Clostridium butyricum Inhibits the Inflammation in Children with Primary Nephrotic Syndrome by Regulating Th17 / Tregs Balance via Gut-Kidney Axis

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Abstract

Background

Primary nephrotic syndrome (PNS) is a common glomerular disease in children. *Clostridium butyricum* (*C. butyricum*), a probiotic producing butyric acid, exerts effective in regulating inflammation. This study was designed to elucidate the effect of *C. butyricum* on PNS inflammation through the gut-kidney axis.

Methods

BALB/c mice were randomly divided into 4 groups: normal control group (CON), *C. butyricum* control group (CON + *C. butyricum*), PNS model group (PNS) and PNS with *C. butyricum* group (PNS + *C. butyricum*). The PNS model was established by a single injection of doxorubicin hydrochloride (DOX) through the tail vein. After 1 week of modeling, the mice were treated with *C. butyricum* or normal saline for 6 weeks. At the end of the experiment, the mice were euthanized and associated indications were investigated.

Results

Since the successfully modelling of the PNS, the 24h urine protein, blood urea nitrogen (BUN), serum creatinine (SCr), urine urea nitrogen (UUN), urine creatinine (UCr), lipopolysaccharides (LPS), pro-inflammatory interleukin (IL)-6, IL-17A were increased, the kidney pathological damage was aggravated, while a reduction of body weights of the mice and the anti-inflammatory IL-10 significantly reduced. However, these abnormalities could be dramatically reversed by *C. butyricum* treatment. The crucial Th17-Tregs axis in PNS inflammation also was proved to be effectively regulated by *C. butyricum* treatment. This probiotic intervention notably affected the expression levels of signal transducer and activator of transcription 3 (STAT3), Heme oxygenase-1 (HO-1) protein, and retinoic acid-related orphan receptor gamma t (RORγt). 16S rRNA sequencing showed that *C. butyricum* could regulate the composition of intestinal microbial community and found *Proteobacteria* was more abundant in urine microorganisms in mice with PNS. Short-chain fatty acids (SCFAs) were measured and showed that *C. butyricum* treatment increased contents of acetic acid, propionic acid, butyric acid in feces and acetic acid, valeric acid in urine. Correlation analysis showed that there was a closely complicated correlation among inflammatory indicators, metabolic indicators, microbiota and associated metabolic SCFAs in gut-kidney axis.

Conclusion

*C. butyricum* regulates Th17 / Tregs balance via the gut-kidney axis to suppress the immune inflammatory response in mice with PNS, which may potentially contribute to a novel, inexpensive, and safe therapeutic reagent for the control of the disease.
Introduction

Primary nephrotic syndrome (PNS) characterized by massive proteinuria, hypoalbuminemia, edema and hyperlipidemia, represents a common glomerular disease, accounting for about 90% of nephrotic syndrome in children\(^1\),\(^2\). It is reported that the incidence of PNS is approximately 2–7/100,000 in children and the prevalence rate is about 16/100,000\(^3\). A large amount of albumin loss in the urine leads to infection, thromboembolism, cardiovascular disease, hypovolemic crisis, anemia and acute renal failure\(^4\). At present, the treatment of primary nephrotic syndrome in children is limited to glucocorticoids and immunosuppressants. Long-term use of glucocorticoids and immunosuppressants may induce multiple adverse reactions in children, such as cushing-like characteristics, obesity, growth retardation, bone marrow suppression\(^4\). Although most children respond well to steroids within four weeks, yet the majority of them will relapse and about half of children will frequently recurrent or result a dependence on steroids\(^5\). Therefore, there is an urgent need to seek safe and effective intervention strategies for children with PNS.

PNS in children is mainly believed in association with immune dysfunction including lymphocytes and podocytes\(^6\),\(^7\). The main manifestations are the abnormal number and functions of T lymphocytes and the imbalance of the proportion of each subset\(^8\). A biased T helper cell 17 (Th17)-Regulatory cells(Tregs) axis in patients with PNS are thought to be closely correlated with the remission and resistance\(^9\),\(^10\). Tregs are reduced in children with PNS and recovered after the remission\(^11\),\(^12\).

*Clostridium butyricum* (*C. butyricum*) is an obligate anaerobic gram-positive bacillus, its main metabolite butyric acid has been proved by many research teams including our academic group to improve atherosclerosis\(^13\), alcoholic liver disease\(^14\), renal ischemia-reperfusion injury\(^15\), airway inflammation\(^16\) and other diseases through anti-oxidative stress and reducing inflammatory response. It is worth noting that the gut dysbiosis has an important impact on the proportion of butyrate-producing bacteria, and once the gut homeostasis is destroyed, it may lead to or participate in the occurrence and development of the disease\(^12\),\(^17\)–\(^19\).

PNS is also known as Idiopathic nephrotic syndrome (INS). It has been found that the proportion of butyrate-producing bacteria in the gut flora of children with INS was decreased, and it was related to the imbalance of Th17 / Tregs in peripheral blood\(^12\), and moreover, studies had established that oral the *C. butyricum* preparations during remission in children with INS can reduce the frequency of recurrence and the need for immunosuppressive agents\(^1\). However, the effect of *C. butyricum* intervention on PNS and its possible mechanism are largely unclear.

This study was design to investigate the effect of *C. butyricum* on PNS and associated underlying mechanism via gut-kidney axis by regulating Th17 / Tregs balance, which may potentially contribute to a therapeutic agent for the control of PNS.
Materials and methods

Experimental animals and PNS model

All experiments were performed according to Animal Use guidelines and approved by the Ethics Committee of Ningxia Medical University (No.2022 – 206). Forty-eight male 6-week-old BALB/c mice (22 ± 1g) were obtained from Beijing Huafukang Bio-Technology. Co., LTD (Beijing, China). Mice were fed in the Laboratory Animal Research Center of Ningxia Medical University (Yinchuan, China) under a 12 h light and dark cycle with free access to food and water. The mouse model of PNS was established by a single injection of doxorubicin hydrochloride (DOX;MCE,USA) 10mg / kg through the tail vein[20]. One week later, the mice were placed in a mice metabolic cage to collect 24-hour urine and measure 24-hour urine protein. After successful modeling, the subsequent experiments were performed.

