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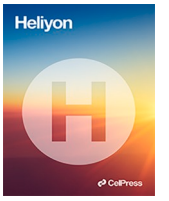

Urinary microbiota and serum metabolite analysis in patients with diabetic kidney disease

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ABSTRACT

Background: Diabetic kidney disease (DKD) is a common and potentially fatal consequence of diabetes. Chronic renal failure or end-stage renal disease may result over time. Numerous studies have demonstrated the function of the microbiota in health and disease. The use of advanced urine culture techniques revealed the presence of resident microbiota in the urinary tract, undermining the idea of urine sterility. Studies have demonstrated that the urine microbiota is related with urological illnesses; nevertheless, the fundamental mechanisms by which the urinary microbiota influences the incidence and progression of DKD remain unclear. The purpose of this research was to describe key characteristics of the patients with DKD urinary microbiota in order to facilitate the development of diagnostic and therapeutic for DKD.

Methods: We evaluated the structure and composition of the microbiota extracted from urine samples taken from DKD patients (n = 19) and matched healthy controls (n = 15) using 16S rRNA gene sequencing. Meanwhile, serum metabolite profiles were compared using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Associations between clinical characteristics, urine microbiota, and serum metabolites were also examined. Finally, the interaction between urine microbiota and serum metabolites was clarified based on differential metabolite abundance analysis.

Results: The findings indicated that the DKD had a distinct urinary microbiota from the healthy controls (HC). Taxonomic investigations indicated that the DKD microbiome had less alpha diversity than a control group. Proteobacteria and Acidobacteria phyla increased in the DKD, while Firmicutes and Bacteroidetes decreased significantly ($P < 0.05$). Acidobacteria was the most prevalent microbiota in the DKD, as determined by the Linear discriminant analysis Effect Size (LEfSe) plot. Changes in the urinary microbiota of DKD also had an effect on the makeup of metabolites. Short-chain fatty acids (SCFAs) and protein-bound uremic toxins (PBUTs) were

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shown to be specific. Then we discovered that arginine and proline metabolism was the primary mechanism involved in the regulation of diabetic kidney disease.
Conclusions: This study placed the urinary microbiota and serum metabolite of DKD patients into a functional framework and identified the most abundant microbiota in DKD (Proteobacteria and Acidobacteria). Arginine metabolites may have a major effect on DKD patients, which correlated with the progression of DKD.

1. Introduction

Diabetic kidney disease (DKD) is a global health hazard that has steadily increased in prevalence over the last few decades. With the ongoing improvement of people's lifestyles and living standards, DKD has become a prevalent consequence of diabetes, estimated to impact 40% of diabetic patients [1]. DKD not only contributes greatly to the development of end-stage renal disease (ESRD) [2] but also raises the chance of developing [3]. The glomerular filtration rate (GFR) and urine albumin excretion rate (AER) are routinely employed to diagnose DKD, and several biomarkers have been connected with the disease. They still lack the specificity and sensitivity necessary for early diagnosis [4]. Despite efforts to slow the progression of DKD, no significant advances in the management of people with DKD have been made.

The microbiota, which consists of around 10 to 100 trillion symbiotic microbial cells, is distributed throughout the entire human body and contains all the genetic groups of microbial cells [5]. The bulk of microbiota can be found in the gut, mouth, skin, and vagina [6]. The gut is home to a vast number of microorganisms that encode about 3 million genes and produce thousands of metabolites, all of which work together to regulate various activities of the human host [7]. The intestines of healthy person are colonized by a diverse array of bacteria, with Firmicutes and Bacteroides accounting for the majority of the gut microbiota [8]. The gut microbiota is crucial for maintaining host homeostasis and for the development of a variety of disorders, including obesity, diabetes, liver disease, cancer, and DKD [9–12].

Due to the technological constraints of normal urine bacterial culture, urine is often assumed to be sterile. Recent investigations using 16S rRNA sequencing techniques have confirmed the presence of a distinct urinary microbiota in the urinary tract [13,14]. The existence of urinary bacteria in urine is not indicative of a urinary tract infection (UTI), as the bacteria that comprise the resident urinary microbiome are distinct from those associated with clinical UTIs [15–17]. Healthy urinary microbiota contains numerous bacterial taxa, primarily Lactobacillus, Corynebacterium, Staphylococcus, Streptococcus, Veillonella, and Prevotella, with sex-specific variations [18]. Numerous studies demonstrate the close connection between urine bacteria and human health and disease [19–21]. Recent research relating kidney diseases and comorbidities (such as chronic kidney disease, diabetes, and urinary tract infections) to urine microbiome dysbiosis has revealed alterations in the variety and abundance of disease-related urinary microbiome microorganisms [22–24].

DKD can impair the function of the intestinal barrier and result in microbiota migration, resulting in endotoxemia and inflammatory reactions and hastening the decline of renal function [25,26]. Although the majority of publications have confirmed the connection between DKD and gut microbiota, there are few investigations on urine microbiota. This study employed 16S rRNA sequencing technology and metabolomics technology to investigate the differences in urinary microbial diversity and metabolites between DKD patients and healthy individuals, as well as to determine the differential microbiota and differential metabolites, which would benefit in uncovering the pathogenesis of DKD and its diagnosis and treatment.

Table 1
Clinical characteristics of healthy controls and diabetic kidney disease patients.

Characteristics	DKD group (n = 19)	Healthy control group (n = 15)	t	*P*-value
Age	59.95 ± 9.38	43.73 ± 7.54	5.443	<0.001
Gender				1.000
Female	10 (52.6%)	7 (46.7%)		
Male	9 (47.4%)	8 (53.3%)		
BMI ($kg/m^{2)}$	24.16 ± 2.55	20.15 ± 1.05	6.22	<0.001
SBP (mmHg)	143.53 ± 22.6	112.67 ± 7.43	5.581	<0.001
MTP (mg/L)	2459.42 ± 676.81	0.00 ± 0.00	15.839	<0.001
PRO (g/L)	0.82 ± 0.89	0.03 ± 0.06	3.864	0.001
Urea nitrogen (mmol/L)	11.57 ± 3.46	4.56 ± 0.35	8.771	<0.001
Creatinine (μmol/L)	141.63 ± 50.46	69.67 ± 7.31	6.136	<0.001
Seruim acid (umol/L)	441.6 ± 142.89	302.1 ± 31.00	4.137	<0.001
ALT (U/L)	21.05 ± 8.35	78.29 ± 14.37	−0.211	0.835
AST (U/L)	21.79 ± 8.89	18.27 ± 4.73	1.482	0.149
FBG (mmol/L)	5.95 ± 1.51	5.45 ± 0.61	1.318	0.200
Cholesterol (mmol/L)	4.72 ± 1.86	4.44 ± 0.38	0.636	0.532
Triglyceride (mmol/L)	2.61 ± 3.95	1.49 ± 0.15	1.233	0.233
HDL-C (mmol/L)	0.92 ± 0.32	1.21 ± 0.08	−3.722	0.001
LDL-C (mmol/L)	2.51 ± 1.29	2.57 ± 0.15	−0.915	0.847
ALB (g/L)	33.32 ± 6.08	43.55 ± 2.90	−6.459	<0.001
HbA1c (mmol/L).	6.57 ± 1.62	4.88 ± 0.31	4.454	<0.001

