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The potential role of the gut microbiota in modulating renal function in experimental diabetic nephropathy murine models established in same environment

Yang Li[a,b,c,1], Xinhuan Su[d,1], Ying Gao[e], Chenxiao Lv[a,b,c], Zhiwei Gao[e], Yipeng Liu[a,b,c], Yan Wang[f], Shujuan Li[g,*,2], Zunsong Wang[a,b,c,**,2]

[a] Department of Nephrology, The First Affiliated Hospital of Shandong First Medical University, Shandong Provincial Qianfoshan Hospital, Shandong University, No.16766 Jingshi Road, Jinan, Shandong 250014, China
[b] Shandong Provincial Key Laboratory for Rheumatic Disease and Translational Medicine, No.16766 Jingshi Road, Jinan, China
[c] Nephrology Research Institute of Shandong Province, No.16766 Jingshi Road, Jinan, China
[d] Department of Endocrinology, Shandong Provincial Hospital affiliated to Shandong University, Shandong Provincial Key Laboratory of Endocrinology and Lipid Metabolism, Institute of Endocrinology and Metabolism, Shandong Academy of Clinical Medicine, Jinan, Shandong 250000, China
[e] Weifang Medical University, No.7166 Baotong West Street, Weifang, Shandong 261053, China
[f] Department of Hematology, The First Affiliated Hospital of Shandong First Medical University, Shandong Provincial Qianfoshan Hospital, Shandong University, No.16766 Jingshi Road, Jinan, Shandong 250014, China
[g] Department of Nephrology, The First people's Hospital of Yancheng, No. 66 Renmin South Road, Yancheng 224005, China.

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ABSTRACT

Recent studies have shown that laboratory murine autoimmunity models under the same environment display different outcomes. We established diabetic nephropathy model mice under the same environment using the classic streptozotocin method. Renal dysfunction was different among the mice. Proteinuria was more significant in the severe proteinuria group (SP) than in the mild proteinuria group (MP). We hypothesized a role for the gut microbiota in the outcome and reproducibility of induced DN models. 16S rDNA gene sequencing technology was used to analyze the differences in the gut microbiota between the two groups. Here, through fecal microbiota transplantation (FMT) and gas chromatography mass spectrometry (GC–MS), we verified the role of the gut microbiota and its short-chain fatty acid (SCFA) generation in DN mouse renal dysfunction. In the SP group, there was a reduced abundance of Firmicutes ($P < 0.0001$), and the dominant genus Allobaculum [linear discriminant analysis (LDA) > 3, $P < 0.05$] was positively correlated with body weight (Rho = 0.767, $P < 0.01$) and blood glucose content (Rho = 0.648, $P < 0.05$), while the dominant genus Anaerosporobacter (LDA > 3, $P < 0.05$) was positively correlated with 24-hour urinary protein content (Rho = 0.773, $P < 0.01$). In the MP group, the dominant genus Blautia (LDA > 3, $P < 0.05$) was negatively correlated with 24-hour urinary protein content (Rho = −0.829, $P < 0.05$). The results indicated that Allobaculum and Anaerosporobacter may worsen renal function, while Blautia may be a protective factor in DN. These findings suggested that the gut microbiota may contribute to the heterogeneity of the induced response since we observed potential disease-associated microbial taxonomies and correlations with DN.

1. Introduction

Diabetes mellitus (DM) has become one of the most prevalent chronic diseases in humans. Diabetic nephropathy (DN) is a critical complication of DM, as approximately 35% of all diabetic patients may develop DN [1]. DN has surpassed variable glomerulonephritis as the first cause of end-stage renal disease (ESRD) [2].

Murine models of DN play an important role in the study of DN

* Corresponding author.
** Correspondence to: Z. Wang, Department of Nephrology, The First Affiliated Hospital of Shandong First Medical University, Shandong Provincial Qianfoshan Hospital, Shandong University, No.16766 Jingshi Road, Jinan, Shandong 250014, China.
E-mail addresses: liuchengyuan@163.com (S. Li), wzsong3@163.com (Z. Wang).
[1] Yang Li and Xinhuan Su contributed equally to first authors.
[2] Zunsong Wang and Shujuan Li contributed equally to corresponding authors.

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pathogenesis and its impact on the body. However, in the model construction process, even when the mouse strain, supplier, feeding environment and diet are completely consistent, the repeatability is still not 100% [3]. In recent years, it has been found that the gut microbiome is inextricably linked to an increasing number of diseases, and it has been suggested that the gut microbiome may be one of the confounding factors for the successful construction of murine models of human disease [4–7]. The gut microbiome is a general term for various microorganisms widely present in the gut of mammals. With the development of next-generation sequencing (NGS) technology, research on the gut microbiome has become increasingly prevalent in recent years. There are > 100 trillion microbial cells in the human body, including bacteria, archaea, yeast and fibrous fungi, which are > 10 times more abundant than human cells, and the number of genes they encode is 100 times the number of human genes [8]. These flora constituents play a pivotal role in metabolism, nutrition and immunity, and we can even treat the gut microbiome as an endogenous organ with active metabolism in the body [1,9]. With technological advancement, the relationship between the composition of the human microbiome, such as that of the gut microbiome, and corresponding diseases is being explored gradually and studied extensively [10]. Variation in the gut microbiome is connected with endocrinopathy, such as polycystic ovarian syndrome (PCOS), obesity, and DM [11–14]. As mammals, mice have a gut microbiome that has a similar distribution and abundance as that of humans, and only *Fusobacteria* is not detected, while *TM7* is unique.

In one of our recent studies, we planned to develop some DN murine models, but ultimately, no > 2/3 of the DN model mice we raised had severe proteinuria, while others developed only DN without significant renal function damage [15]. We wondered why there were different outcomes in the mice under the same environments. We hypothesized that the gut microbiome plays a crucial role in the induction of DN in murine models. Therefore, we designed this study to compare the intestinal microbial composition of mice with different outcomes during induction of DN to investigate whether differences in the gut microbiome could help explain the different outcomes of disease model construction and their impact on biochemical indexes of disease.

2. Materials and methods

2.1. DN mouse models

The mouse samples used in the present work were obtained from one of our recent studies, as described in the Introduction section. Seventeen male specific-pathogen-free (SPF) C57BL/6 mice (40 days old) were supplied by the Experimental Animal Center of Shandong University and raised in a temperature- and humidity-controlled laminar flow room under a 12 h light/12 h dark cycle with free access to water and food. After 3 days of adaptive feeding, the DN model was established by intraperitoneal injection of streptozotocin (STZ, 80 mg/kg/d, Sigma-Aldrich, St. Louis, MO, USA) for 3 days. All mice were maintained on a high-fat diet (19.7 kJ/g, 45% of energy as fat, Research Diets, New Brunswick, NJ, USA) for 12 weeks, in accordance with the study by Liu et al. [16,17]. During this period, the parameters including body weight (BW), blood glucose (BG) and 24-h urinary protein content (24 h-UP) were measured weekly. Tail blood samples were used to perform BG measurements with a Glucometer Elite (Bayer, Leverkusen, Germany). At the end of this procedure, 11 DN mice with severe proteinuria (with BG ≥ 16.67 mmol/L and 24 h-UP ≥300 mg/24 h) and 6 DN mice with mild proteinuria (with BG ≥ 16.67 mmol/L and 24 h-UP < 300 mg/24 h) were obtained and divided into the SP group and MP group, respectively. The pre- and post-STZ parameters of the mice are described in Table 1.

2.2. Biospecimen collection, DNA extraction and sequencing

A total of 51 fecal pellets were obtained from the intestinal lumen after sacrifice of the mice, with 3 pellets per mouse (SP group and MP group). Seventeen of the fecal pellets were used for sequencing, 17 were used to analyze metabolites, and the remaining 17 were used to obtain microbiota-containing supernatants that were stored in a freezer (Haier, Qingdao, Shandong, CN) at −80 °C (Fig. 1a). The method for preparing microbiota-containing supernatants is described in detail in the FMT section. Genomic DNA was extracted using the cetyl trimethylammonium bromide (CTAB) method [18]. A NanoDrop 2000 (Thermo Electron Corporation, Waltham, MA, USA) spectrophotometer was used to determine the concentration of the extracted DNA. The V1-V2 regions of the 16S rRNA gene were amplified and sequenced on an Illumina HiSeq 2500 system (Illumina, Hayward, CA, USA). According to the reported bacterial 16S rRNA gene sequences in GenBank, we used DNAMAN V6 (Chinese version) software (Lynnon Biosoft Company, San Ramon, CA, USA) to compare the homology of the bacteria and chose the highly conserved V1-V2 region to design a pair of primers (F: 5′-AGAGTTTGATCMTGGCTCAG-3′ and R: 5′-GCTGCCTCCCGTAGGAGT-3′) according to the basic principles of primer design [19]. PCR (polymerase chain reaction) amplification was performed on the target fragments. Follow-up experiments were carried out according to the sequencing manual.