Bacterial preparation

*C.butyricum* was a vacuum freeze-dried strain provided by China General Microbiological Culture Collection Center (CGMCC, strain number 1.5205). PYG MEDIUM (modified, Shandong, China) was used to resuscitate the bacteria. The bacteria were cultured in an anaerobic incubator (5% carbon dioxide) at 37°C for about 24 h, and the fully grown bacteria were visible. The colonies were placed in 10% skim milk to make a freeze-dried powder and stored at -80°C. During the intervention, PYG MEDIUM was used daily to resuscitate *C.butyricum* lyophilized powder in an anaerobic incubator (5% carbon dioxide) at 37°C for 24 h, centrifuged at 3000 × g for 5 min, and resuspended in sterile saline. The experimental final concentrations were $1 \times 10^8$ CFU / mL.

Experimental design

The time diagram of the experimental design is shown in Figure.1A. The mice were randomly divided into 4 groups (12 mice/group): CON, CON + C.butyricum, PNS and PNS + C.butyricum. The CON + C.butyricum and PNS + C.butyricum groups were given 200µl C.butyricum by gavage once a day for 6 weeks. Meanwhile, mice in CON and PNS groups were treated with 200µl normal saline in the same time. The 24-hour urine was collected every 2 weeks using a mice metabolic cage, and the 24-hour urine protein was measured by Bradford protein detection kit (Thermo Fisher, USA) to observe the changes of urine protein in mice. During the experiment, the body weights (BWs) of the mice were monitored weekly. After 6 weeks of gavage, fresh stool and urine samples were collected and immediately frozen at -80°C for subsequent analysis. At the termination of the experiment, mice were anesthetized with isoflurane inhalation (0.41ml / min at 4L / min Fresh gas flow, application concentration 2%) was performed under the IACUC protocol, and euthanized via transcardiac perfusion. Blood samples were rapidly collected by orbital bleeding and centrifuged at 4°C (1500 × g for 10 min) to obtain plasma samples, which were stored at -80°C for the further study.

Plasma and Urine biochemistry tests
Blood and urine samples were measured for the following biochemical properties: Blood urea nitrogen (BUN), serum creatinine (SCr), urine urea nitrogen (UUN) and urine creatinine (UCr). BUN and UUN were detected by urease method with urea nitrogen test box; SCr and UCr were measured by creatinine assay kit using sarcosine oxidase method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

**Kidney tissue pathological section staining**

After sacrifice of the mice, the kidney tissues were immediately fixed in 4% paraformaldehyde, washed with tap water and dehydrated with ethanol, paraffin-embedded sections (5µm) were placed on slides.

Hematoxylin & eosin (HE) staining was performed according to the standard procedure for HE staining, paraffin sections were routinely dewaxed and rehydrated, stained with hematoxylin solution (Servicebio, NO.G1004, Wuhan, China) for 1 min, and then differentiated with 1% hydrochloric acid alcohol for 1 s. After washing with tap water, it was immersed in eosin solution (Servicebio, NO.G10001, Wuhan, China) for 30 s. The sections was finally washed with tap water and after air-dried or naturally air-dried cell climbing slices, the neutral gum was sealed.

Masson staining using Masson's trichrome stain kit (Solarbio, NO.G1340, Beijing, China). According to the kit instructions, sections were conventionally dewaxed to water. Firstly, the prepared Weigert iron hematoxylin staining solution was used to stain for 5 min, and then the acidic ethanol differentiation solution was used to differentiate for 5 s. After washing with water, the Masson blue solution was returned to blue for 3 min, and then washed again with distilled water for 1 min, and stained with ponceau acid fuchsin staining solution for 5 min. In the above operation process, the weak acid working solution was prepared according to the ratio of distilled water: weak acid solution = 2:1, and washed with weak acid working solution for 1 min. After the sections washed with phosphomolybdic acid solution for 1 min, the weak acid working solution was washed for 1 min and then directly put into aniline blue staining solution for 1 min, and were washed with weak acid working solution for 1 min. Finally, 95% ethanol was used for rapid dehydration for 2 s, and anhydrous ethanol was dehydrated for 3 times, 5 s each time. Then, transparent with xylene 3 times, each time for 1 min after neutral gum sealing.

Periodic Acid Schiff (PAS) staining was performed using the glycogen PAS staining kit (including hematoxylin) (Solarbio, NO.G1281, Beijing, China). According to the instructions, paraffin sections were routinely dewaxed and rehydrated. The paraffin sections were primarily rinsed with tap water for 2 min and immersion cleaned with distilled 2 times. They were placed in an oxidizing agent and placed at room temperature for 5 min. Subsequently, rinsed again with tap water and immersion cleaned with distilled 2 times. The sections were placed in Schiff staining solution and the dark of room temperature, stained for 10 min and rinsed with tap water for 10 min. Then, the sections were placed in a hematoxylin staining solution and the nuclei were stained for 1 min. They were used acid differentiation liquid to differentiate for 2 s, rinsed with tap water for 10 min and return to blue. Lastly, the sections were subjected to conventional ethanol dehydration step by step, transparent with xylene and neutral glue fixation.
Periodic acid-silver methenamine (PASM) staining was stained with Methenamine Silver Plating Stain Kit For Basement Membranes (Solarbio, NO.G1790, Beijing, China). According to the kit instructions, paraffin sections were deparaffinized and rehydrated, the sections were oxidized with oxidant for 15 min and washed with distilled water for 2 min. Next, they were dyed with iron alum solution for 10 min and washed with distilled water for 2 min. After the sections were thoroughly cleaned, the excess water was poured out and the preheated methenamine silver working solution was dropwise added, the metal bath at 60°C was used for constant temperature dyeing for 40 min until the sections were tobacco yellow and washed with distilled water for 1 min. By now, the sections were re-stained with a light green solution for 1 min and washed with distilled water for 1 min. In the end, the sections were dehydrated with 95% ethanol 5 s, 100% ethanol I 5 s, 100% ethanol II 30 s, transparent with xylene 2 times, and sealed with neutral glue.

After completing the above staining steps, the sections were observed and photographed under a microscope (Aomori Olympus glass slide, Japan) at 40 ×.

**Determination of lipopolysaccharides (LPS) levels in kidney and gut**

Limulus reagent detection kit (Xiamen Boaoduo Technology Co., Ltd., Xiamen, China) was used to detect LPS levels in the intestine and kidney of mice according to the manufacturer’s instructions.

