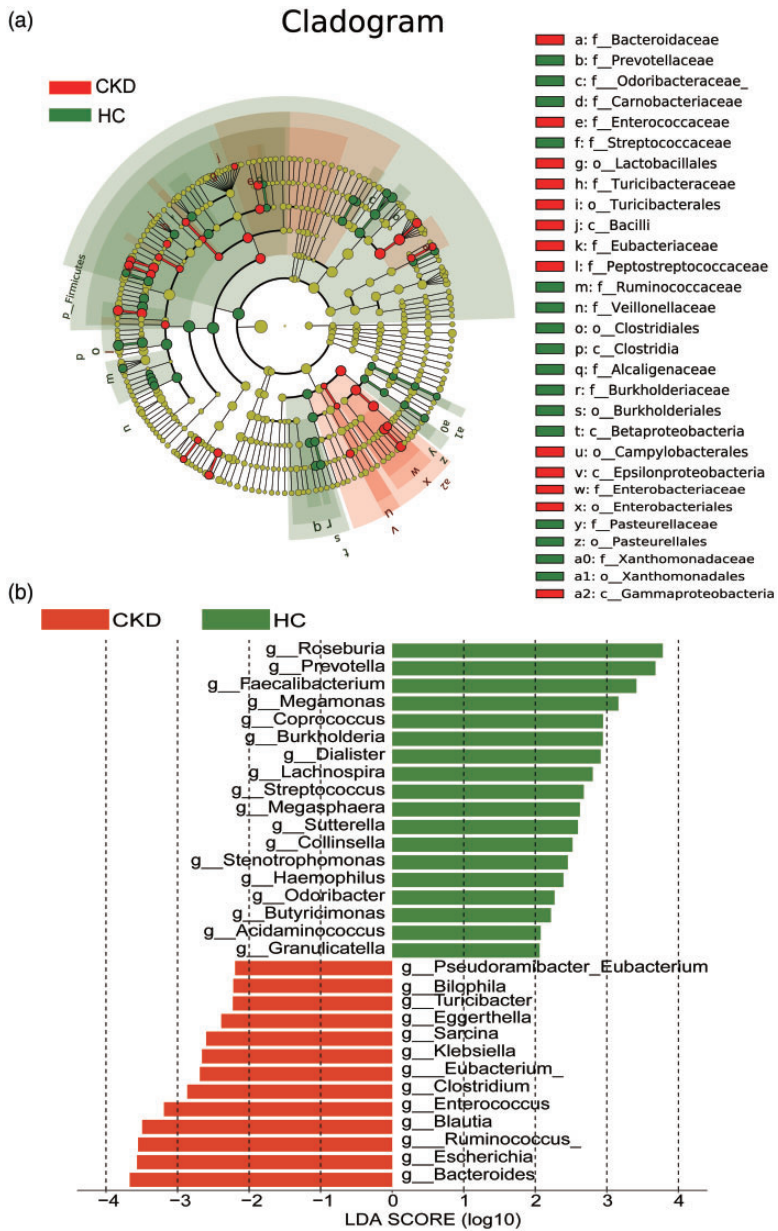


Figure 3. Species that differed significantly between the chronic kidney disease (CKD) patients and healthy controls based on LEfSe. (a) Species with significantly different relative abundances (red dot, CKD group; green dot, healthy controls). (b) Species with significantly different abundances (red, CKD group; green, healthy controls). Only the taxonomic clusters with linear discriminant analysis >2.0 at the genus level are shown.

Table 1. Second-level entries of the Kyoto Encyclopedia of Genes and Genomes pathways with significant differences between the chronic kidney disease (CKD) group and the healthy controls (HC).