2. Materials and methods

2.1. Study cohort and recruitment of subjects

To examine the urinary microbiota and serum metabolite in DKD patients, we recruited 19 patients diagnosed with the disease and 15 healthy participants for a traditional physical examination at Jinan University's First Clinical Medical College. The following criteria were used to identify patients with DKD: urine albumin creatine ratio (UACR) $\geq$ 30 mg/g or urinary albumin excretion rates (UAER) $\geq$ 30 mg/24 h ($\geq$20 μg/min) in at least 2 of 3 tests within 3–6 months; (eGFR $<$ 60 mL min^{-1}·$(1.73\ m^2)^{-1}$ for more than 3 months; renal biopsy consistent with the pathological changes of DKD. If concomitant with diabetic retinopathy (DR), accompanied by microalbuminuria or complicated with chronic kidney disease (CKD). Exclusion criteria were lack of clinical data, patients with type I diabetic nephropathy and other special types of diabetic nephropathy, combined with acute and chronic infectious diseases, acute exacerbation of cardiovascular and cerebrovascular diseases, antibiotics, probiotics or corticosteroid having been taken 3 months prior to sample collection. All healthy volunteers were over the age of 18 and had no history of sickness. The subjects were divided into two groups: those with diabetic kidney disease (DKD) and those with healthy controls (HC). Each patient was thoroughly informed about the goal of the trial and the experimental procedures. They all agreed to engage in the program on their own will and signed the informed consent form.

This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Jinan University's First Affiliated Hospital.

2.2. Metabolite extraction from urine and serum samples

Following an overnight period, all participants were assessed the following morning ($\geq$8 h). All patients had their peripheral blood and urine samples taken concurrently. 100 μL of the sample was transferred to an EP tube. After adding 400 μL of extract solution (1: 1 acetonitrile-methanol-water, comprising an isotopically labeled internal standard mixture), the samples were vortexed for 30 s, sonicated for 10 min in an ice-water bath, then incubated at −40 °C for 1 h to precipitate proteins. The material was then centrifuged at 12,000 rpm per minute for 15 min at 4 °C. The supernatant was then transferred to a new glass vial and analyzed. The quality control (QC) sample was created by combining an equal quantity of each sample's supernatants.

2.3. DNA extraction and 16S rRNA gene sequencing

We extracted genomic DNA from the samples using a PowerSoil DNA Isolation Kit (Qiagen, Germany). Then, we amplified the V3 and V4 sections of the 16S rRNA gene using universal primers (forward: 5′-ACTCCTACGGGAGGCAGCA-3'; reverse: 5′GGACTACHVGGGTWTCTAAT-3′). Following amplification, polymerase chain reaction products were combined with AMPure XP beads (Beckman Coulter, UK), and fragments screened and cleaned. The amplicons were quantified using a Qubit fluorometer (Invitrogen, USA) and sequenced using the HiSeq PE2500 sequencing instrument (Illumina, California, USA).

2.4. 16S rRNA sequencing data analysis

Trimmomatic version 0.33 [27] was used to filter the original data's quality, and Cutadapt version 1.9.1 [28] was used to locate and eliminate primer sequences. Additionally, reads were combined using FLASH version 1.2.7 [29]. Then, using UCHIME software version 4.2 (18), we were able to eliminate chimeric sequences and obtain high-quality clean tags. We assigned sequences with a similarity greater than 97% to the same operational taxon (OTU) using USEARCH version 10.0 [30]. Based on the 16S SILVA online database release 132 [31], the Naive Bayes classifier was used to perform taxonomic annotations on the feature sequence. The investigation of alpha diversity was carried out using the QIIME2 software (https://qiime2.org/). We calculated beta diversity using unweighted principal coordinate analysis (PCoA). Linear discriminant analysis (LDA) effect size (LEfSe) was used to compare microbiota between groups and to quantify the differentially abundant taxon (http://huttenhower.sph.harvard.edu/lefse/) [32]. To determine the difference in microbial community abundance between the two sets of samples, we utilized the Metastats program (http://metastats.cbcb.umd.edu/) to conduct a T-test on the species abundance data between the groups.

2.5. Liquid chromatography-tandem mass spectrometry data acquisition

The LC-MS/MS studies were conducted utilizing a UHPLC system (Vanquish, Thermo Fisher Scientific) equipped with a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm) and a Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo). The liquid contained two phases: mobile phase A (25 mmol/L ammonium acetate and 25 mmol/L ammonia hydroxide in water, pH = 9.75) and mobile phase B (acetonitrile). 3 μL of each sample was put into the system, and the temperature of the auto-sampler was set at 4 °C [33]. The QE HFX mass spectrometer was chosen because of its capacity to acquire MS/MS spectra using the information-dependent acquisition (IDA) mode under the control of the acquisition software (Xcalibur, Thermo). In this mode, the acquisition software constantly examines the complete scan MS spectrum. The following conditions were set for the ESI source: sheath gas flow rate of 30 Arb, auxiliary gas flow rate of 25 Arb, capillary temperature of 350 °C, full MS resolution of 60,000, MS/MS resolution of 7500, collision energy of 10/30/60 in NCE mode, and spray voltage of 3.6 kV (positive) or −3.2 kV (negative).