**Enzyme-linked immunosorbent assay (ELISA)**

Kidney tissue homogenate supernatants were collected to determine the concentration of inflammatory cytokines. Interleukin (IL)-6, IL-10, IL-17A in kidney, were determined by using an ELISA kit (Proteintech, Wuhan, China) according to the manufacturer’s instructions. The optical density at a wavelength of 450 nm was measured with an automatic microplate reader (Thermo Fisher Science Inc., USA).

**Quantitative real-time PCR**

According to the manufacturer’s protocol, total RNA was extracted from kidney tissue using the RNA Extraction kit (Omega, United States), and the UEIris RT mix with DNase (All-in-One) (UE, China) was used to synthesize cDNA. Then, RT-qPCR was performed using Universal SYBR Green qPCR Supermix (UE, China). The expression of the target gene was normalized by GAPDH. All experiments were carried out three independent experiments. Primer sequence (Sangon Biotech, Shanghai, China) was as follows: Keap1-F: 5′-GCTCAACCAGCTTGCTGTAGC-3′, Keap1-R: 5′-CATCCGCCACTTCTCCATTCTCTG-3′; Nrf2-F: 5′-AGCACAGCCACATTCTCC-3′, Nrf2-R: 5′-GACCAGAATCTACGGAACTTCC-3′; HO-1-F: 5′-AAGACCCTTCCTTGCTCAAC-3′, HO-1-R: 5′-TCTGACGAAGTGACGCATCTG-3′; JAK2-F: 5′-AGTGTGGAGATGTGCGCTATG-3′, JAK2-R: 5′-AGGTGCTTCCAGTGTGTGCG-3′; STAT3-F: 5′-CGATGCCCTTGAGGGAAGTCTC-3′, STAT3-R: 5′-TGTCACTACGGCGGGTGTG-3′; RORγt-F: 5′-TCTACAGGCGCTGGTTCT-3′, RORγt-R: 5′-ATGTTCACCTCCTCTTTCTTTCTTTG-3′; GAPDH-F: 5′-GGTTGCTCTCAGGCGATTCA-3′, GAPDH-R: 5′-TGGTCCAGGTTTCTTACTCC-3′.

**Western blot**
The kidney tissue protein was extracted using the whole protein extraction kit (Keygen, NO.KGP250, China) according to the manufacturer's protocol. Protein concentrations were detected with a BCA protein assay kit (Keygen, No.KGP902, China). 30 µg protein was added to each well of SDS-polyacrylamide gel (SDS-PAGE) for electrophoresis. The voltage of the concentrated gel was 90 V, and the voltage was changed to 120 V when the protein was transferred to the separation gel. After electrophoresis, the protein was transferred to the methanol-soaked polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, USA). Cell membranes were then blocked with 5% skimmed milk at room temperature for 2 h and incubated overnight at 4°C with primary antibody including: Heme oxygenase-1 (HO-1) rabbit mAb (1:5000 dilution, ABclonal, China), signal transducer and activator of transcription 3 (STAT3) rabbit mAb (1:1000 dilution, ABclonal, China), purified anti-retinoic acid-related orphan receptor gamma t (RORγt) Antibody (1:500, BioLegend, USA), janus kinase 2 (JAK2) rabbit mAb (1:1000 dilution, CST, USA) and mouse monoclonal GADPH (1:5000 dilution, Proteintech, China). After washing with TBST buffer for 3 times, they were incubated with HRP-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) (1:5000 dilution, Proteintech, China), HRP labeled Goat Anti-Mouse IgG (1:5000 dilution, Abbkine, China), or HRP-conjugated AffiniPure Goat Anti-Rat IgG (H + L) (1:5000 dilution, Proteintech, China) at room temperature for 1 h. After washing membranes with TBST buffer for 3 times, ECL chemiluminescence kit (Keygen NO.KGP1127, China) and SH-Compact 523 (Shenhua Technology Co., Ltd., USA) were used for imaging and detection. Image J software (National Institutes of Health Bethesda, MD) was used to analyze the gray value of the image.

Flow cytometry analysis

The cell suspension of spleen was prepared. The spleen was ground firstly, centrifuged at 400 × g, 4°C for 5 min after passing through a 300-mesh filter membrane and the supernatant was discarded. Appropriate amount of red blood cell lysate was added. After standing on ice for 5 min, the sample was centrifuged at 400 × g for 5 min, the supernatant was discarded, and lysed again according to the situation. Then RPMI1640 medium was used to wash the cells. Finally, the cell concentration was adjusted to 1×10^6 cells/mL for subsequent detection.

Peripheral blood mononuclear cell (PBMC) suspension was prepared as described below. The peripheral blood of mice was collected in an anticoagulant tube containing EDTA-K2, centrifuged at 600 × g, 4°C for 10 min and the plasma was frozen at -80°C. The remaining blood cell-containing liquid was transferred to the centrifuge tube and mixed with an appropriate amount of erythrocytes lysis solution. After standing on ice for 10 min, centrifuged at 4°C, 400 × g for 5 min, discarded the supernatant, and lysed again according to the situation. The cells were washed with RPMI 1640 medium. Finally, the cell concentration was adjusted to 1×10^6 cells/mL for subsequent measurement.

The colon cell suspension was prepared. After the colon was removed, the colon was washed with normal saline, the adipose tissue and feces were removed, and the longitudinal dissection was performed. The intestinal segment was cut into about 1cm, added with RPMI 1640 medium, and placed in a shaker at 37°C for 30 min (shaken every 10 min for 10 s). After shaking for 15 s, the intestinal
segment was allowed to sink for a moment to collect all the liquid, passing through a 200 mesh filter membrane (2 times) and collected into a centrifuge tube. At the same time, nylon wool column was prepared: glass wool was loosely filled in a 10 mL syringe and the column was firstly infiltrated with RPMI 1640 medium, the filtrate was continuously filtered for 3 times. Then the collected intestinal cell fluid was filtered and the column was washed with RPMI 1640 medium. All the liquid was collected and centrifuged for 500 × g, 4°C for 5 min, and the supernatant was discarded. The RPMI 1640 medium was added and centrifuged for 600 × g, 4°C for 20 min to collect the white membrane in the middle part. The RPMI 1640 medium was added and centrifuged for 400×g, 4°C for 5 min. The supernatant was discarded and an appropriate amount of RPMI 1640 was added to re-suspend the cells. The cell concentration was adjusted to 1×10⁶ cells/mL for subsequent detection.