2.3. 16S rRNA gene sequence analysis

The 16S sequencing paired-end data set was compiled and quality filtered using the Laser FLASH (fast length adjustment of short reads) method [20]. All sequences were analyzed using the Quantitative Insights into Microbial Ecology (QIIME, version 1.9.1) software suite [21]. Sequences were clustered against the Greengenes (13.8 release) ribosomal database's 97% reference data set (http://greengenes.secondgenome.com/downloads). Sequences that remained unmatched with any of the entries in this reference were subsequently clustered into de novo OTUs at 97% similarity with the UCLUST algorithm. Taxonomy was assigned to all OTUs using the ribosomal database project (RDP) classifier within QIIME and the Greengenes reference data set [22]. Rarefaction and rank abundance curves were calculated from OTU tables using alpha diversity and rank abundance scripts within the QIIME pipeline. Hierarchical clustering based on population profiles of the most common and abundant taxa was performed using UPGMA (unweighted pair group method with arithmetic mean, also known as average linkage) clustering on the distance matrix of OTU abundance. This method resulted in a Newick-formatted tree, which was obtained utilizing the QIIME package. Furthermore, Calypso online software (version 8.20, http://cgenome.net/wiki/index.php/Calypso) and R software (version 3.5.1) were used to analyze alpha diversity (Shannon, ACE, and Chao1), beta diversity [weighted UniFrac distances, principal coordinate analysis (PCoA)], linear discriminant analysis effect size (LEfSe), and correlations between biomarkers and biochemical indexes.

2.4. Fecal microbiota transplantation (FMT)

Forty naive male SPF C57BL/6 mice (6–8 weeks old) were obtained from the Experimental Animal Center of Shandong University, and the raising environments and methods were the same as those described previously. After 3 days of adaptive feeding, all the mice were randomly divided equally into two cohorts (cohort 1 and cohort 2). In cohort 1, the 20 mice were randomly divided equally into two subgroups (FMT-SP group and FMT-MP group) and then received antibiotic treatment before transplantation of fecal microbiota from the SP group and MP group, respectively. At the time of FMT completion, intraperitoneal injection of STZ was performed as described previously. During this process, the living environments of the mice were kept as clean as

Table 1
The pre- and post-STZ parameters of mice.

Group	Pre-STZ parameters			Post-STZ parameters		
	BW (g)	BG (mmol/L)	24 h-UP (mg/24 h)	BW (g)	BG (mmol/L)	24 h-UP (mg/24 h)
SP	29.80 (28.20–30.40)	9.40 (8.70–9.70)	42.60 (37.47–60.36)	21.80 (20.80–24.00)	30.10 (28.60–33.30)	530.81 (386.38–816.72)
MP	29.85 (27.35–31.45)	8.95 (8.65–9.13)	53.85 (44.31–62.67)	21.25 (20.60–22.30)	24.10 (22.43–29.05)	220.79 (207.70–239.95)
P	0.73	0.27	0.27	0.45	0.10	0.001

Note: STZ: streptozotocin, BW: body weight. GLU: blood glucose. 24 h-UP: 24-hour urinary protein content. SP: severe proteinuria. MP: mild proteinuria.

possible.

Unlike those in the first cohort, mice in cohort 2 received an STZ injection before FMT when antibiotic treatment was complete. At the end of the 12th week after STZ injection, the 20 DN mice were randomly divided equally into two subgroups (P1-FMT-SP group and P2-FMT-MP group). The living environments and raising methods were the same as those in cohort 1.

After FMT was complete, the relative abundance of several major microbiota constituents was analyzed using quantitative real-time polymerase chain reaction (qPCR). The methods of antibiotic treatment and FMT were described below.

Antibiotic-treated mice were given the following cocktail for 2 weeks: 500 mg of ampicillin (Sigma-Aldrich, St. Louis, MO, USA), 250 mg of vancomycin (Sigma-Aldrich, St. Louis, MO, USA), 500 mg of neomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA), 500 mg of metronidazole (Sigma-Aldrich, St. Louis, MO, USA), and 10 g of grape Kool-Aid (Kraft Foods Group Inc., Northfield, Ill, USA) in 500 mL of water, which was sterile filtered through a 0.22 μm filter. Water bottles were changed once per week [23,24].

Feces from mice in the previously described SP and MP groups were collected as described above. Each fecal sample was diluted in phosphate-buffered saline (PBS, 15 mL/mouse, Sigma-Aldrich, St. Louis, MO, USA) to obtain a solution with a concentration of 120 mg/mL, then homogenized by vortexing and minimally clarified by low-speed centrifugation for 5 min at 300 $\times g$ to obtain the supernatants. Glycerol (Sigma-Aldrich, St. Louis, MO, USA) was added to each supernatant, and the concentration of the supernatant was adjusted to 20%. Then, all supernatants were stored at −80 °C. Before FMT, all the supernatants were mixed in one container. Mice were then gavaged three times with 150 μL of the mixture each time. Gavages occurred on day 1, day 2 and day 5 post-4 antibiotic treatments [24].

2.5. qPCR

The gDNA from the feces of the mice in the FMT groups was obtained by the CTAB method. Then, SYBR Green qPCR technology was used to analyze the changes in the phylum Firmicutes and the genera Allobaculum and Blautia (Anaerosporobacter was not analyzed because there were few references or data about primers for this microbiome constituent). The qPCR primers were purchased from Sangon Biotech (Shanghai, CN) Co., Ltd., and the 16S rRNA gene was used as a reference gene (internal reference). A TB Green™ Premix Ex Taq™ kit was purchased from TaKaRa (Shiga Prefecture, Kusatsu City, JPN), and q-PCR was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). Three replicates of each sample were analyzed. The relative changes in expression were calculated by the $2^{-\Delta\Delta\ CT}$ value. The primers used are as follows: Univ337-F: 5′-ACTCC TACGGGAGGCAGCAGT-3′ and Univ518-R:5′-GTATTACCGCGGCTGCT GGCAC-3′; p-Firm934-F: 5′-GGAGYATGTGGTTTAATTCGAAGCA-3′ and p-Firm1060-R: 5′-AGCTGACGACAACCATGCAC-3′; g-Blau-F: 5′-GTGA AGGAAGAAGTATCTCGG-3′ and g-Blau-R: 5′-TTGGTAAGGTTCTTCGC GTT-3′; g-Allo-F: 5′-ACCTGCGGTGCATTAGYTGG-3′ and g-Allo-R: 5′-GCATYGCTCGTTCAGGCTTGC-3′.

2.6. Analysis of lipopolysaccharide, trimethylamine oxide and SCFA

Serum lipopolysaccharide (LPS) and trimethylamine oxide (TMAO) were analyzed using an EC Endotoxin Test Kit (Bioendo, Xiamen, Fujian, China) and a Mouse TMAO ELISA Kit (Jianglai, Shanghai, China), respectively. Analysis of SCFA in mouse feces was performed by GC–MS. Samples were analyzed by a 7890A gas chromatography system coupled to a 5975C inert XL EI/CI mass spectrometer (Agilent Technologies, Santa Clara, CA). The concentration of SCFA was standardized with fecal mass.

2.7. Statistical analysis

The biochemical indexes of the subjects are represented as the median (Q1 – Q3). The differences in the biochemical indexes were compared by the Mann-Whitney *U* test. Moreover, alpha diversity was determined using the Mann-Whitney U test, and beta diversity was acquired by ANOSIM (analysis of similarities). LEfSe combines the Kruskal-Wallis test or pairwise Wilcoxon rank sum test with LDA, whose threshold value on the logarithmic LDA score equals 3.0. Spearman's rank correlation method was used to analyze the relationship between the microbiome and biochemical indexes. Analyses were performed using the SPSS statistical package (version 17.0), R software (version 3.5.1), Calypso online software (version 8.20) and GraphPad Prism 7 software. P values < 0.05 were considered statistically significant.

3. Results

3.1. DN mouse models construction

After model construction, all 17 mice developed DN with mild or severe proteinuria. The changes in BG, BW, and 24-h UP of the mice are shown in Fig. 1b.

3.2. Sequencing metrics

From 16S rRNA gene sequencing (V1-V2 regions), the averaged coverage and subsampling were sufficient to describe gut bacterial communities according to sequence-based rarefaction curves after quality filtering.