Pathway	CKD (M ± SD)	HC (M ± SD)	P value	FDR
Digestive System	0.0033 ± 0.0013	0.0056 ± 0.0034	<0.0001	<0.0001
Cell Motility	0.0032 ± 0.0014	0.0057 ± 0.0038	<0.0001	<0.0001
Endocrine System	0.0037 ± 0.0010	0.0055 ± 0.0027	<0.0001	0.0001
Environmental Adaptation	0.0036 ± 0.0011	0.0055 ± 0.0031	<0.0001	0.0001
Immune System	0.0038 ± 0.0012	0.0055 ± 0.0027	0.0001	0.0005
Translation	0.0039 ± 0.0011	0.0055 ± 0.0027	0.0001	0.0005
Glycan Biosynthesis and Metabolism	0.0040 ± 0.0012	0.0054 ± 0.0026	0.0002	0.0010
Metabolism of Cofactors and Vitamins	0.0040 ± 0.0012	0.0054 ± 0.0027	0.0002	0.0010
Metabolism of Terpenoids and Polyketides	0.0040 ± 0.0012	0.0054 ± 0.0026	0.0003	0.0010
Metabolic Diseases	0.0039 ± 0.0010	0.0054 ± 0.0027	0.0003	0.0010
Folding, Sorting and Degradation	0.0040 ± 0.0012	0.0054 ± 0.0026	0.0003	0.0010
Biosynthesis of Other Secondary Metabolites	0.0039 ± 0.0011	0.0054 ± 0.0028	0.0003	0.0010
Nucleotide Metabolism	0.0040 ± 0.0012	0.0054 ± 0.0026	0.0004	0.0013
Replication and Repair	0.0040 ± 0.0012	0.0054 ± 0.0026	0.0005	0.0014
Cell Growth and Death	0.0040 ± 0.0011	0.0054 ± 0.0026	0.0005	0.0014
Cancers	0.0040 ± 0.0014	0.0054 ± 0.0026	0.0007	0.0017
Enzyme Families	0.0040 ± 0.0013	0.0054 ± 0.0026	0.0009	0.0023
Genetic Information Processing	0.0041 ± 0.0013	0.0054 ± 0.0026	0.0011	0.0023
Immune System Diseases	0.0039 ± 0.0013	0.0054 ± 0.0030	0.0011	0.0023
Metabolism of Other Amino Acids	0.0041 ± 0.0014	0.0054 ± 0.0026	0.0012	0.0024
Amino Acid Metabolism	0.0040 ± 0.0012	0.0054 ± 0.0027	0.0012	0.0024
Nervous System	0.0040 ± 0.0011	0.0054 ± 0.0027	0.0014	0.0027
Energy Metabolism	0.0041 ± 0.0013	0.0054 ± 0.0026	0.0017	0.0030
Infectious Diseases	0.0042 ± 0.0019	0.0053 ± 0.0026	0.0020	0.0034
Signal Transduction	0.0041 ± 0.0019	0.0054 ± 0.0029	0.0023	0.0037
Cellular Processes and Signaling	0.0042 ± 0.0016	0.0054 ± 0.0026	0.0031	0.0047
Poorly Characterized	0.0042 ± 0.0014	0.0054 ± 0.0026	0.0031	0.0047
Lipid Metabolism	0.0041 ± 0.0013	0.0054 ± 0.0027	0.0033	0.0048
Transport and Catabolism	0.0041 ± 0.0011	0.0054 ± 0.0028	0.0037	0.0052
Transcription	0.0042 ± 0.0015	0.0054 ± 0.0027	0.0086	0.0117
Carbohydrate Metabolism	0.0042 ± 0.0014	0.0053 ± 0.0027	0.0110	0.0145
Xenobiotics Biodegradation and Metabolism	0.0043 ± 0.0017	0.0053 ± 0.0026	0.0121	0.0155
Signaling Molecules and Interaction	0.0042 ± 0.0011	0.0053 ± 0.0026	0.0150	0.0187
Metabolism	0.0044 ± 0.0017	0.0053 ± 0.0026	0.0246	0.0297
Membrane Transport	0.0044 ± 0.0019	0.0053 ± 0.0027	0.0364	0.0427

Notes: M ± SD, mean ± standard deviation; FDR, false discovery rate.