Flow cytometry was used to detect the percentage of Treg and the percentage of Th17 cells in diverse tissues. For Treg staining, CD4-FITC (eBioscience, NO.2344796) was used for surface labeling and eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Thermo Fisher, USA) was elicited to fix and penetrate the cells. After that, the transcription factor forkhead box p3 (Foxp3)-PE (eBioscience, NO.4307350, USA) was added for intracellular labeling, and incubated at 4°C for 30 min in the dark. For Th17 cell staining, cells were stimulated at 37°C for 1 h using a 500 × cell stimulation cocktail (Thermo Fisher, USA), followed by cell staining, including CD4-FITC (eBioscience, NO.2344796, USA) and IL-17A-PE (eBioscience, NO.4306419, USA), incubated at 4°C in the dark for 30 min. Finally, prepared samples were measured and analyzed by Beckman Cyto FLEX flow cytometer (Beckman Bioscience, USA).

**Gut and Urethra microbiota analysis**

After 6 weeks of intervention with *C. butyricum* or normal saline, six mice in each group were randomly selected to collect fresh feces in sterile cages and three mice in each group were randomly selected to collect fresh urine in sterile metabolic cages. The collected feces and urine were immediately stored at -80°C until DNA was extracted.

The total DNA was extracted by Omega Mag-bind soil DNA kit (Omega M5636-02) and the DNA was quantified by Nanodrop, the quality of DNA extraction was detected by 1.2% agarose gel electrophoresis. UsingO5® High-Fidelity DNA Polymerase of TransGen Biotech (Beijing, China) with the primers 338F-5′ ACTCCTACGGGAGGCAGCAG 3′, 806R-5′ GGACTACHVGGGTWTCTAAT 3′ to amplified the hypervariable region V3-V4 of 16S rRNA sequence in bacterial DNA samples by PCR amplification instrument (ABI 2720, USA). PCR amplification system (25 µL) : 5 × reaction buffer 5 µL, 5 × GC buffer 5 µL, dNTP (2.5mM) 2 µL, forward primer (10 µM) 1 µL, reverse primer (10 µM) 1 µL, DNA Template 2 µL, ddH₂O 8.75 µL, Q5 DNA Polymerase 0.25 µL. Amplification parameters: initial denaturation 98°C 2 min, denaturation 98°C 15 s, annealing 55°C 30 s, extension 72°C 30s, final extension 72°C 5min, 10°C hold,25–30 cycles. Quant-iT PicoGreen dsDNA Assay Kit fluorescence reagent and Microplate reader (BioTek, FLx800) quantitative instrument were used for fluorescence quantification of PCR amplification recovery products. The sequencing library was prepared using Illumina’s TruSeq Nano DNA LT Library Prep Kit, and the library was subjected to final fragment selection and purification by 2% agarose gel electrophoresis. Finally, the
sequencing was performed on a MiSeq sequencer by Suzhou Panomix Biomedical Technology Co., Ltd., China.

**Measurement of the feces and urine SCFAs Concentrations**

Ether was obtained from Titan (Shanghai, China). Phosphoric acid was obtained from Sinopharm (Shanghai, China). Acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, 4-methylvaleric acid and caproic acid were all obtained from Sigma-Aldrich (Shanghai, China). The 100 mg/mL stock solutions of 6 SCFAs (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid) and caproic acid were prepared by water and ether methods, respectively and a series of working standard solutions were obtained by dilution.

Fecal standards were prepared and metabolites were extracted. The internal standard (4-methylvaleric acid) was prepared with ether to 375 µg/mL, 200 µL series of working standard solutions of 6 acids, 100 µL 15% phosphoric acid, 20 µL series of working standard solutions of hexanoic acid, 20 µL internal standard and 260 µL ether were mixed to prepare ten standard curve points, covering 0.02 to 500 µg / mL (0.02, 0.1, 0.5, 2, 10, 25, 50, 100, 250, 500 µg / mL). A total of 50 mg fecal samples were homogenized with 100 µL of 15% phosphoric acid, 20 µL of 375 µg / mL internal standard (4-methylpentanoic acid) solution and 280 µL ether for 1 min, centrifuged at 4°C 12000 rpm for 10 min. The supernatant was analyzed by gas chromatograph-Mass spectrometric (GC-MS).

Urine standards were prepared and metabolites were extracted. The internal standard (4-methylvaleric acid) was prepared with ether to 75 µg/mL. A series of working standard solutions of 200 µL six acids, 100 µL 15% phosphoric acid, 20 µL caproic acid, 20 µL internal standard and 260 µL ether were mixed to prepare ten standard working solutions, covering from 0.02 to 100 µg/mL (0.02, 0.1, 0.5, 1, 2, 5, 10, 25, 50, 100 µg/mL). Appropriate amount of sample was taken in a 2 mL centrifuge tube, added with 50 µL 15% phosphoric acid, and then added with 10 µL of 75 µg / mL internal standard (isocaproic acid) solution and 140 µL of ether for homogenization for 1 min, centrifuged at 4°C 12000 rpm for 10 min, and the supernatant was analyzed by GC-MS.

The GC analysis was performed on trace 1310 gas chromatograph. The chromatographic column was Agilent HP-INNOWAX capillary column (30 m×0.25 mm ID×0.25µm)(Thermo Fisher Scientific, USA). MS detection of metabolites was performed on ISQ LT (Thermo Fisher Scientific, USA).

**Statistical analysis**

GraphPad Prism version 9.0 (GraphPad Software Inc., La Jolla, CA, USA) and the statistical package for the social sciences (SPSS) 23.0 software (IBM Inc., Armonk, NY, USA) were used for statistical analyses. All experimental data were expressed as the mean ± SD of at least three independent experiments. One-way analysis of variance was used to compare the mean values of variables between groups. After that, Tukey post hoc test was used to determine the significance of pairwise comparison of mean values between groups. In addition, the expression correlation was analyzed by Spearman correlation coefficient assay. \( P < 0.05 \) was considered statistically significant.
Results

C. butyricum treatment improved BWs, 24-hour Urinary Protein and kidney insuciciency in mice with PNS

At the beginning of the experiment, there was no significant difference in BWs among the 4 groups of mice. After successful modeling, the mice in the model group were given C. butyricum or normal saline for 6 weeks. It was found that compared to the CON group, the BWs of the mice in the PNS group decreased significantly in the experiment ($P < 0.0001$), while the weights in the PNS + C. butyricum group were increased gradually after 2 weeks of dietary C. butyricum supplement ($P < 0.01$, Figure.1B). 24-hour Urinary Protein was significantly increased ($P < 0.01$) at 1 week after DOX injection, whereas C. butyricum treatment significantly reduced about 70% of urine protein after 3 weeks and 80% of urine protein at 5 weeks (Figure.1C).