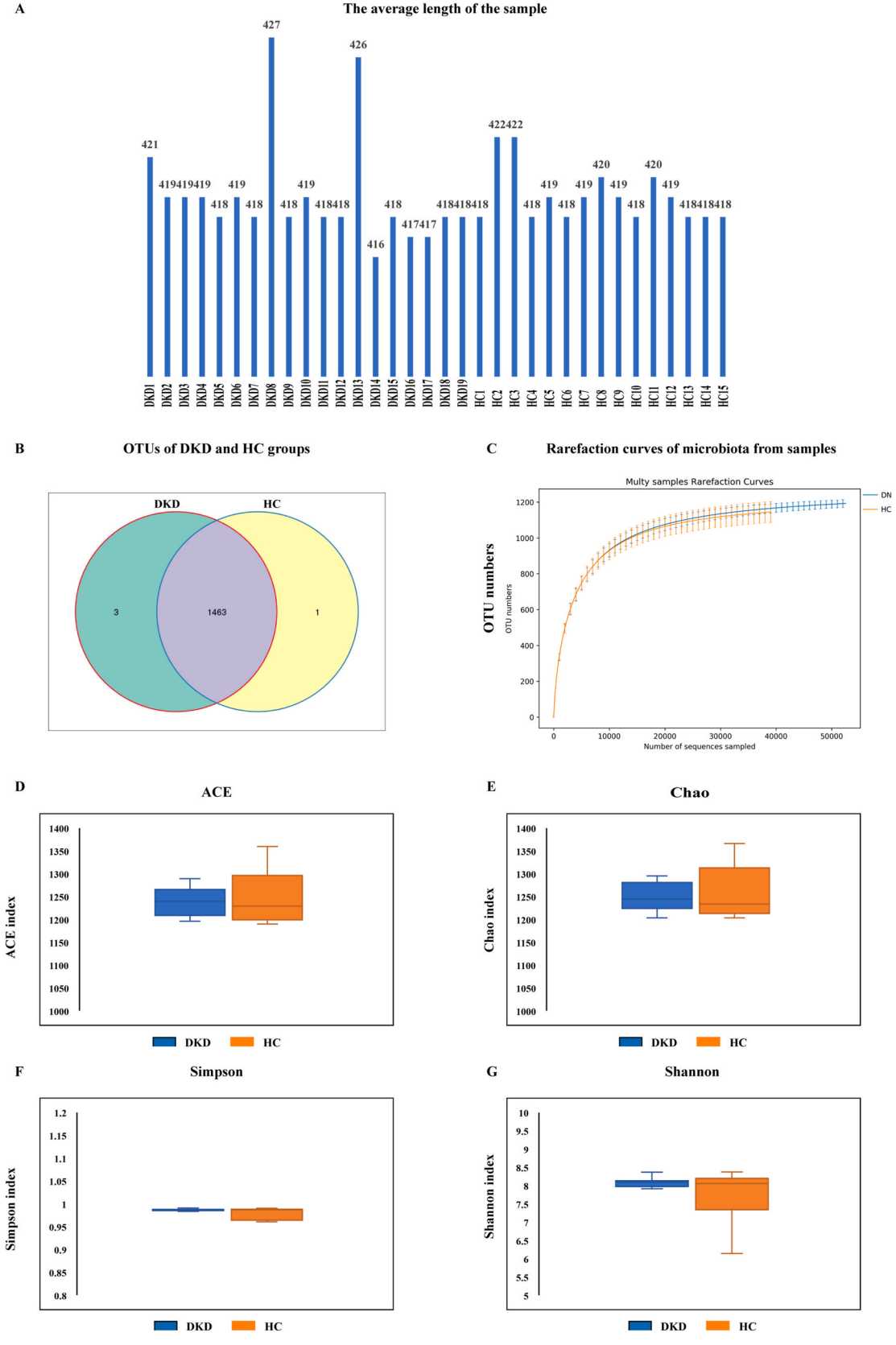

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Fig. 1. Microbiota diversity analysis. (A) The length of reading (B) Operational taxonomic units (OTUs) of diabetic kidney disease (DKD) and healthy control (HC) groups. C) Rarefaction curves of microbiota from serum samples. Means ± 95% confidence intervals are shown. (D–G) Abundance-based coverage estimator (ACE), richness estimator (Chao1) and diversity indices (Simpson and Shannon) of microbiota from the serum samples of DKD and HC groups.

2.6. Metabolomics data preprocessing and annotation

The QE platform's ionization source was ESI in both positive and negative ion modes. The raw data were converted to the mzXML format using ProteoWizard and processed using an in-house peak identification, extraction, alignment, and integration tool created in R and based on XCMS. Then, metabolite annotation was performed using an in-house MS2 database (Biotree DB). The annotation cutoff was set at 0.3.

2.7. Metabolomics analysis

Orthogonal projections to latent structures-discriminant analysis (OPLS-DA) was used for statistical analysis to evaluate global metabolic differences between comparable groups [34]. This technique was crucial for preventing overfitting of the test model and determining the statistical significance of the model. In the OPLS-DA model, variable significance in the projection (VIP) in projections was calculated. The value of p was obtained using the paired Student's t-test for analysis in a single dimension. When VIP > 1 and $P < 0.05$, metabolites were deemed statistically significant. Metabolic enrichment and pathway analysis based on Kyoto Encyclopedia of Genes and Genomes pathway database (KEGG, http://www.genome.jp/kegg/) were used to summarize and map biochemical pathways for metabolites that differed between the two groups [35–37]. After obtaining the matching information of differential metabolites, the pathway database of the corresponding species Homo sapiens (human) was searched and metabolic pathway analysis was performed. Through a complete examination of the pathways containing the differential metabolites (including enrichment analysis and topological analysis), we could then screen the pathways and identify the main pathways that are most closely associated with the metabolite discrepancies.

2.8. Statistical analyses

The results of this study were expressed as the mean ± standard deviation of continuous normally distributed variables and the median (interquartile range, IQR) of non-normally distributed continuous variables. The Student's t-test or the Pearson chi-square test were used to test for significant differences between the two groups. All data analyses were performed using R 4.0.3 statistical software. The Spearman rank correlation coefficient was used to calculate the correlation between different metabolites and various microbes, then it was visualized by heatmap in R version 3.5.3 (https://www.r-project.org), using the "heatmap" package. And Cytoscape version 3.7.1 (https://cytoscape.org/) was used to construct the relational network.