3.3. Gut microbiome composition of the SP and MP groups

To assess whether the gut microbiome has an effect on the biochemical indexes of DN mice, we compared the differences in the gut microbiome between the SP group ($n = 11$) and the MP group ($n = 6$). The comparison of α diversity showed (Fig. 2) that there was no significant difference in species abundance (Chao1 and ACE) or Shannon index between the two groups ($P > 0.05$). Ward hierarchical clustering (Fig. 3) suggested that there were some differences in the composition of the gut microbiome between the two groups. At the same time, in terms of β diversity, principal coordinate analysis (PCoA) based on UniFrac distance (Fig. 4a) further indicated that the gut microbiome samples of the SP group and MP group were from different populations. LEfSe (Fig. 4b) based on UniFrac distance further revealed the types of

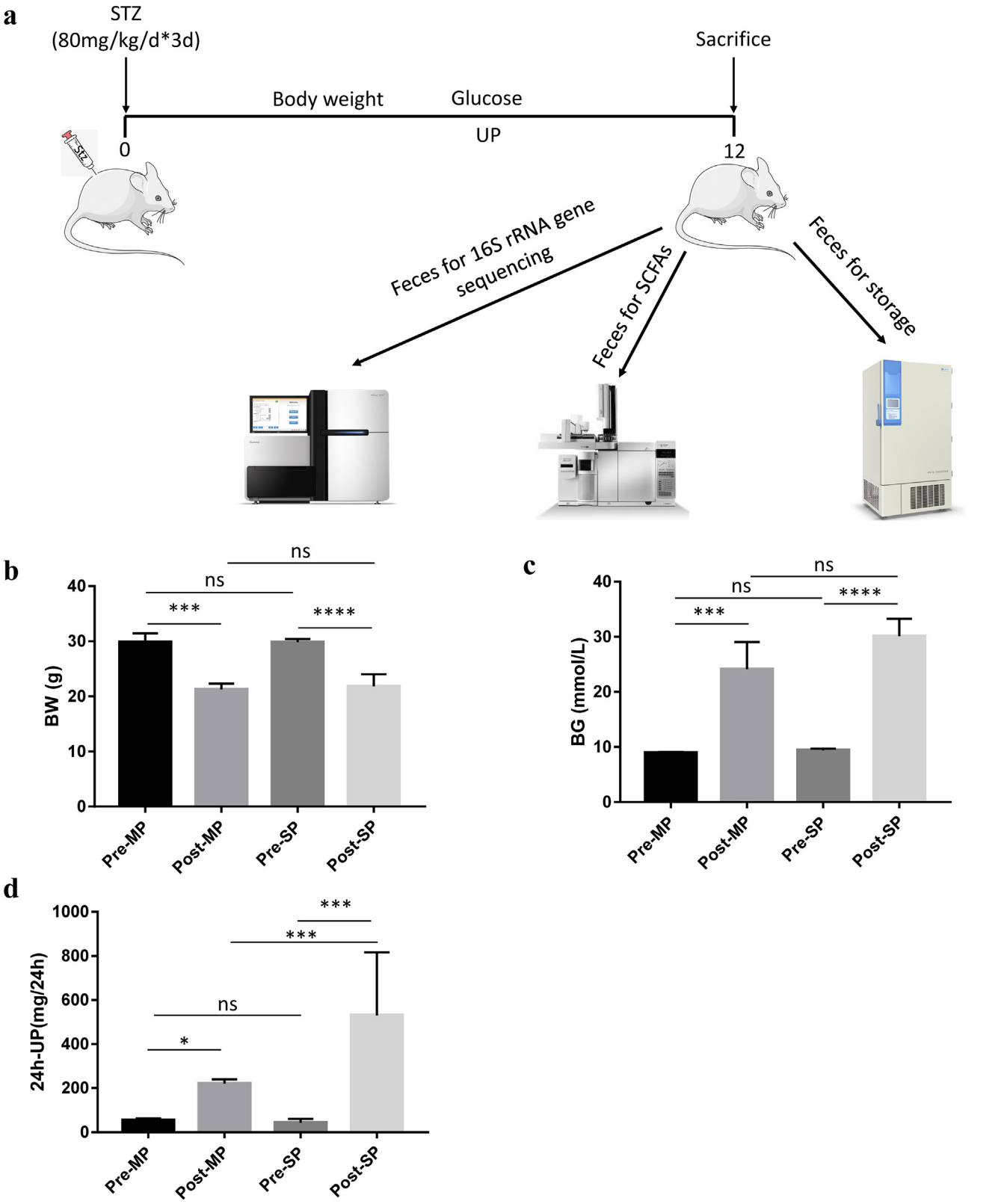

Fig. 1. The DN mice models and their biochemical indexes. a. The process of DN mice models construction. b–d. The biochemical indexes of the DN mice models. BW: body weight. BG: blood glucose. UP: urinary protein.

differentially abundant bacteria between the two groups. At the phylum level, the abundance of Firmicutes in the MP group was higher than that in the SP group, and the difference was statistically significant (Fig. 5a, $P < 0.01$), while Bacteroides abundance did not show significant differences between the two groups (Fig. 5a, $P = 0.157$). The Firmicutes/Bacteroides ratio was higher in the MP group than in the SP group (Fig. 5b, $P < 0.01$). At the genus level, *Allobaculum* and *Anaerosporobacter* were more abundant in the SP group, while *Blautia*

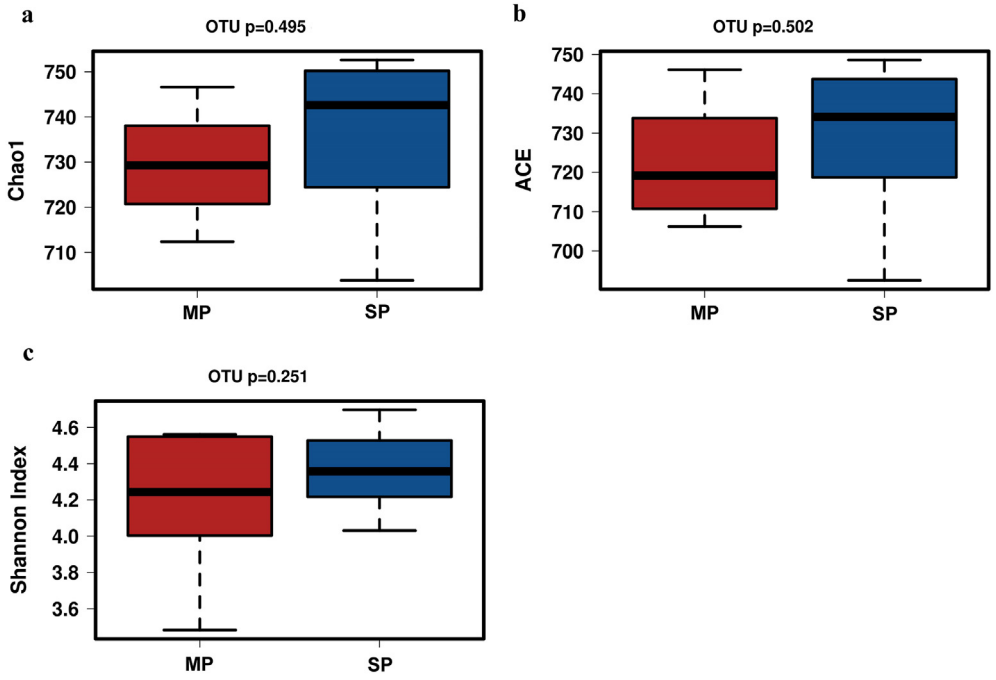

Fig. 2. Box plot of the alpha diversity indices for richness (a. Chao1 and b. ACE indices) and evenness (c. Shannon index) of the bacterial communities in SP group (blue) and MP group (red), respectively.

was more abundant in the MP group (Fig. 5c–e).

3.4. The gut microbiome of DN mice was associated with biochemical indexes

Spearman's rank correlation coefficient method was used to evaluate the correlation between each subject's gut microbiome and biochemical indexes, including BW, BG and 24-h UP. The details are shown in Fig. 6. Significant negative correlations ($r = -0.829$, $P < 0.05$) were found between Blautia and 24-h UP in MP subjects (Fig. 6a). Similarly, a relationship between the biomarkers and biochemical indexes in SP subjects was established (Fig. 6b). BW ($r = 0.767$, $P < 0.01$) and BG ($r = 0.648$, $P < 0.05$) exhibited a significant positive correlation with the genus Allobaculum while 24-h UP ($r = 0.773$, $P < 0.01$) showed a significant positive correlation with the genus Anaerosporobacter.