large amounts, thus damaging renal tubular epithelial cells, leading to renal interstitial fibrosis.^{21,22} The CKD-colon axis theory states that CKD can alter the intestinal flora and cause gut barrier dysfunction, leading to intestinal flora translocation, excessive uremic toxins and systemic inflammatory reactions. This condition further accelerates CKD

development, ultimately forming a vicious cycle between the kidney and the gut.²³ A study in California, USA, found that the relative abundances of Proteobacteria (primarily Gammaproteobacteria), Actinobacteria and Firmicutes (particularly *Clostridium subphylum*) were increased in patients with ESRD.¹¹ In ESRD patients from South

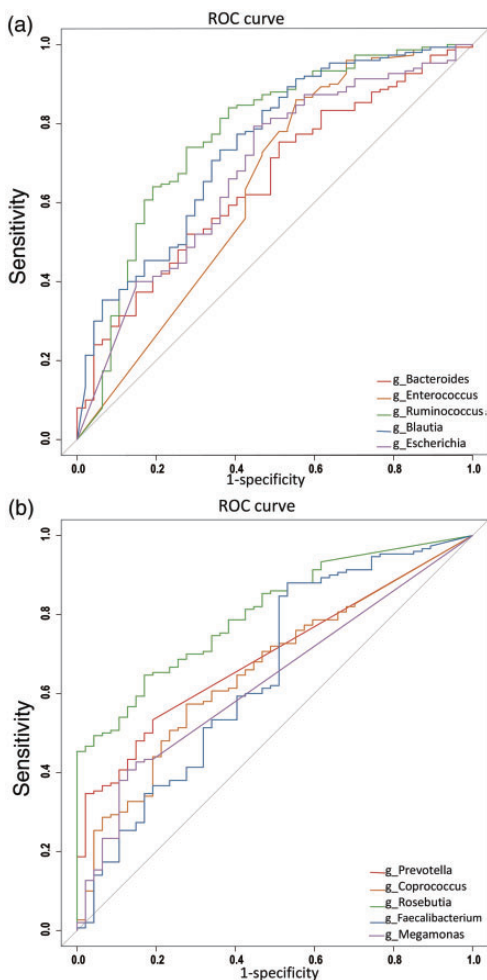


Figure 4. Receiver operating characteristic (ROC) curves for microbial markers. (a) ROCs for the top genera in the chronic kidney disease group according to linear discriminant analysis (LDA) scoring. (b) ROCs for the top genera in the healthy controls according to LDA scoring.

China, the absolute amounts of all bacteria were significantly decreased, and the abundances of butyrate-producing *Roseburia*, *Faecalibacterium*, *Clostridium*, *Coprococcus* and *Prevotella* were decreased.²⁴ In the present study, the α -diversity of the intestinal flora in the CKD group was significantly lower than that in the healthy controls. β -diversity analysis showed that the UniFrac

distance between the CKD group and the healthy controls tended to separate, indicating an altered intestinal flora composition in patients with early-stage CKD. This finding was consistent with previous literature.^{25,26}

Firmicutes, Bacteroides, Proteobacteria and Actinomycetes are the dominant bacterial phyla in the intestines, and a proportional imbalance in the intestinal flora plays an important role in the development of chronic inflammatory diseases.^{27,28} In children with ESRD, the relative abundance of Proteobacteria is increased, while the relative abundances of Firmicutes and Actinomycetes are decreased. Ingested iron supplements can promote an increase in the Proteobacteria abundance in ESRD patients.²⁹ In patients with colitis-associated colorectal cancer, Proteobacteria occupy a dominant position over other intestinal bacterial phyla. In mice, high abundances of Proteobacteria cause local or systemic inflammation and metabolic dysfunction, which increase susceptibility to chronic colitis.³⁰ In our study, the CKD group displayed higher relative abundances of Proteobacteria and Actinobacteria than did the healthy controls, further suggesting a possible association between intestinal flora alterations and CKD.