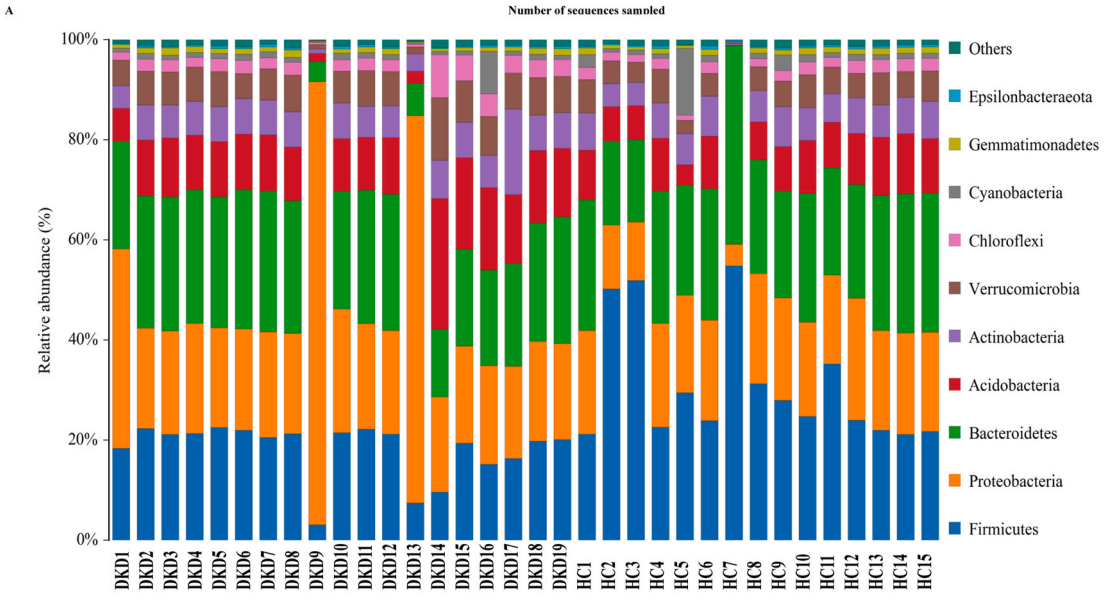
3. Results

3.1. The baseline data and clinical indicators of all participants

There were 34 participants in this study, including 19 patients with DKD (mean age 59.95 ± 9.38 years) and 15 HCs (mean age 43.73 ± 7.54 years). With significant age difference between the two groups ($P < 0.001$). And the blood pressure and body mass index (BMI) of the DKD group were higher than those of the HC group. MTP, proteinuria, serum albumin, creatine, urea nitrogen, serum uric acid and HbA1c were significantly different between the HC and DKD groups after 24 h ($P < 0.001$) (Table S1). There was no significant difference in ALT, AST, FBG, HDL-C, LDL-C, TG, TC and ALB between the two groups ($P > 0.05$). MTP, proteinuria, creatine, ureanitrogen, serumuric acid, HDL-C, ALB and HbA1c were significantly different between the HC and DKD groups after 24 h ($P < 0.001$). There was no sig-nificant difference in ALT, AST, FBG, LDL-C, TG and TC between the two groups ($P > 0.05$) (Table 1). The clinical indicators of all patients were shown in Supplementary Table S1.

3.2. Bacterial OTUs and diversity analyses

We acquired 2,685,485 high-quality 16S rDNA readings from the 34 samples, with a median read count of 79,974. (range from 79,726 to 80,313). After quality control, the sample averaged 419bp in length. 1467 OTUs were obtained following taxonomic assignment (Fig. 1A). The Venn diagram revealed three distinct OTUs in the DKD group and one distinct OTU in the HC group. Both groups shared 1463 OTUs (Fig. 1B). The rarefaction curves indicated that the depth and coverage of the sequencing were sufficient (Fig. 1C). Paired t-tests were used to compare Alpha diversity indices between treatments. The Shannon and Simpson indices demonstrated statistically significant variations in alpha diversity ($P < 0.01$), however the Chao and abundance-based coverage estimator (ACE) index did not differ significantly between DKD and HC (Fig. 1D–G). PCA, PCoA, and NMDS plots were used to compare the microbial communities from each DKD and HC group, and the results indicated a distinct separation of the two groups.



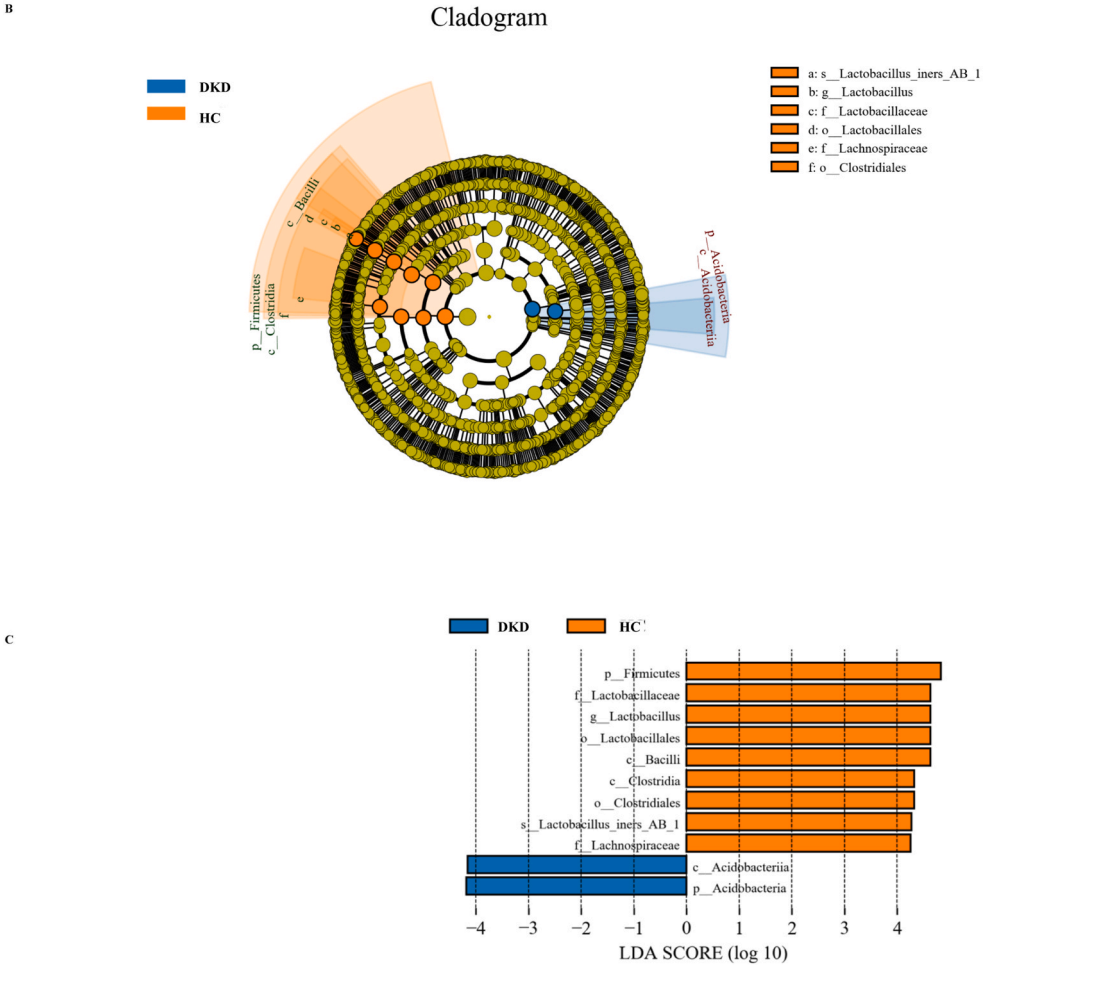


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Fig. 2. Bacterial Abundance Changes at Phylum Level and Linear Discrimination Analysis (LDA) (A) Ten major groups of bacteria in fecal samples from DKD and HC groups at the phylum level. (B) Cladogram of the phylogenetic distribution of microbes. Each circle represents a classification level from phylum to species from the inner to outer circles. The size of each circle is proportional to relative abundance. Microbes with no significant difference in abundance are shown in green. Microbes with an LDA value > 4 in DKD and HC groups are marked with orange and blue, respectively. (C) Differences in the relative abundance of bacteria in DKD and HC groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Abundance distribution and differential analysis of different biologic classifications