3.5. The potential function of the microbiome in the SP and MP groups

The effect of FMT on the two cohorts is shown in Figs. 7a and 8a. In

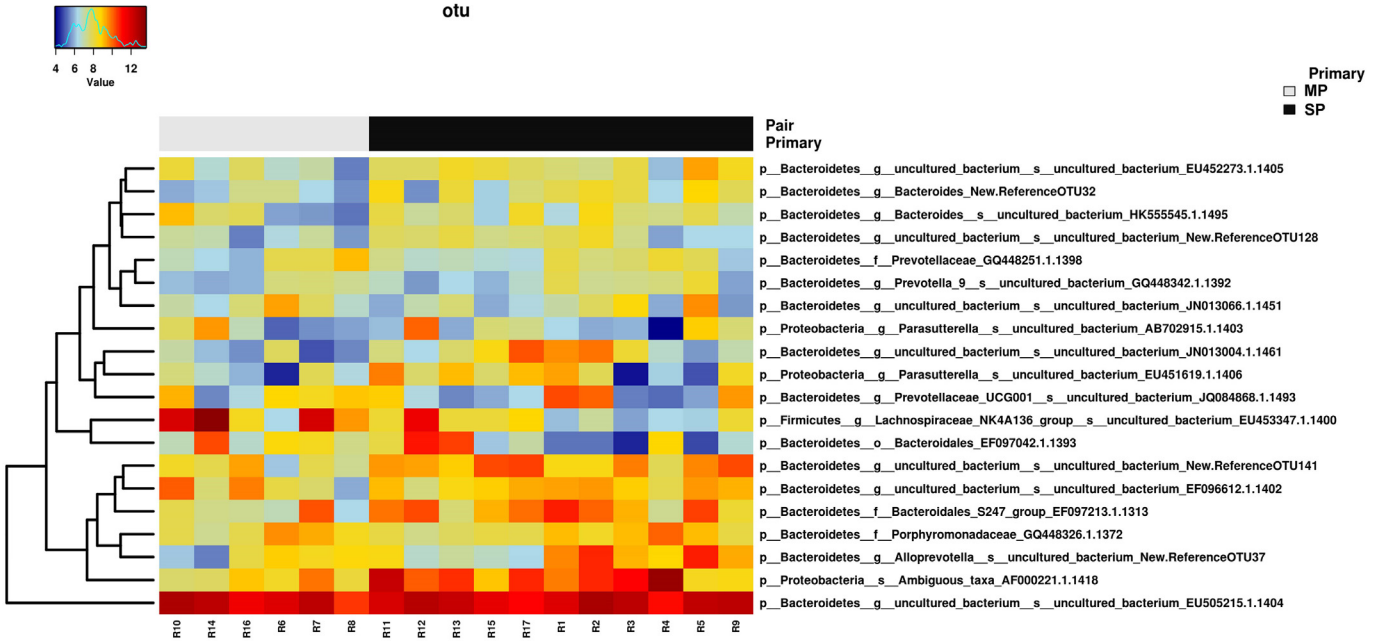

Fig. 3. Annotated heat map based on UniFrac distance and Ward hierarchical clustering of the mainly different microbiome shows how well SP and MP groups cluster together. Taxonomy explanation includes species, genera, family, and phylum, which are entered in order of abundance. Genus abundance and groups are described by the change in the intensity of the different color, as annotated.

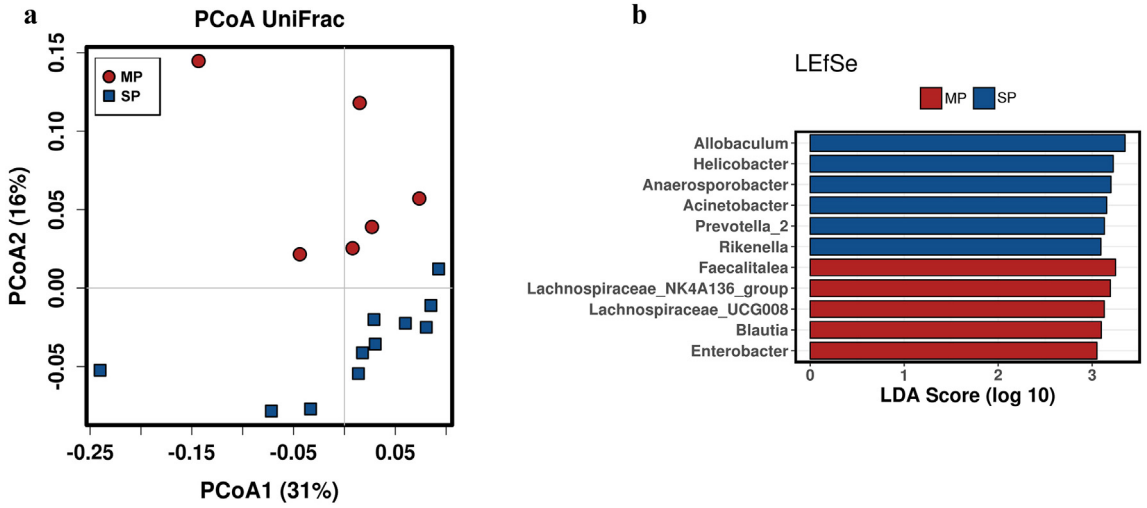

Fig. 4. Box plot of the beta diversity indices of the bacterial communities in SP group (blue) and MP group (red), respectively. a. Overall gut microbial structure. Principal co-ordinates analysis was performed on the basis of the weighted Unifrac distance. PCoA1: principal co-ordinates analysis 1; PCoA2: principal coordinates. b. Linear discriminant analysis Effect Size (LEfSe) on genus level.

cohort one, qPCR (Fig. 7b) and 24-h UP analyses (Fig. 7c) were performed to determine the changes in 3 major microbiota constituents and the effect of the microbiome on renal function in the SP and MP groups at 48 h after FMT and 12 weeks after STZ injection. There was no difference in 24-h UP between the FMT-SP group [46.40 (41.90–50.15) mg/24 h] and FMT-MP group [45.97 (43.09–50.38) mg/24 h] before FMT and STZ injection ($P = 0.571$). However, at the end of the 12th week after STZ injection, the 24 h-UP in both groups increased, and it was higher in the FMT-SP group [574.34 (489.38–701.11) mg/24 h] than in the FMT-MP group [213.02 (201.16–236.87) mg/24 h] ($P < 0.0001$).

In cohort two, at the end of the 12th week after STZ injection, the 24 h-UP of the two subgroups was analyzed (Fig. 8b). There was no difference between these two subgroups [330.67 (301.90–356.48) mg/24 h vs 331.56 (302.22–360.27) mg/24 h; $P = 0.821$]. From the time of FMT completion (week 0), we observed the dynamics of 24-h UP in these two subgroups for three consecutive weeks (week 1 – week 3). It was found that the 24-h UP in both subgroups increased and the increase was more obvious in the P2-FMT-SP group than in the P1-FMT-MP group. The dynamics of 24-h UP were shown in Table 2. qPCR was performed to determine the changes in 3 major microbiota constituents in both subgroups at 48 h after FMT.

3.6. LPS, TMAO, and SCFA in mice

LPS and TMAO contents were measured by corresponding kits. The concentrations of LPS and TMAO were higher in the SP group and in the groups that received FMT from the SP group than in the MP group and the groups that received FMT from the MP group. GC–MS was performed to analyze the SCFA in the feces of the mice in the two cohorts. It was found that propionic acid and butyric acid contents were higher in the MP group than in the SP group. After FMT, in both cohorts, the concentrations of propionic acid and butyric acid were higher in the subgroups that received fecal transplantation from the MP group than in the subgroups that received fecal transplantation from the SP group, whereas there was no difference in acetic acid content between the subgroups in both cohorts (Fig. 9). The details are shown in Tables 3 to 8.

4. Discussion

Murine models of human disease play a vital role in helping people study the occurrence, development, diagnosis and treatment of diseases. However, the preparation of murine models often produces certain heterogeneity, i.e., their representations of diseases are not always reproducible [3]. Many factors may be involved, and our research has found that the gut microbiome may be an important part of this phenomenon.