Urea nitrogen and creatinine in plasma and urine were measured to evaluate renal function. Compared with the CON group, the levels of BUN and SCr in the PNS group were significantly increased ($P < 0.0001$), while the levels of BUN and SCr in the PNS + C. butyricum group were significantly decreased ($P < 0.0001$) (Figure.1D, E). Similarly, compared with the CON group, the levels of UUN ($P < 0.001$) and UCr ($P < 0.0001$) in the urine of the PNS group showed increased, and the levels of UUN ($P < 0.001$) and UCr ($P < 0.0001$) in the PNS + C. butyricum group were attenuated significantly (Figure.1F, G). Collectively, C. butyricum could ameliorate the functional impairment of the kidney in mice with PNS.

C. butyricum treatment alleviated the pathological progress of PNS mice

To confirm whether C. butyricum treatment could effectively affect the pathological changes of PNS, renal sections were stained with HE, PAS, Masson and PASM. As expected, compared to the CON group, HE staining of the kidney in the PNS group showed increased tubular epithelial cell volume, irregular lumen, interstitial edema and inflammatory cell infiltration (Figure.2A) and Masson staining showed increased kidney interstitial fibrosis (Figure.2B), PAS staining showed glycogen deposition and basement membrane thickening in the glomeruli and tubules (Figure.2C). In addition, we also observed partial disruption of glomerular capillary basement membrane in the PNS group by PASM staining, but these changes were improved by dietary C. butyricum administration (Figure.2D). Taken together, C. butyricum treatment possessed the capacity for attenuating the pathological lesions of PNS mice.

C. butyricum treatment reduced endotoxin levels in gut-kidney axis of PNS mice

LPS derived from the gut pathogenic bacteria play a vital role in the promotion of inflammation. Thus, the level of LPS in gut-kidney axis was measured and found that compared with the CON group, LPS in the intestine and kidney of the PNS mice was significantly increased ($P < 0.001, P < 0.0001$), but significantly decreased after C. butyricum treatment ($P < 0.01, P < 0.0001$), indicating that C. butyricum could weaken LPS generation and translocation from gut to kidney (Figure.3A,B).

C. butyricum suppressed the inflammation by regulating the balance of Th17 / Tregs in mice with PNS
Clinical studies have demonstrated that there is an imbalance of Th17 / Tregs in peripheral blood of PNS children[12]. In order to further evaluate the effect of *C. butyricum* on Th17 / Tregs balance in PNS, we used flow cytometry to detect Treg cells and Th17 cells in different tissues. Compared with the CON group, the expression of IL-17A in the spleen of PNS was significantly increased (*P* < 0.01), while the expression of IL-17A after *C. butyricum* treatment was significantly decreased (*P* < 0.01) (Figure.4A). Meanwhile, we found that compared to the CON group, the expression of Foxp3 in the spleen, peripheral blood and colon of the PNS group was significantly decreased (*P* < 0.01, *P* < 0.05, *P* < 0.01), while which was conversed by dietary *C. butyricum* administration (*P* < 0.05) (Figure.4B-D). The above suggested that probiotic *C. butyricum* could effectively attenuated impeded Th17 - Tregs balance in mice with PNS.

Next, we identified the anti-inflammatory effect of *C. butyricum* on PNS. The results showed that the level of anti-inflammatory IL-10 in the kidney of the PNS model was significantly decreased (*P* < 0.0001, Figure.5A) and the levels of pro-inflammatory IL-6 and IL-17A in the kidney were increased (*P* < 0.01, *P* < 0.05, Figure.5B,C). After the treatment with *C. butyricum*, the kidney levels of IL-10 (*P* < 0.0001) and IL-6 (*P* < 0.0001) and IL-17A (*P* < 0.001) were significantly ameliorated (Figure.5A-C). In summary, *C. butyricum* could suppressed the inflammation in mice with PNS.

**C. butyricum treatment alleviated the inflammation through HO-1 / STAT3 / RORγt signaling pathway in mice with PNS**

In order to elucidate the anti-inflammatory mechanism of *C. butyricum*, we detected the transcriptional expression levels of Kelch-like ECH-associated protein 1 (Keap1), nuclear factor erythroid2-related factor 2 (Nrf2), HO-1, STAT3 and JAK2 in kidney tissues. The results showed that compared with the CON group, the mRNA expression levels of Keap1, HO-1 and RORγt in the PNS were significantly increased (all *P* < 0.0001, Figure.6A, C, F), while the mRNA expression levels of Nrf2 and STAT3 were significantly decreased (all *P* < 0.0001) (Figure.6B, E). However, *C. butyricum* treatment significantly alleviated the mRNA expression levels of Keap1, HO-1 and RORγt (all *P* < 0.001), as well as elevated the mRNA expression levels of Nrf2 and STAT3 (*P* < 0.01, *P* < 0.0001). In addition, the expression of JAK2 showed no significant change in the mice of diverse groups (Figure.6D).

The protein expressions of HO-1, JAK2, STAT3 and RORγt were further measured by western blot. The results showed that compared with the CON group, the expression levels of HO-1 (*P* < 0.01, Figure.6G, H) and STAT3 (*P* < 0.05, Figure.6G, J) in the kidney tissue of the PNS group were notably decreased, which could be rectified by *C. butyricum* treatment. In addition, abnormal elevated RORγt in PNS model was completely restored by *C. butyricum* persistent intervention(*P* < 0.05, Figure.6G, K). The expression of JAK2 was not significantly altered among diverse groups (*P* > 0.05, Figure.6G, I), indicating that *C. butyricum* predominantly interacted with STAT3 rather than JAK2.

It is concluded that *C. butyricum* may lead to the instability of Keap1-Nrf2 binding by inhibiting the expression of Keap1 gene, and promote Nrf2 into the nucleus, then enhancing the expression of downstream HO-1 gene, thereby inhibiting the phosphorylation of STAT3, and then reducing the
expression of RORγ (Figure 6L). Therefore, _C. butyricum_ may reduce the immune inflammatory response of PNS through the HO-1 / STAT3 / RORγ signaling pathway.