At the phylum level, ten major groups of bacteria have been found, including Firmicutes, Proteobacteria, Bacteroidetes, Acidobacteria, and Actinobacteria (Fig. 2A). Proteobacteria had a greater relative abundance in the DKD group (median value: 28.00%) than in the HC group (median value: 18.00%). Firmicutes were lower in the DKD group (median value: 18.20%) than in the HC (median value: 30.80%). Additionally, we quantified the relative abundance of OTU at the class, order, family, genus, and species levels and highlighted the top ten microbial groups (Supplementary Fig. S1). We used LEfSe analysis to determine the difference between the HC and DKD groups (Fig. 2B). There were 11 bacteria showed difference between the two groups, including 2 phylum, 3 classes, 2 orders, 2 families, 1 genus, and 1 species. (all LDA values (log10) > 4) (Fig. 2C). p_Firmicutes was the most prevalent taxon in the microbiota in HC when compared to DKD. Conversely, p_Acidobacteriia was more abundant in the DKD group. c_Bacilli and c_Clostridia were substantially more abundant than c_Acidobacteriia in the HC group, but c_Acidobacteriia was more abundant in the DKD group. o_Lactobacillales and o_Clostridiales were the most prevalent taxon in the microbiota at the order level in the HC. At the family level, f_Lactobacillaceae and f_Lachnospiraceae were notably numerous in the HC (Supplementary Table S2). Then, the *t*-test was used to identify species with significant differences ($P < 0.05$). p_Firmicutes、c_Bacilli、f_Lactobacillaceae、g_Lactobacillus、s_Lactobacillus_iners_AB-1 showed a significant difference between two groups (Supplementary Table S3).

3.4. Relationship between clinical indicators and microbiota in the DKD group

By using spearman correlation analysis, correlations between various species and various clinical indicators were estimated (Fig. 3A, Supplementary Table S4). The results showed that serum albumin was positively correlated with p_Firmicutes (r = 0.526, P = 0.001), o_Lactobacillales (r = 483, P = 0.004), c_Bacilli (r = 0.481, P = 0.004), f_Lactobacillaceae (r = 0.468, P = 0.005) and g_Lactobacillus (r = 0.458, P = 0.006). Estimated glomerular filtration rate (eGFR) was positively correlated with p_Firmicutes (r = 0.478, P = 0.005), o_Clostridiales, c_Clostridia, f_Lactobacillaceae and g_Lactobacillus ($P < 0.05$). LDL was positively correlated with g_Lactobacillus (r = 0.465, P = 0.006) and f_Lactobacillaceae (r = 0.451, P = 0.007). Glycosylated hemoglobin (HbA1c) was positively correlated with p_Acidobacteria (r = 0.520, P = 0.001) and c_Acidobacteriia (r = 0.504, P = 0.002). Age was positively correlated with p_Acidobacteria (r = 0.446, P = 0.008). On the contrary, age, BMI, systolic pressure, MTP, Creatinine (CR) and urea nitrogen (BUN) were negatively correlated with p_Firmicutes, f_Lactobacillaceae, g_Lactobacillus, c_Bacilli, o_Lactobacillales and s_Lactobacillus_iners_AB-1($P < 0.05$). According to the results of LEfSe analysis, p_Acidobacteria and c_Acidobacteriia were the most discrepant and abundant microbiota in the DKD group. Then, receiver operating characteristic (ROC) curve analyses were performed to further explore the application of the microbiota in the diagnosis of DKD. Area under the curve (AUC) were 0.765, and 0.758 for p_Acidobacteria and c_Acidobacteriia (Fig. 3B).

3.5. Identification of metabolites in serum samples

We used score plots and the principal component analysis (PCA) model to examine data. PCA was used to differentiate the DKD and HC groups. The orthogonal projections to latent structures-discriminant analysis (OPLS-DA) score chart revealed that the two sets of samples were statistically different and that the samples were essentially within the 95% confidence interval (Hotelling's T-squared ellipse) (Fig. 4A–B). Additionally, the permutation test for OPLS-DA demonstrated that the model is robust and does not exhibit overfitting (Fig. 4C–D). Differential serum metabolites (291) were obtained (VIP >1, $P < 0.05$) based on the OPLS-DA model (Supplementary Table S5). And 73 differential metabolites had a higher concentration between the two groups using the screening criteria VIP > 2, $P < 0.05$ and log fold change > 2 (Supplementary Table S6). The DKD group identified Organic acids and derivatives, Organoheterocyclic compounds, Organic oxygen compounds, Lipids, Lipid-like molecules, and Benzenoids.

3.6. Metabolic pathway analysis of the differential metabolites

To gain a better understanding of the differential metabolites' activities, we performed KEGG analysis on the differential metabolites in the DKD (Fig. 4E). The major metabolic pathways were those for arginine and proline (impact value: 0.27), histidine metabolism (impact value: 0.22) and pantothenate and CoA biosynthesis (impact value: 0.18). Nine microbiota-related metabolites enriched on the arginine and proline pathway, including Citrulline, L-Arginine, D-Proline, Hydroxyproline, Guanidoacetic acid, Creatinine, 5-Guanidino-2-oxopentanoate, 4-Aminobutyraldehyde, and 5-Aminopentanoic acid.