Regarding the composition of the gut microbiome, there was no significant difference in the α diversity between the initial SP and MP groups, i.e., the species abundance and diversity of the two ecosystems were similar, which may be due to the high level of blood glucose available for the growth of microorganisms. Glucose makes all kinds of microorganisms proliferate excessively, which is different from the previous concept that increased overall diversity implies improved health, but it is similar to result of a recent study [25,26]. For β diversity, there were significant differences between the SP group and the MP group, i.e., the microbial species composition differed between the two groups. In the SP group, the abundance of Firmicutes was reduced at the phylum level, and there was no significant difference between the two groups in Bacteroides abundance. In healthy mammals, the F/B ratio is relatively stable, and an increase or a decrease in the F/B ratio often implies a disease state [27]. Researchers reached a consistent conclusion for patients with type 2 diabetes mellitus (T2DM), i.e., T2DM patients have fewer beneficial bacteria in the intestine than normal people, with an abnormal F/B ratio [28,29]. In our experiment, we found that the MP group had an increased abundance of Firmicutes, which resulted in higher F/B ratio than that in the SP group. This finding seems to contradict the existing view that an increased F/B ratio indicates an inferior health condition. However, as mentioned before, the ratio of F/B in healthy bodies is relatively stable, and abnormal increases or decreases indicate disease status. Some studies have noted that in healthy people, the F/B ratios of infants, adults and the elderly are 0.4, 10.9 and 0.6, respectively [27]. Similar conditions may exist in mice. In our analysis, the F/B ratios of the SP and MP groups were both abnormal, but the ratio of the MP group may have been closer to the normal level than that of the SP group, although we have not found a report about the normal F/B ratio of mice. At the same time, the MP group had an increased abundance of Firmicutes, which partly explained the elevated SCFA content in this group because most SCFA are produced by Firmicutes.

We analyzed different microbiota constituents of the SP and MP groups using LEfSe and found that at the genus level, Allobaculum in the SP group was more abundant than in the MP group. Allobaculum has been shown to be associated with diseases such as diabetes and obesity [30]. Studies have shown that Allobaculum is a fairly active

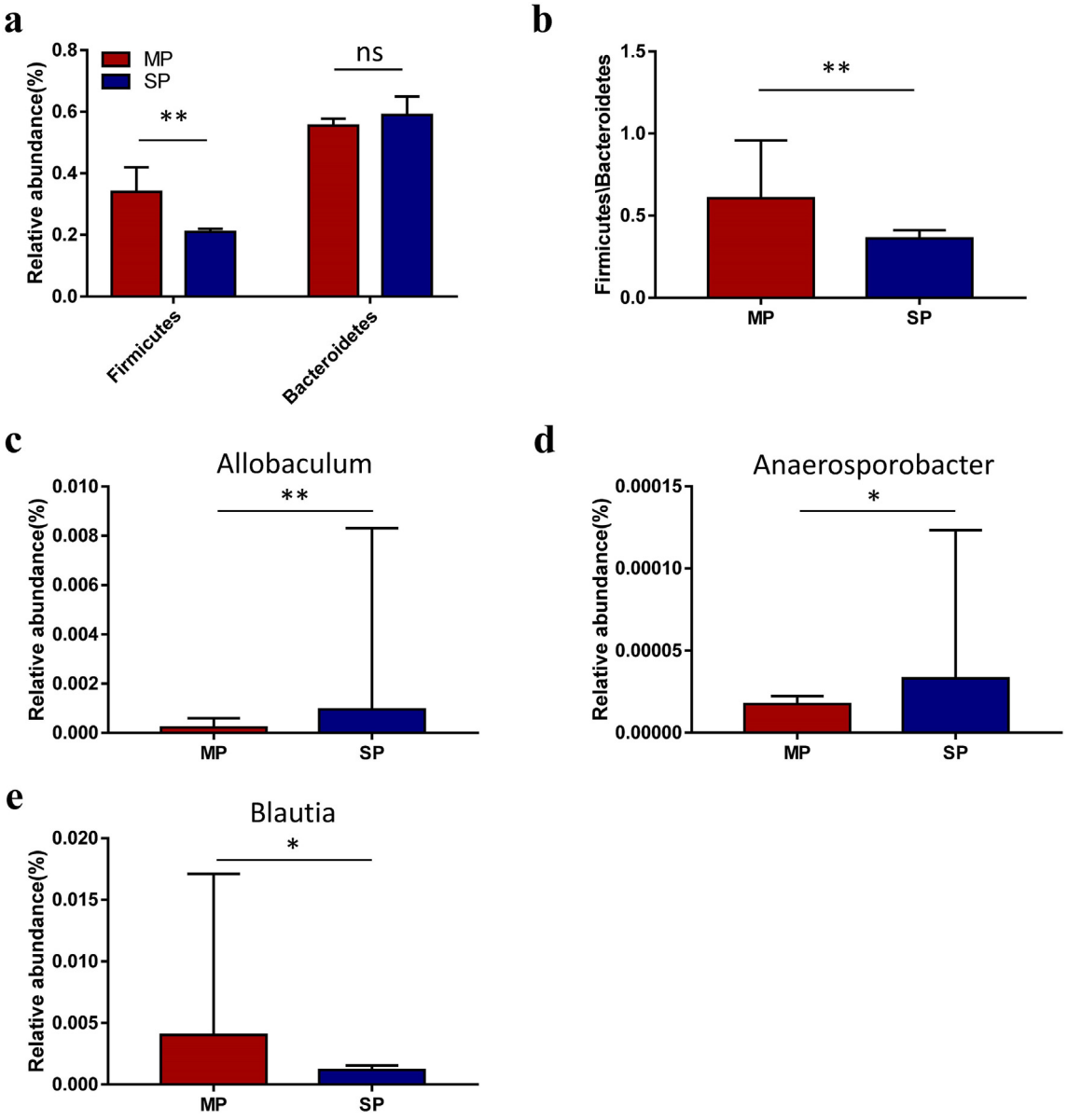

Fig. 5. Different characteristics of the microbial community composition in SP and MP groups. a. Difference of the relative abundance of phylum of Firmicutes and Bacteroidetes between SP and MP groups. b. The compare of the Firmicutes/Bacteroidetes ratio between SP and MP groups. c–e. Relative abundance of 3 major taxa between the SP and MP subjects was compared (Mann–Whitney *U* test). $^{*}P < 0.05$, $^{**}P < 0.01$.

glucose utilizer and yields mainly lactate during glucose metabolism [6,31,32]. Turnbaugh et al. found that obese rats had significantly higher levels of acetic acid in the intestine than lean mice, i.e., the increase in acetic acid-producing bacteria may increase the ability of the host to absorb energy from food, leading to obesity [33]. Based on the findings of Elena et al., Allobaculum species are optimized for energy harvesting, as they rapidly consume simple sugars, and their relatives are linked to an adiposity-favoring microbiota [31]. Our study found that in the SP group, Allobaculum was significantly positively correlated with BW and BG, which means that the increase in Allobaculum abundance led to elevated glucose utilization and absorption and a further increase in body weight and blood glucose, thus aggravating diabetes and diabetic nephropathy.

At the genus level, another significantly enriched bacterium in the SP group was Anaerosporobacter. Studies have shown that Anaerosporobacter is associated with an increase in trimethylamine oxide (TMAO) content in vivo [34]. When the gut microbiome metabolizes choline and lecithin (often called phosphatidylcholine), it produces trimethylamine (TMA). After absorption into the blood, TMA is modified by hepatic flavin monooxygenase (FMO) to produce TMAO, a metabolite of amine. The presence of TMAO in the blood upregulates macrophage scavenger receptors, macrophage cholesterol accumulation and foam cell formation and promotes the formation of vascular plaques, thereby damaging blood vessels and the intestinal mucosa [35,36]. Because the kidney is rich in blood vessels, it is highly vulnerable to TMAO. Moreover, intestinal mucosal destruction accelerates the whole-body transport of TMAO from bacteria and causes oxidative stress damage to the kidney, cardiovascular system and endocrine system [37]. Our study found that the concentrations of LPS and TMAO in the SP group and groups that received FMT from the SP group were higher than those in the MP group and groups that received FMT from the MP group. Furthermore, through Spearman analysis, it was found that the level of Anaerosporobacter in the SP group was significantly increased and was positively correlated with 24-h UP. We speculate that the vascular damage caused by Anaerosporobacter and its generation of TMAO may be one of the reasons for the significant increase