**C. butyricum treatment improved the imbalance of gut microbiome in mice with PNS**

In recent years, accumulating studies have confirmed that microbiota has an important impact on the occurrence and development of PNS through the gut-kidney axis\[^{12,17,21}\]. Due to study the effect of _C. butyricum_ on the composition of gut microbiota in PNS, we detected fecal samples in different groups by 16S rRNA sequencing. Here, alpha-diversity was firstly analyzed. The rarefaction curve showed that the sequencing depth of samples among different samples was consistent (Figure 7A). Compared with the CON group, the observed species and Chao 1 of PNS mice were significantly increased ($P < 0.0001$, $P < 0.001$), while _C. butyricum_ treatment significantly reduced the observed species characteristics and Chao 1 (all $P < 0.01$), but there was no significant difference in Shannon index among the groups ($P > 0.05$) (Figure 7B). Jaccard-based principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) were used to analyze the overall composition of the bacterial community. A cluster represented a group. We found that the bacterial community between the PNS group and the CON group was significantly different and different clusters were formed after the supplementation of _C. butyricum_ in PNS (Figure 7C).

Next, we studied the abundance changes of intestinal flora in different groups at the phylum and genus levels. The results showed that at the phylum level, _Firmicutes_ and _Bacteroidetes_ were dominant in different groups. The proportion of _Firmicutes_ or _Bacteroidetes_ in PNS group was separately higher than that in CON group and the ratio of _Firmicutes_ to _Bacteroidetes_ was significantly increased ($P < 0.05$). However, in the PNS + _C. butyricum_ group, the proportions of _Firmicutes_ and _Bacteroidetes_ in gut microbial community was restored, the ratio of _Firmicutes_ to _Bacteroidetes_ also decreased significantly ($P < 0.05$) (Figure 7D, E). At the genus level, the top 20 genera were also analyzed and found differences among groups (Figure 7F). The heat map of the abundance data of the top 50 genera with average abundance, showed that the abundance distribution in the PNS group was different from that in the other three groups (Figure 7G). In addition, LEfSe analysis found that there were significant differences in species among groups (Figure 7H). We observed that the abundances of _Bacteroides, Parabacteroides_ and _Bacillus_ in the PNS group were elevated compared with those in the CON group ($P 0.05$), but the abundance of _Adlercreutzia_ was lower in PNS model ($P 0.01$). After dietary probiotic _C. butyricum_ treatment, the abundances of _Bacteroides, Parabacteroides_ and _Bacillus_ were reduced ($P 0.05$), whereas _Adlercreutzia_ was increased ($P 0.05$) (Figure 7I-L). Moreover, community analysis using the venn diagram showed that the number of OUTs shared by the four groups was 416, and the number of OUTs unique to the CON group, CON + _C. butyricum_ group, PNS group and PNS + _C. butyricum_ group was 1041, 823, 2607 and 1961, respectively (Figure 7M). In summary, _C. butyricum_ significantly changed the initial proportion of OTUs at the genus level, mainly including _Bacteroides, Parabacteroides, Bacillus_ and _Adlercreutzia_.

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\[^{12}\] Z. C. \[^{17}\] W. \[^{21}\] L.
**C. butyricum** treatment modulated urinary microbiota in PNS mice

Studies have shown that there are changes in the urinary microbiota in many urinary system diseases, such as interstitial cystitis, bladder cancer\[^{22, 23}\]. Thus, we speculated a close relationship between urinary microbiota and PNS with or without dietary *C. butyricum* treatment. After 16S rRNA sequencing and analysis of the urine microbiota in mice of 4 groups. The sparse curve showed that the sequencing depth among different samples was consistent, indicating that the amount of sequencing data was reasonable (Figure. 8A). There was no significant difference in alpha diversity between the PNS group and the CON group (Observation species, Chao1 index, shannon index) (Figure. 8B). PCoA and NMDS were used to analyze the overall composition of urinary microorganisms. PCoA result showed that mice in the CON group had different bacterial community composition from the PNS group. However, the total bacterial community were dramatically altered by *C. butyricum* treatment (Figure. 8C). NMDS analysis also obtained similar results (Figure.8D).

At the phylum level, we found that *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were dominant. *Firmicutes* were more abundant in the CON, CON + *C. butyricum* and PNS + *C. butyricum* groups, but *Proteobacteria* was more abundant in the PNS group (Figure.8E). At the genus level, we analyzed the top 20 genera of urinary microorganisms and found that there was no significant difference in species abundance between the groups (Figure.8F). Further, the abundance data of the top 50 genera with average abundance were plotted to show that the species abundance distribution of the PNS group was different from that of other groups (Figure.8G). In addition, the Venn diagram showed that there were 51 common microorganisms among diverse groups. There were 191 OUTs in the CON group, 205 OUTs in the CON + *C. butyricum* group, 187 OUTs in the PNS group, and 584 OUTs in the PNS + *C. butyricum* group (Figure.8H).

**C. butyricum** treatment increased the content of SCFAs in feces of PNS mice

The concentrations of acetic acid, propionic acid, butyric acid and valeric acid in feces of mice in each group were determined by GC-MS. The total ion current chromatogram (TIC) showed that the methodology was stable (Figure.9A) and the relative standard deviation (RSD) was less than 10%, indicating that the data was reasonable (Figure.9B). Then, the relative contents of SCFAs in each group were displayed using the overall metabolite clustering heat map (Figure.9C), suggesting a significant reduction of the contents of SCFAs including acetic acid (*P* < 0.05, Figure.9D), propionic acid (*P* < 0.001) (Figure.9E), butyric acid (*P* < 0.001) (Figure.9F) and caproic acid (*P* < 0.05, Figure.9G) in mice with PNS. These decreased SCFAs were remarkably restored by continuous dietary *C. butyricum* intervention except caproic acid. Meanwhile, there was no significant difference in the contents of valeric acid, isovaleric acid and isobutyric acid among different groups (all *P* > 0.05) (Figure.9H-J). The protective effect of *C. butyricum* on PNS may be due to the promotion of SCFAs production in the intestine.
The effect of *C. butyricum* treatment on the contents of SCFAs in urine of PNS mice

The above data that *C. butyricum* could promote the contents of SCFAs in the feces of PNS mice convinced us that the contents of SCFAs in the urine also may probably be influenced by dietary *C. butyricum* administration. Firstly, the method was proved to be stable according to TIC and the RSD was less than 10% /15%, indicating that the data performed well (Figure.10A,B). The overall heat map showed the relative contents of SCFAs in mice of diverse groups kept considerable difference (Figure.10C). However, there was no significant difference in the contents of SCFAs between PNS group and CON group (*P* > 0.05) (Figure.10D-J). Importantly, the treatment of *C. butyricum* increased the contents of acetic acid and valeric acid (all *P* < 0.05) (Figure.10D,G). Therefore, dietary *C. butyricum* intervention significantly increased the content of acetic acid and valeric acid in urine.