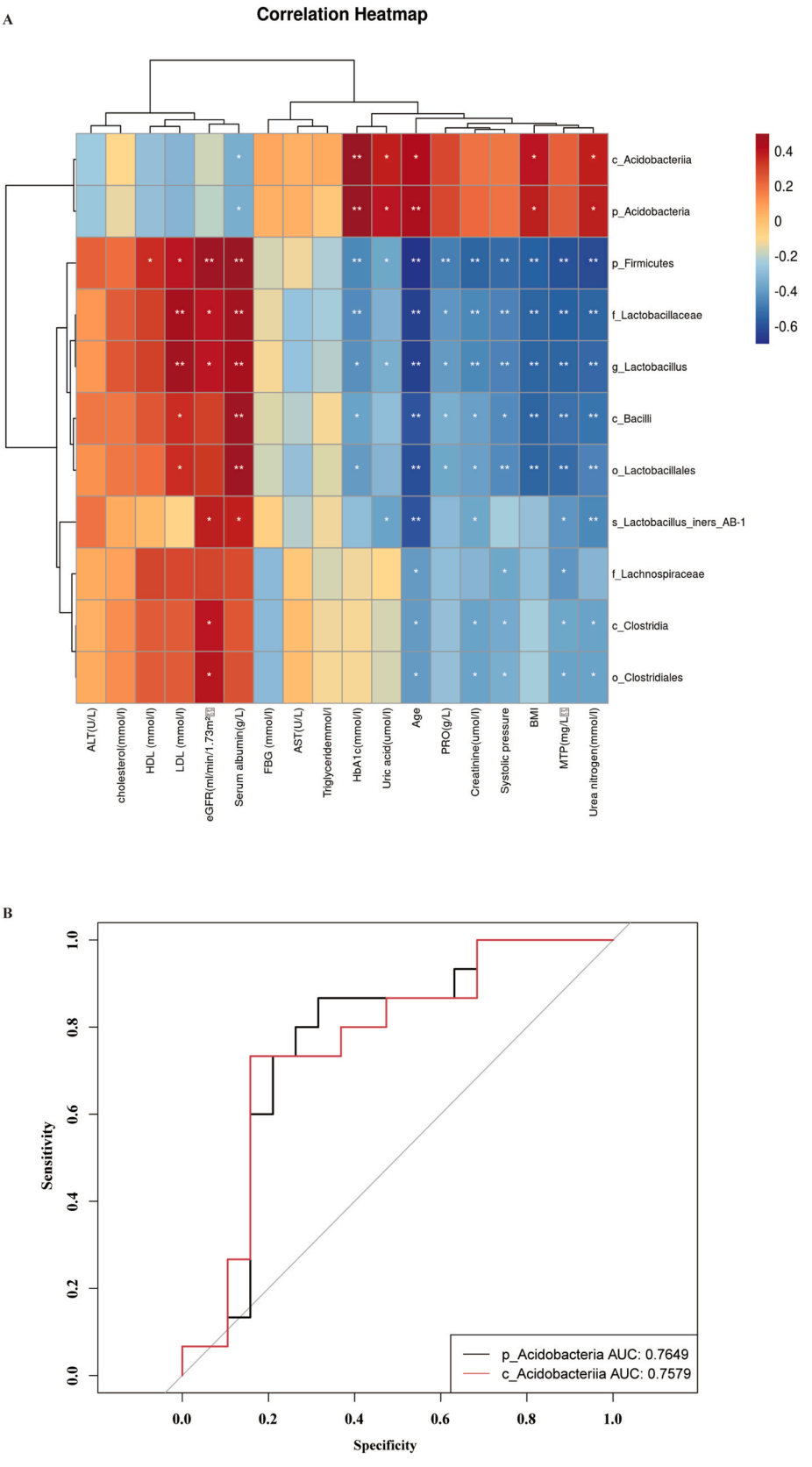

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Fig. 3. A. Correlation heatmap analysis between the various species and clinical indicators. Red represents a positive correlation and blue represents a negative correlation. B. ROC curve analyses are performed to assess the diagnostic of the microbial biomarkers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.7. Analysis of the correlation between differential serum metabolites and urinary microbiota in the DKD group

We then used spearman correlation analysis to examine the relationship between 73 differential serum metabolites and 11 differential urinary microbiotas. There was a clear distinction between serum metabolites and urine microbiota. p_Acidobacteria and c_Acidobacteriia were positively related to metabolites (Fig. 5). And other bacteria showed negative relationship with serum metabolites.

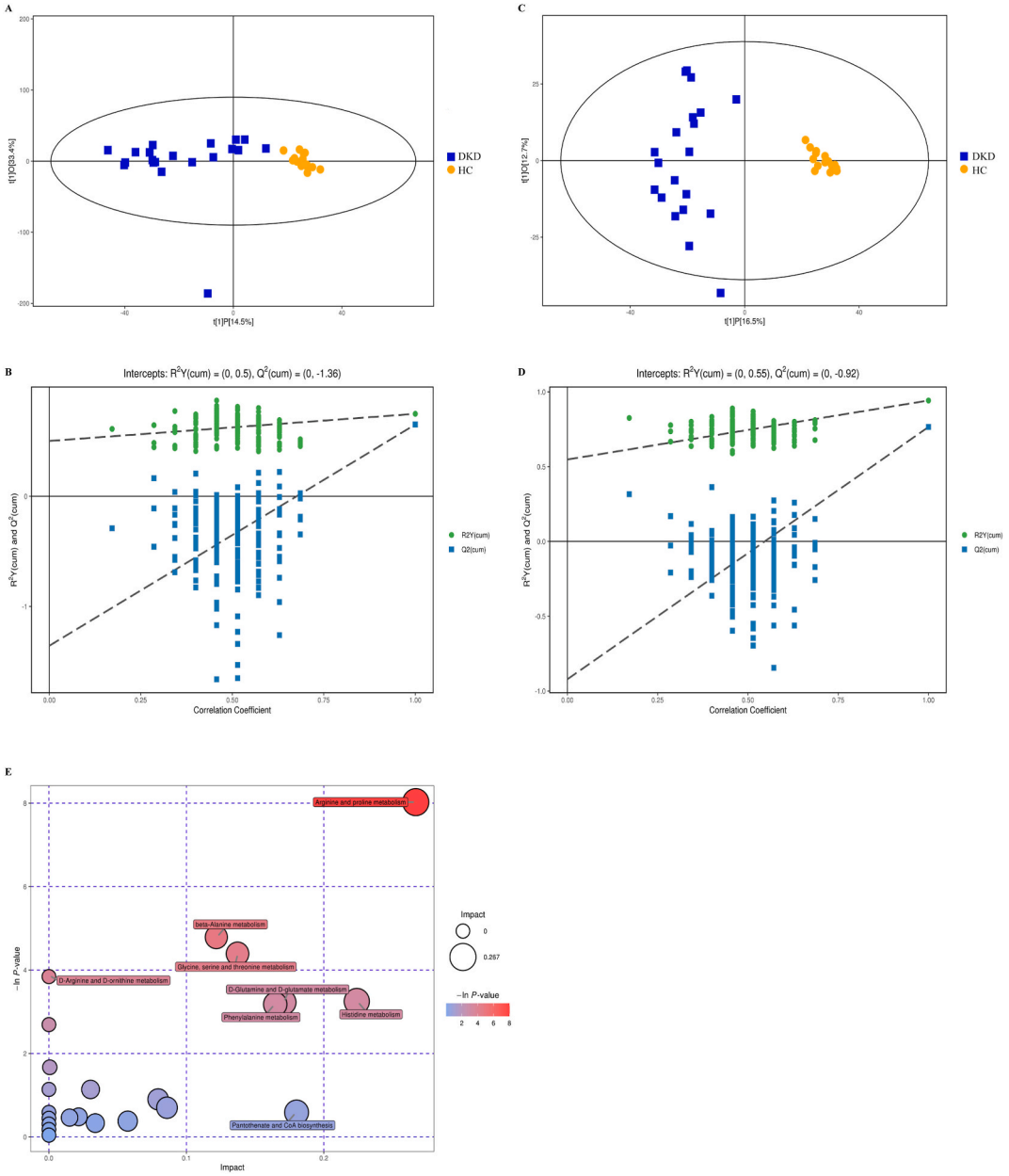

Fig. 4. Serum metabolomics analysis between groups in DKD patients. Orthogonal partial least squares discriminate analysis (OPLS-DA) score plots of serum metabolic profiling in positive mode(A-B) and negative mode (C-D); The bubble plot of KEGG analysis. Each bubble in the figure represents a KEGG pathway. The major metabolic pathways were Arginine and proline, Beta-alanine, and Glycine, serine, and threonine (E).