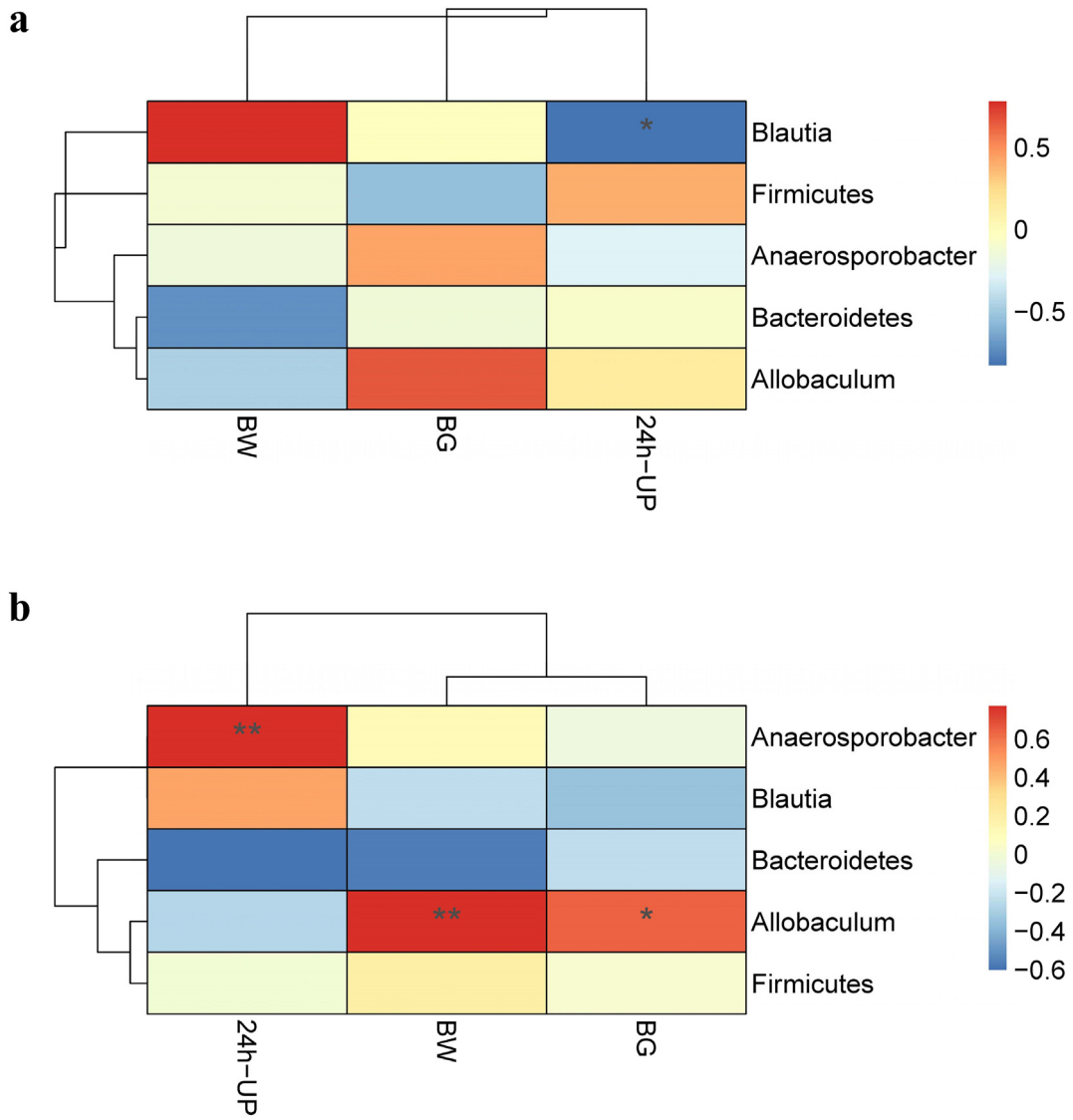

Fig. 6. Heat map of Spearman's correlation analysis between the gut microbiome of SP and MP groups and the biochemical indexes. a. Heat map of Spearman's correlation analysis between the gut microbiome of MP subjects and the biochemical indexes. b. Heat map of Spearman's correlation analysis between the gut microbiome of SP subjects and the biochemical indexes. $^{*}P < 0.05$, $^{**}P < 0.01$. BW: body weight. BG: blood glucose. UP: urinary protein.

in urinary protein. Additionally, LPS produced by harmful bacteria can damage the intestinal barrier so that additional LPS and TMAO enter the blood and aggravate renal damage.

In the MP group, at the genus level, the dominant microbiota constituent was Blautia. The study of Blautia has revealed diversity. Zhao et al. found that Blautia abundance increased in the intestine of unilateral ureteral obstruction (UUO) mice and CKD mice and was related to an increase in uremic toxins, such as indole sulfate (IS) and p-cresol sulfate (PCS) [38,39]. However, another study reported that there was an increased abundance of Blautia in healthier people than in people with poorer health [40]. At the same time, Barcenilla et al. found that Blautia had the ability to consume acetic acid and synthesize butyric acid and some other SCFA, which is similar to our results [41]. Because intestinal microecology is complex, some bacteria may play different roles with changes in the environment. Here, we agree with Zhao's research results. However, perhaps because of the different microenvironments of diseases and the different functions of bacteria we are interested in, we came to a conclusion that seems to be the opposite. In our study, we found that Blautia may reduce the urine protein content of DN mice by producing SCFA (propionic acid and butyric acid). Propionic acid and butyric acid can bind to G-protein-coupled receptors (GPCRs) on intestinal L-cells to stimulate the release of glucagon-like peptide and YY peptide, resulting in increased insulin and decreased glucagon release, both of which lower blood glucose [42]. SCFA have also been shown to dampen inflammation via a decrease in intestinal permeability and a reduction in circulating endotoxins and systemic inflammation [42]. Some studies have suggested that GPCR 43, a receptor of SCFA, mediates the effects of SCFA in regulating inflammatory responses [43]. Studies have shown that a lack of SCFA can aggravate the condition of kidney disease patients and is related to the mortality of kidney disease patients [44,45]. Butyrate can be used as the preferred energy source for colon cells to improve the intestinal barrier and reduce the influx of toxins, such as LPS and TMAO, into the blood, thus alleviating DN [46–49]. The nutritional and anti-inflammatory effects of Blautia may be helpful to explain the reduced UP content in the MP group.

To verify the function of the microbiome in the SP and MP groups, fecal microbiota transplantation was performed. We designed two cohorts to receive FMT. In cohort one, mice received FMT before STZ injection to induce the DN model to clarify that the microbiomes from the SP and MP groups have the ability to modulate the renal function of STZ-induced DN mice and that STZ injection had no effect on the