**Correlation Analysis**

In order to evaluate the relationship among gut microbiome / urine microbiome, inflammation, metabolic indicators and SCFAs in PNS, we performed a correlation analysis. We found in the gut microbial genus level that *Rikenellaceae*, *RF39* and *AF12* were positively correlated with IL-10 and SCFAs. But it was inversely proportional to IL-6, IL-17, LPS and metabolic indicators. On the contrary, *Prevotella*, *Bacteroides* and *Bacteria* were negatively correlated with IL-10 and SCFAs, and positively correlated with IL-17, LPS and metabolic indicators. The remaining bacteria including *Clostridiales*, *Adlercreutzia*, *Desulfovibrio* and *F16* were positively correlated with SCFAs. In addition, *Parabacteroides* was directly proportional with urinary metabolism indicators, IL-17 and LPS, but inversely proportional to IL-10 (Figure.11A). At the level of urinary microbial genera, only *Acinetobacter* was proportional to UCr, *Enterococcus* was inversely proportional to SCr and IL-6, and *Enterobacteriaceae* was inversely proportional to Acetic acid (Figure.11B). These results indicated that there was a closely complicated correlation between gut microbiome / urine microbiome, PNS metabolic indicators and inflammatory indicators.

**Discussion**

In this study, the protective effect of *C. butyricum* on PNS was investigated by measuring 24-hour urinary protein, metabolites, kidney pathological damage, inflammation and gut microbial community. Our results suggest that supplementation of *C. butyricum* could effectively alleviate PNS, which may be due to the regulation of Th17 / Tregs balance by *C. butyricum* through HO-1 / STAT3 / RORγt signaling pathway to inhibit the immune inflammatory response of PNS and restore gut microbial disorders in mice. Herein, our study may provide a safe, effective and inexpensive intervention for the treatment and prevention of recurrence of PNS in children.

PNS in children is characterized by a series of pathological and physiological changes caused by a large amount of urinary protein loss[24]. The ultrastructure of podocytes is the ultimate barrier leading to the loss of urinary protein[2,25]. Long-term proteinuria can lead to progressive decline in glomerular filtration
function, kidney interstitial damage, kidney tubular sclerosis, and ultimately kidney dysfunction, development of chronic kidney disease, and poor prognosis\textsuperscript{[26]}. In this study, it was observed that the BWs in mice with PNS induced by tail vein injection of DOX was lower, while \textit{C. butyricum} treatment could slow down the weight loss of PNS. In addition, \textit{C. butyricum} treatment could reduce proteinuria in PNS, which was consistent with our conjecture. Importantly, compared to PNS model, we found that after 6 weeks of intragastric administration of \textit{C. butyricum}, urea nitrogen and creatinine in blood and urine were reduced, and pathological damage was alleviated, indicating that \textit{C. butyricum} had a significant recovery effect on PNS injury.

At present, the pathogenesis of PNS are poorly understood. It is generally believed that immune abnormalities are the initial factors, and inflammatory response plays an important role in the occurrence and development of PNS\textsuperscript{[27, 28]}. LPS is a highly inflammatory component of the cell wall of Gram-negative bacteria, which is the causal relationship between gut microbiota and systemic low-grade inflammation\textsuperscript{[29]}. In this study, the levels of LPS in the gut and kidney were significantly reduced in the PNS + \textit{C. butyricum} group, indicating that \textit{C. butyricum} reduced the translocation and circulation of LPS from the intestine to the kidney in PNS, thereby helping to reduce the renal inflammatory response, but the cellular and molecular mechanism needs further study. Tregs is a kind of significant immunosuppressive cells, represented by naturally generated thymus-derived CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} Tregs (nTregs) and inducible Tregs cells (iTregs)\textsuperscript{[30]}. Under physiological conditions, Th17 / Tregs is in a state of dynamic immune balance. When the body is abnormal, Th17 / Tregs imbalance can cause a series of inflammatory immune responses to damage the body\textsuperscript{[9, 12, 31]}. Studies have shown that Foxp3\textsuperscript{+} Treg cells in peripheral blood and renal tissue of children with PNS are down-regulated, and the expression of IL-23p19, IL-17, IL-6 and IL-1\textbeta in kidney tissue is increased\textsuperscript{[9]}. Butyric acid can significantly enhancing the acetylation of histone h3 in the promoter of Foxp3 site and the conserved non-coding sequence region which is a key marker of Tregs, suggesting that butyric acid plays an important role in the induction of Treg cell differentiation\textsuperscript{[15, 18]}. Our study also confirmed the down-regulation of Treg cells and the up-regulation of Th17 cells in PNS, importantly, both of which were restored after the intervention of \textit{C. butyricum}. In addition, our study also observed that the anti-inflammatory factor IL-10 of PNS was significantly reduced and the pro-inflammatory factor IL-6 and IL-17A was significantly increased, which was significantly improved after \textit{C. butyricum} intervention. In summary, \textit{C. butyricum} can regulate the balance of Th17 / Tregs in PNS and reduce the immune inflammatory response of PNS.