Thirty-one species differed significantly between the CKD group and the healthy controls and may serve as biomarkers for distinguishing CKD patients from healthy individuals. ROC curves showed that *Ruminococcus* and *Roseburia* had the best diagnostic performance. *Ruminococcus* belongs to an important group of intestinal probiotic microbial flora that degrade and transform complex polysaccharides into nutrients for the host.³¹ In individuals with Crohn's disease, *Ruminococcus* affects CKD development by producing inflammatory polysaccharides.³² *Roseburia* is another important component of the intestinal flora; it produces butyrate, which has anti-inflammatory and immunoregulatory

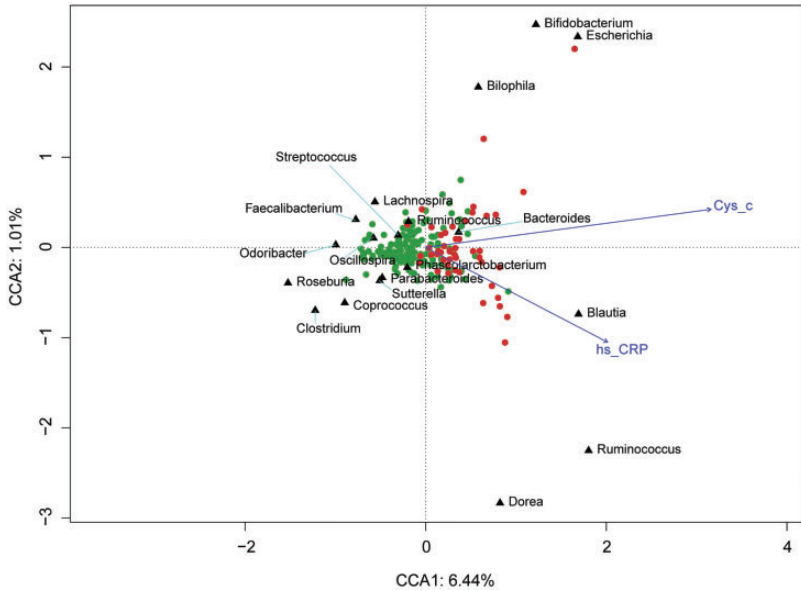


Figure 5. Associations between internal environmental factors and intestinal microflora in the chronic kidney disease (CKD) patients and healthy controls. Black triangles represent different microbial types; the red and green dots represent the CKD group and healthy controls, respectively. The explained variances of the primary axes (axis 1 [horizontal] and axis 2 [vertical]) are 6.44% and 1.01%, respectively. The length of the environmental parameter arrow indicates the intensity of the environmental parameter effect on all microflora.

functions. In patients with metabolic and inflammatory diseases, the relative abundance of intestinal *Roseburia* is decreased.³³ In mice with different genetic backgrounds, *Roseburia* correlated negatively with atherosclerosis development.³⁴ Decreased *Roseburia* might damage local gastrointestinal tract function, thereby aggravating inflammation in CKD patients.³⁵ The results of this study were consistent with those reported in the literature.^{32–35}

Amino acids are the main precursors of uremic solutes, such as indoxyl sulfate and cresol sulfate, and metabolism of intestinal amino acids becomes imbalanced with CKD development.³⁶ In this study, the CKD patients had significantly lower average relative gene abundances in 35 KEGG pathways, including amino acid metabolism, lipid metabolism and the endocrine system, suggesting that CKD patients experience metabolic dysfunction.

This study had some limitations. Because of the small sample size, we did not explore the association between different CKD stages and the intestinal flora composition. Therefore, further studies with larger sample sizes should validate the use of different intestinal flora compositions as markers for diagnosing CKD. Furthermore, the combination of clinical data relative to CKD pathological staging should be analyzed to determine the diagnostic value of the intestinal flora composition for CKD, and the mechanisms by which the intestinal flora composition influences CKD development should be explored.

Conclusions

The intestinal flora composition is greatly altered in CKD patients. Detection of different intestinal flora compositions may be a useful diagnostic measure for early CKD.

Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

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