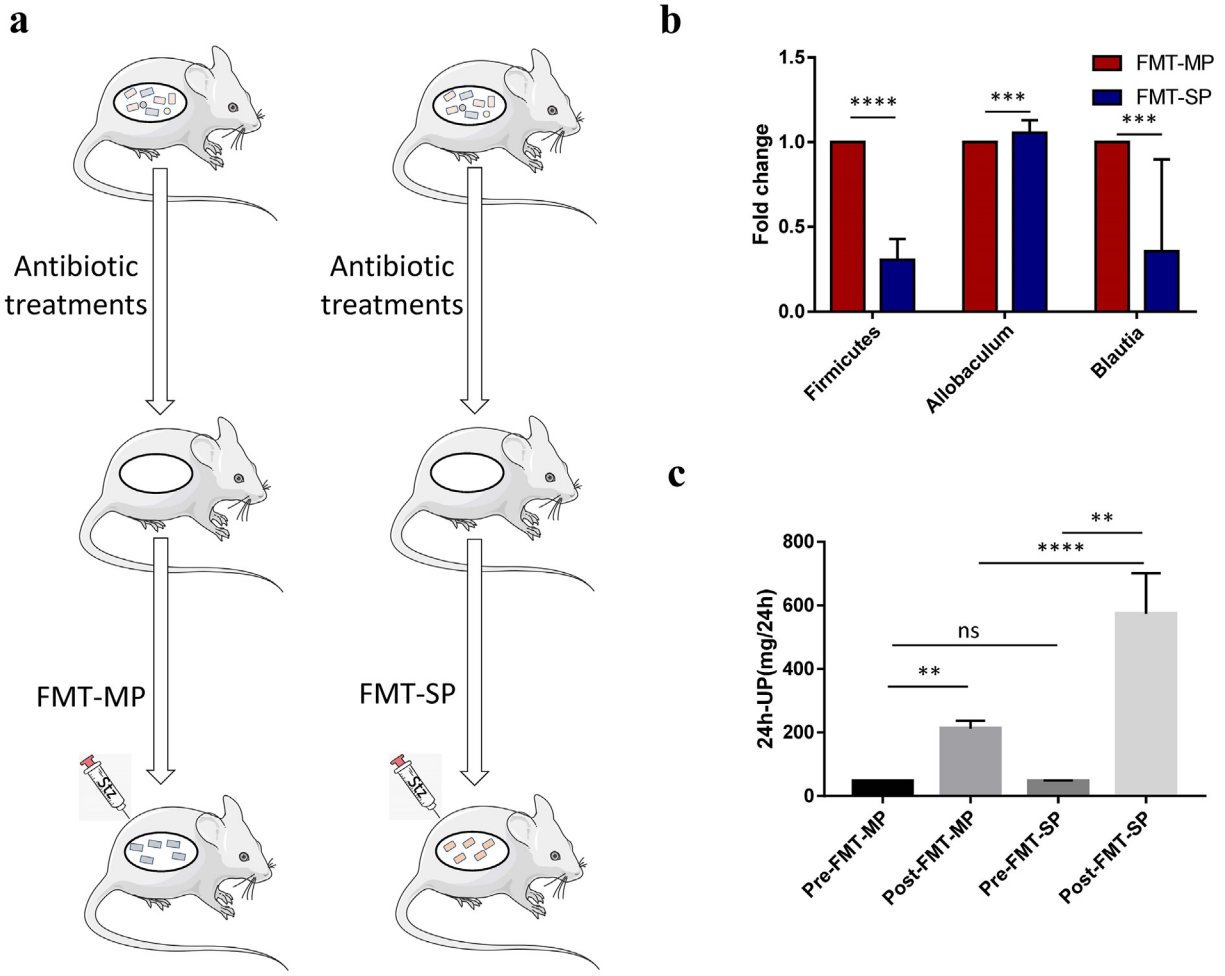

Fig. 7. FMT in cohort one. a. Mice were treated with antibiotics to eliminate the previous microbiota and received FMT, and then were injected with STZ to construct DN models. b. The four major taxa of microbiota in feces transplanted from SP and MP groups were measured by qPCR 12 weeks after STZ injection. c. The changes of 24 h-UP of the two groups in cohort one before and after FMT. FMT: fecal microbiota transplantation, MP: mild proteinuria group, SP: serve proteinuria group, STZ: streptozocin, 24 h-UP: 24 hour urinary protein. **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$.

microbiome. In cohort two, mice received STZ injection to induce DN before FMT to illustrate the exact effect of the microbiome from the SP and MP groups. After FMT, we observed consistent results in the two cohorts. Regardless of the cohort, acetic acid-producing Allobaculum was more abundant in the groups that received the microbiome from the SP group, while butyric acid-producing Blautia was more abundant in the groups that received the microbiome from the MP group. Unfortunately, we did not find any references or data about primers to detect TMAO-producing Anaerosporobacter, so we could not perform qPCR for this bacterium; we even tried to design primers ourselves but ultimately finally. We also observed an increase in 24-h UP in mice transplanted with microbiomes from the SP group and MP group. However, the increase was higher in groups that were transplanted with a microbiome from the SP group than in groups that were transplanted with a microbiome from the MP group. We hypothesized that the microbiome and the related SCFA play a role in modulating renal function. To clarify why these results were obtained, we analyzed the serum LPS, TMAO, and the SCFA contents in the feces of the two cohorts and found that the concentration of LPS and TMAO were lower, while the levels of beneficial SCFA (especially propionic acid and butyric acid) were higher in the groups transplanted with a microbiome from the MP group than in the groups transplanted with a microbiome from the SP group. This result was consistent with our hypothesis.

Our study suggests that differences in the gut microbiome may be one of the keys to modulating the renal function of DN murine models, and we verified this hypothesis by FMT. However, there are still several limitations in our study. On the one hand, limited by laboratory conditions, we used antibiotic-treated mice instead of germ-free mice, which may affect our results to some extent. Antibiotic treatment is a widely used method to obtain pseudo-germ-free mice in gut microbiota research because the condition of germ-free mouse maintenance is strict and hard to achieve in most laboratories. Antibiotic treatment cannot kill all microorganisms in the mouse gut, but it can keep the microbiota at a very low level [50]. Furthermore, we used the method in all the FMT groups to eliminate the impact of this treatment. In addition, a large amount of antibiotics could damage the kidneys of mice, but we used this method in all FMT groups, so the groups were homogeneous. On the other hand, the absence of further mechanistic research is a weakness. In our future experiments, we will focus on the mechanism of the interaction between the microbiome and diseases and try to artificially regulate the interaction to promote the disease to provide new targets for the diagnosis and treatment of diabetic nephropathy.

5. Conclusions

Our results indicate that the gut microbiome plays an important regulatory role in the renal function modulation of DN mouse models, which can explain the heterogeneity of the results to some extent. Our findings also suggest potential pathogens active in diabetic nephropathy. These results may be essential for clinical diagnosis and could be

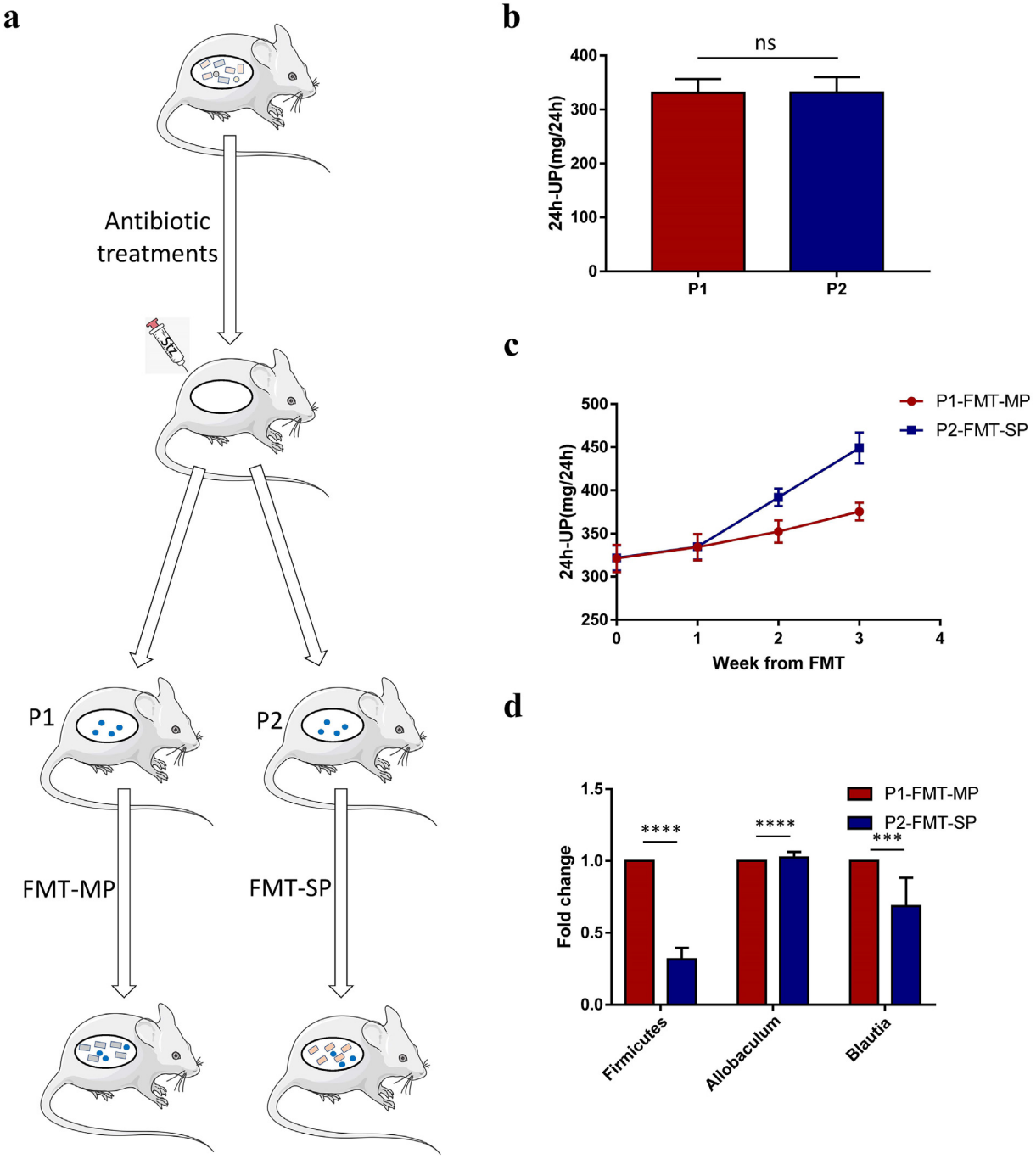

Fig. 8. FMT in cohort two. a. Mice were treated with antibiotics to eliminate the previous microbiota and then were induced into DN models using STZ before FMT. b. The difference of 24 h-UP between P1-FMT-MP and P2-FMT-SP groups at the 12th week after STZ injection. c. The dynamic changes of 24 h-UP between P1-FMT-MP and P2-FMT-SP groups. d. The major microbiota in feces transplanted from SP and MP groups were measured by qPCR 48 h after FMT. P1-FMT-MP: phase 1-fecal microbiota transplantation-mild proteinuria group; P2-FMT-SP: phase 2-fecal microbiota transplantation-serve proteinuria group; STZ: streptozocin, 24 h-UP: 24 hour-urinary protein.