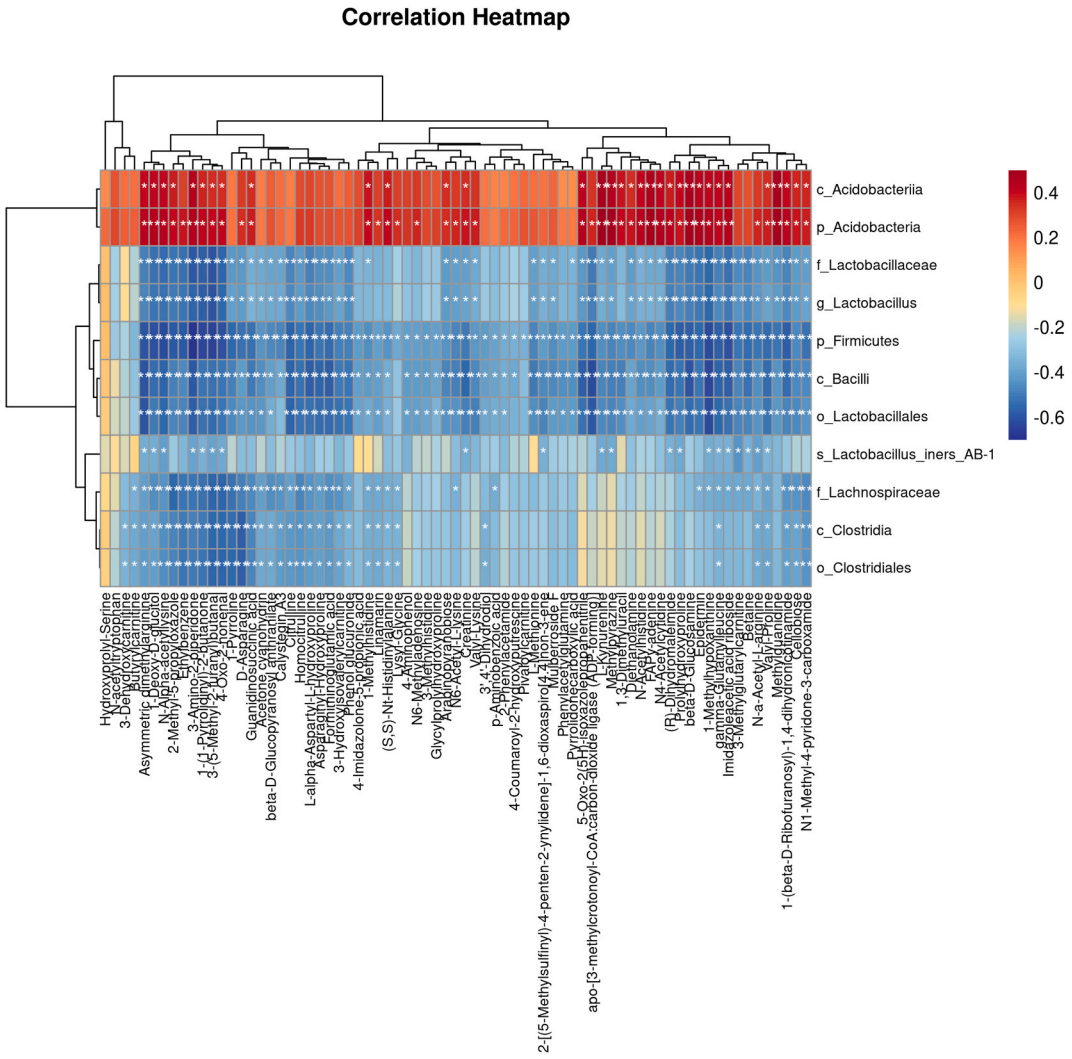

Fig. 5. Correlation heatmap analysis between differential serum metabolites and differential urine microbiota using Spearman rank correlation. Red represents a positive correlation and blue represents a negative correlation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

We employed 16S rRNA sequencing technology and metabolomics technology to investigate the differences in urinary microbial diversity and metabolites between DKD patients and healthy individuals. 11 significantly different microbiota and 73 significantly different metabolites were identified between the DKD group and the HC group. Our results demonstrated that healthy urine was not sterile, consistent with earlier findings, and that DKD patients had distinct urinary microbiomes than healthy person. The intestinal microbial ecology is involved in the digestion and absorption of nutrients, energy metabolism, immunological control, and a variety of other physiological functions [38–40]. Due to the significant attention on gut microbiota, there have been numerous findings confirming that a contributory role of gut microbiota in the process of DM and DKD [41,42]. Changes in the diversity of gut microbiota and the resulting metabolites can cause gut dysbiosis, leading to increased gut wall permeability, leakage of pro-inflammatory bacterial products such as lipopolysaccharide (LPS), and insulin resistance, and can even help accelerate the progression of kidney disease in DKD patients. Our findings indicated that the DKD and HC groups had significantly different microbial counts and relative abundances. Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria were the top four most abundant bacteria, which was consistent with the outcomes of a couple of earlier studies on the urine microbiome, where Proteobacteria was among the most abundant bacteria [23,43]. Firmicutes and Bacteroidetes are the two major bacterial groupings found in healthy humans, together accounting for around 90% of the phylum level [44]. Interestingly, we discovered that the DKD group possessed a larger relative abundance of Proteobacteria, Acidobacteria, and Actinobacteria, but lower levels of Firmicutes and Bacteroidetes than the healthy group. Nosratola D.Vaziri found that Actinobacteria, Firmicutes, and Proteobacteria were abundant in patients with ESRD than healthy controls [45]. Numerous

studies have demonstrated that the number of proteobacteria increases at the phylum level in obese and diabetic patients, while the Bacteroidetes phylum decreases [46,47]. Proteobacteria rise promotes the formation of Lipopolysaccharides (LPS), which results in an increase in pro-inflammatory factors and an inflammatory response [48].

Meanwhile, we performed a differential statistical analysis of the clinical characteristics. p_Acidobacteria and c_Acidobacteriia were positively correlated with clinical indicators in DKD patients. The results revealed distinctions in the compositional and functional characteristics of microbiota between the DKD and HC, suggesting that alterations in the intestinal microbiota may play a key role in the pathogenesis of DKD. We detected a considerable decrease in the relative abundance of Lactobacillus at the genus level in the DKD, while Escherichia-Shigella increased. Lactobacillus is high in probiotics, which aid in the maintenance of the intestinal functional barrier's integrity. Tae-Hee Lee et al. confirmed that BP121 boosted the number of good intestinal microbiota Lactobacillus and decreased kidney inflammation, oxidative stress, and uremic toxins [49]. Lactobacillus may create helpful organic acid lactate, which is metabolized in the colon to butyric acid [50]. As a result, we hypothesized that reducing Lactobacillus would potentially result in an increased inflammatory response. Additionally, butyrate contributes to the reduction of the pro-inflammatory effects of LPS activation. Escherichia-Shigella was shown to be abundant in stool samples of patients with DKD and reduced renal function after crossing the intestinal epithelial barrier [51]. Escherichia coli may create more indoxyl sulfate (IS) in people with advanced chronic kidney disease [52].