Table 2
The dynamics of 24 h-UP in the P1-FMT-MP group and the P2-FMT-SP group.

	24-h UP (W1)	24-h UP (W2)	24-h UP (W3)
P1-FMT-MP (mg/24 h)	344.80 (318.06–366.44)	361.89 (332.40–382.29)	378.15 (359.26–401.73)
P2-FMT-SP (mg/24 h)	342.97 (314.38–373.15)	339.41 (375.56–416.64)	458.92 (414.74–500.37)
P value	0.88	0.016	0.007

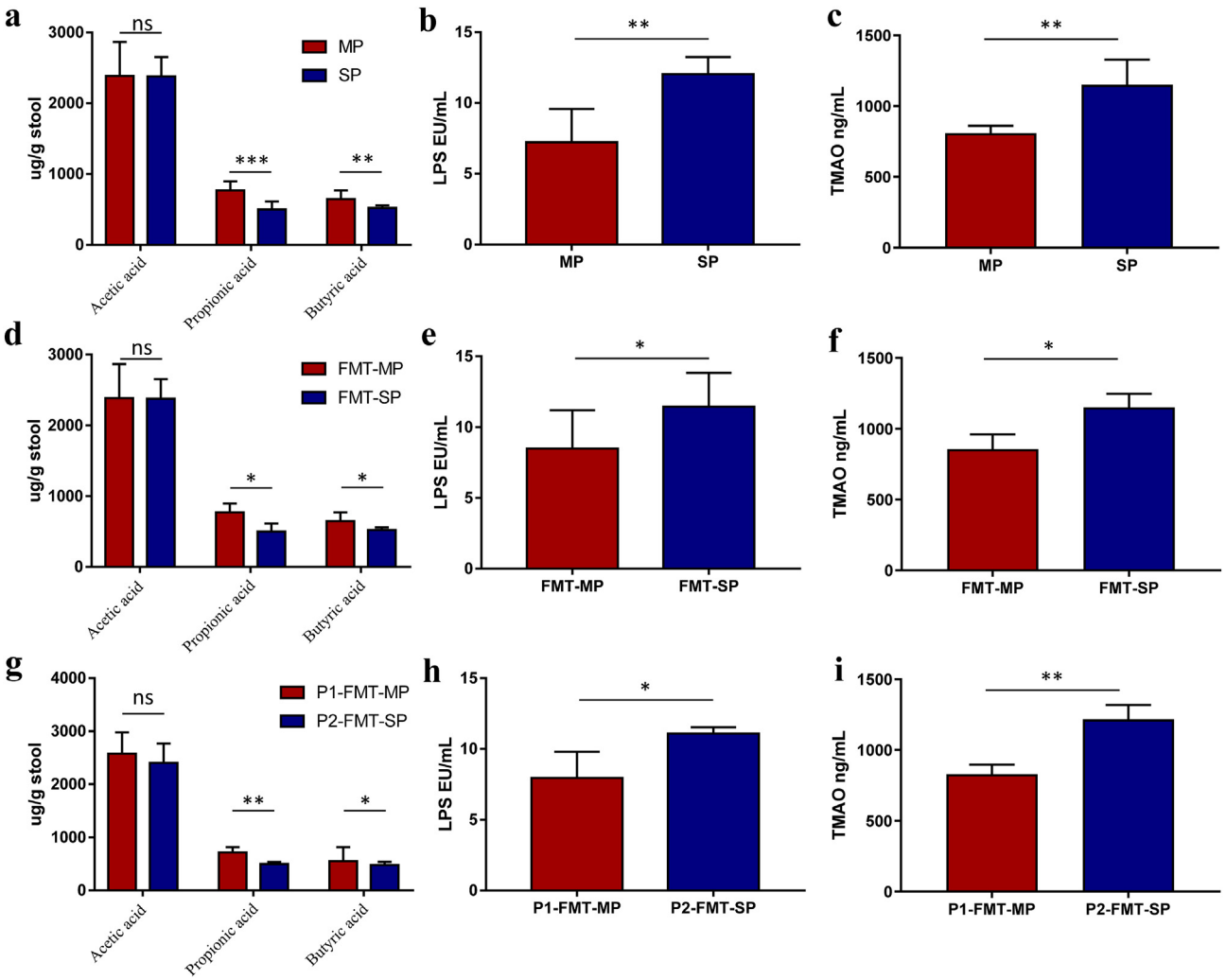

Fig. 9. The concentration of SCFAs, LPS, and TMAO in different groups. a–c. The concentration of SCFAs, LPS, and TMAO in SP and MP groups. d–f. The concentration of SCFAs, LPS, and TMAO in cohort one. g–i. The concentration of SCFAs, LPS, and TMAO in cohort two. LPS: lipopolysaccharide. TMAO: trimethylamine oxide.

Table 3
The concentrations of SCFA in SP and MP groups.

	Acetic acid	Propionic acid	Butyric acid
MP (μg/g)	2603.86 (2166.79–3479.23)	858.78 (772.72–973.46)	877.07 (697.85–990.77)
SP (μg/g)	2239.98 (1984.32–2673.54)	429.56 (412.71–504.46)	547.15 (455.42–658.52)
P value	0.191	0.001	0.005

Table 4
The concentrations of LPS and TMAO in the SP and MP groups.

	LPS (EU/mL)	TMAO (ng/mL)
MP	7.22 (5.82–9.56)	808.56 (765.37–860.95)
SP	12.03 (9.57–13.24)	1150.68 (906.57–1327.94)
P value	0.003	0.002

Table 6
The concentrations of LPS and TMAO in cohort one.

	LPS (EU/mL)	TMAO (ng/mL)
FMT-MP	8.50 (7.07–11.21)	856.63 (824.89–960.09)
FMT-SP	11.46 (9.47–13.83)	1151.81 (958.59–1248.51)
P value	0.019	0.029

Table 5
The concentrations of SCFA in cohort one.

	Acetic acid	Propionic acid	Butyric acid
FMT-MP (μg/g)	2385.62 (2190.97–2865.42)	772.05 (559.36–895.85)	648.82 (520.77–772.50)
FMT-SP (μg/g)	2378.06 (1819.69–2654.11)	502.23 (447.81–613.04)	523.87 (437.62–557.75)
P value	0.545	0.013	0.028

Table 7
The concentrations of SCFA in cohort two.

	Acetic acid	Propionic acid	Butyric acid
P1-FMT-MP (μg/g)	2574.72 (2363.29–2978.82)	715.28 (539.18–816.65)	550.47 (504.97–815.54)
P2-FMT-SP (μg/g)	2400.86 (1915.24–2769.34)	497.79 (442.25–536.33)	476.83 (402.53–536.85)
P value	0.174	0.003	0.028

Table 8
The concentrations of LPS and TMAO in cohort two.

	LPS (EU/mL)	TMAO (ng/mL)
P1-FMT-MP	7.95 (7.07–9.81)	828.09 (798.61–895.31)
P2-FMT-SP	11.10 (9.11–11.54)	1215.65 (932.30–1318.36)
P value	0.029	0.002

further used to develop potential probiotics that could facilitate the treatment of diabetic nephropathy. It remains unknown whether renal disorders cause dysbiosis or vice versa, but we performed FMT to explain the function of the microbiome from DN mice to some extent. This question may be solved by isolating and transplanting a specific single bacterial strain in the future. Further studies need to scrutinize and explore the in-depth mechanisms of the interaction between the gut microbiome and diabetic nephropathy using models in more advanced organisms.

Animal ethics

All animal experiments were approved by the Ethics Committee for Animal Experiments of Shandong Provincial Qianfoshan Hospital (NO. 2015S002), and complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

CRediT authorship contribution statement

Yang Li: Methodology, Software, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Xinhuan Su**: Methodology, Software, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Ying Gao**: Investigation. **Chenxiao Lv**: Investigation. **Zhiwei Gao**: Investigation. **Yipeng Liu**: Resources, Funding acquisition. **Yan Wang**: Conceptualization, Funding acquisition. **Shujuan Li**: Conceptualization, Writing - review & editing, Project administration. **Zunsong Wang**: Conceptualization, Supervision, Project administration, Funding acquisition.

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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