More and more data from trials in mice and humans suggests that metabolites produced by the gut microbiota play a critical role in the development and progression of renal disorders. Numerous studies have demonstrated that trimethylamine-oxide (TMAO), short-chain fatty acids (SCFAs), protein-bound uremic toxins (PBUTs), bile acids (BAs), tryptophan-derived metabolites, and branched-chain amino acids (BCAAs) were abundant in DKD patients, all of which play a significant role in the progression of DKD [53–59]. We observed an increasing rise in several organic nitrogen compounds, including TMPO, L-Carnitine, and Choline, in serum samples from DKD patients. Trimethylamine (TMA) is primarily generated from choline, phosphatidylcholine, and L-carnitine by intestinal bacteria and is subsequently oxidized to TMAO in the liver by monooxygenase 3 enzymes before being distributed to various tissues or eliminated via the kidney [60]. The TMAO pathway was the first to establish a relationship between gut microbe-produced compounds and the risk of cardiovascular and renal illness [61]. High TMAO levels were reported to worsen DKD in animal models, and supplementation with TMAO inhibitors (3,3-dimethyl-1-butanol) was found to mitigate the exacerbation of DKD symptoms [41,62]. Posada-Ayala et al. detected seven distinct metabolites in s from 16 chronic kidney disease patients and 15 healthy controls, with chronic kidney disease patients having higher plasma TMAO levels, consistent with our finding [63].

Numerous PBUTs (for example, indoxyl sulfate, phenyl sulfate, and phenylacetylglutamine) are derived from the results of microbial metabolism of food chemicals in the intestine, including aromatic amino acids, tyrosine, phenylalanine, and tryptophan [64]. Barrios et al. established that indoxyl-sulfate, p-cresyl-sulfate, and phenylacetylglutamine were all early indicators of renal function decrease [65]. Numerous investigations have demonstrated that phenyl sulfate may impair the barrier function of the glomerular basement membrane, resulting in increased urine protein levels [66]. Increased serum phenylacetylglutamine levels have been shown to be an independent risk factor for cardiovascular disease [67,68], implying that DKD patients in this study may be at an increased risk of developing cardiovascular disease. Certain uremic toxins, such as Phenylacetylglutamine, *N*-acetyl-L-arginine, Methylguanidine, and Guanidinosuccinic acid, can be found as metabolites in patients with end-stage renal failure. They demonstrated an upward trend in DKD in our study, implying that it may advance to ESRD. These uremic solutes have the potential to impair endothelium repair following injury and to cause direct vascular damage, hence increasing the risk of cardiovascular problems. In our study, they showed an uptrend in the DKD, which suggested that it might progress to ESRD. These uremic solutes have the potential to impair endothelium repair following injury and to cause direct vascular damage, hence increasing the risk of cardiovascular problems.

We analyzed the KEGG database to gain a better understanding of the probable function of differential metabolism. We discovered an enrichment in arginine and proline metabolism in serum samples from DKD patients. Citrulline, L-Arginine, D-Proline, Hydroxyproline, Guanidoacetic acid, Creatinine, 5-Guanidino-2-oxopentanoate, 4-Aminobutyraldehyde, and 5-Aminopentanoic acid were all constituents of this pathway. Arginine and its metabolites are involved in a variety of metabolic processes. In adults, glutamine and proline are metabolized to citrulline in the colon via pyrroline-5-carboxylate (P5C), and subsequently to arginine in the kidneys. L-arginine is a precursor to the formation of nitric oxide (NO), polyamines, and agmatine. These metabolites play a role in the progression of renal disease [69,70]. Additionally, NO metabolism is critical for endothelial dysfunction in DKD [71]. Although animal models of kidney disease have demonstrated that supplementing with L-arginine is good for diabetic nephropathy, the mechanism by which it works is yet unknown [72]. Numerous traditional Chinese remedies, including Cicada Cordyceps Polysaccharide, Tangshen Recipe, and Shenyankangfu Pian, have demonstrated that DKD can be treated by modifying the intestinal microbiota's composition or function.

However, our research was not without flaws. There was a confounding bias between DKD patients and healthy controls, and the sample size was insufficient and must be increased in future research. The experiment used serum samples for differential metabolite analysis and did not include urine samples for comparison. We used the 16S RNA gene sequencing technique to determine bacterial taxa at a low resolution (genus) level in order to investigate the urinary microbiota. As a result, the absence of comprehensive characterization of the entire microbiome precluded us from examining the taxonomic and functional potentials of species and subspecies.

5. Conclusion

In general, we detected significant differences in the composition and function of the urinary microbiota in patients with DKD. Compared with healthy controls, the DKD group had fewer total bacteria and their urine microbiota shifted from Firmicutes to

Proteobacteria and Acidobacteria. The urinary microbiota was found to be associated with DKD's inflammatory state and renal function. The accumulation of metabolites stimulates the immune system continually, resulting in increased production of inflammatory factors and kidney damage. Additionally, we noticed a correlation between arginine and proline metabolism and DKD. Patients with diabetic nephropathy may need to adapt their diet and arginine intake correctly, which may bring additional therapy options.

Ethics approval and consent to participate

Approved by the Ethics Committee of the First Affiliated Hospital of Jinan University (KY-2021-017), all samples were obtained from the First Affiliated Hospital of Jinan University. We fully explained the purpose and experimental procedures to each patient. They all volunteered to participate in the program and signed the informed consent.

Consent for publication

Not applicable.

Data availability statement

The mass spectrometry proteomics data have been deposited to the figreshare database (https://figshare.com/DOI 10.6084/m9.figshare.20239662).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Abbreviations

DKD	diabetic kidney disease
HC	healthy controls
LEfSe	Linear discriminant analysis Effect Size
ERSD	end-stage renal disease
GFR	glomerular filtration rate
AER	albumin excretion rate
UACR	urine albumin creatine ratio
UAER	urinary albumin excretion rates
eGFR	estimated glomerular filtration rate
HbA1c	glycosylated hemoglobin
CR	creatinine
BUN	urea nitrogen
SCFAs	Short-chain fatty acids
PBUTs	protein-bound uremic toxins
OUT	operational taxon
ACE	abundance-based coverage estimator
PCoA	principal coordinate analysis
LDA	linear discriminant analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
ROC	receiver operating characteristic

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17040.

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