Studies have found that butyrate may activate Nrf2 at the transcriptional level, thereby triggering anti-inflammatory and antioxidant responses to prevent diabetes-induced body damage and slow down the damage of diabetic nephropathy\textsuperscript{[32]}. Similar studies have demonstrated that butyrate blocks liver injury and cerebral ischemia injury caused by various factors by regulating the Keap1 / Nrf2 pathway\textsuperscript{[33, 34]}. Therefore, we speculate that \textit{C. butyricum} may play a regulatory role in the Keap1 / Nrf2 pathway through its metabolites. Further search for the downstream of Nrf2, in many tissues and organs of the organism, the antioxidant response element (ARE) maintains the redox state and reduces the oxidation pathway in oxidative stress under the normal conditions. When the body is stimulated by oxidative stress, Nrf2 and
Keap1 are separated from each other and enter the nucleus and bind to ARE to activate the expression of antioxidant enzyme genes including Nicotinamide adenine dinucleotide phosphate (NADPH) and HO-1[35–38]. More interestingly, HO-1 can inhibit IL-6-induced STAT3 phosphorylation pathway. HO-1 promotes the formation of JAK2-STAT3 complex by binding to JAK2, inhibits the phosphorylation of STAT3, thereby down-regulating the expression of RORγt, and induces the differentiation of Treg cells by inhibiting Th17 cell differentiation[16, 39, 40]. In the present study, as we judged, the metabolite butyric acid of C. butyricum binds to keap1 to inhibit the expression of keap1 gene, making the binding of keap1 to Nrf2 unstable, Nrf2 detaches from the nucleus and binds to ARE, increasing the expression of downstream antioxidant enzyme HO-1 protein, thereby inhibiting the phosphorylation of STAT3, not JAK2, regulating the IL-6-STAT3-RORγt pathway to reduce the expression of RORγt, inhibiting the differentiation of initial CD4+ T cells into Th17 cells and promoting the converse differentiation into Treg cells (Figure.12). In our study, the decrease of HO-1 protein expression level in PNS mice was in line with expectations, while the gene expression level of HO-1 was increased. In summary, dietary C. butyricum supplementation reduced the immune inflammatory response of PNS through the HO-1 / STAT3 / RORγt signaling pathway.

The importance of intestinal microbes in nephrotic syndrome has been widely recognized[12, 41, 42]. At the level of gut microbacteria, we found that Firmicutes and Bacteroidetes were dominant in different groups, which was consistent with previous studies[21]. Moreover, the increase in the ratio of Firmicutes/Bacteroidetes in the PNS group was a feature identified with gut dysbiosis, which was closely related to renal injury[43]. At the genus level, our results showed that C. butyricum restored intestinal homeostasis of PNS by up-regulating Adlercreutzia and down-regulating Bacteroides, Parabacteroides, and Bacillus. Studies have shown that Adlercreutzia is depleted in patients with liver diseases such as NAFLD, and its abundance also decreases with the progression of the disease, indicating a close correlation with the severity of the disease[44]. In this study, Adlercreutzia was positively correlated with IL-10 and SCFAs, but it is inversely proportional to IL-17, LPS and PNS metabolic indicators, indicating that Adlercreutzia may contribute to anti-inflammation effect on PNS in mice.

SCFAs are the main metabolites produced by bacterial fermentation of dietary fiber in the gastrointestinal tract[45]. Numerous evidences have suggested that SCFAs play a key role in regulating mental function[46], metabolism[47, 48], inflammatory response[49] and other diseases. The highest contents of SCFA in the intestine were acetate, propionate and butyrate. Acetic acid was mainly produced by anaerobic bacteria, such as A. muciniphila and Bacteroides spp. Propionate was mainly produced by Bacteroides and Butyrate was mainly produced by Clostridium cluster IV and XIVa, and F. prausnitzii[45]. A study have shown that butyric acid is less in the feces of chronic kidney disease (CKD) mice and prausnitzii could increase the level of butyric acid in feces. No effect of prausnitzii on acetic acid or propionic acid was found[44]. More interestingly, in our study, it was found that Bacteroides was negatively correlated with SCFAs. The abundance of Bacteroides in PNS group was significantly increased, while the contents of acetic acid, propionic acid and butyric acid were significantly decreased. The contents of acetic acid, propionic acid and butyric acid were significantly increased after C. butyricum treatment. Additionally,
there may be other microbial metabolites in the role of PNS to be further studied using metabolomics methods.

Studies have shown that urinary microbes play an important role in the development of urolithiasi, interstitial cystitis, bladder cancer and attention deficit hyperactivity disorder (ADHD)\cite{22, 23, 50–52}. However, the role of urethral microbes in the development of PNS has rarely been reported. In this study, we found that there was no significant difference in alpha diversity between PNS group and CON group, and beta diversity showed different bacterial community composition. At the phylum level, we found that Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes were dominant, and Proteobacteria was more abundant in PNS group. Proteobacteria is a human opportunistic pathogen that mainly causes infections in people with impaired immune systems and cause complex urinary tract infections\cite{53}. Whereas, in the genus level, we found no significant difference in species abundance between groups. Our study also showed that there was no statistically significant difference in the content of short-chain fatty acids in PNS urine, but \textit{C.\,butyricum} treatment increased the content of acetic acid and valeric acid in urine. The deficiency of our research on urine microorganisms is that there were less urine samples. Indeed, the exact role of urinary microorganisms in PNS is largely unknown and needs further study.

**Conclusion**

In summary, \textit{C.\,butyricum} regulates Th17 / Tregs balance via the gut-kidney axis to reduce the immune inflammatory response of PNS through the HO-1 / STAT3 / ROR\(\gamma\)t signaling pathway, which may be a safe, inexpensive intervention for the treatment and prevention of PNS recurrence.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PNS</td>
<td>Primary nephrotic syndrom</td>
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<tr>
<td>\textit{C.,butyricum}</td>
<td>\textit{C.,butyricum}</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin hydrochloride</td>
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<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
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<tr>
<td>SCR</td>
<td>Serum creatinine</td>
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<td>LPS</td>
<td>Lipopolysaccharides</td>
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<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid2-related factor 2</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1</td>
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HO-1  Heme oxygenase-1
STAT3  Signal transducer and activator of transcription 3
JAK2  Janus kinase 2
RORyt  Retinoic acid-related orphan receptor gamma t
SCFAs  Short-chain fatty acids

Declarations

Ethics approval and consent to participate

The animal study was approved by the Ethics Committee of Ningxia Medical University (No.2022-206). The study was conducted in accordance with the local legislation and institutional requirements.

Consent for publication

Not applicable.

Data Availability Statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, PRJNA1028525.

Competing interests

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

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Author Contributions

WH, LT, MXL, ZXX1 and MJH designed and wrote the paper. LT, MXL, TWY, LJ, WT, SWK, ZXX2, LYY, LYW and MJB performed research. All authors have read and approved the final manuscript. (ZXX1:Xiaoxia
Acknowledgements

Not applicable.

References


Figures
Figure 1

The impacts of *C. butyricum* treatment on BWs, 24-hour urinary protein and kidney function in DOX-induced PNS mice. Experimental design time diagram (A). BWs: Body weights (B). 24-hour urine protein of mice in diverse groups (C). BUN: Blood urea nitrogen (D). SCr: Serum creatinine (E). UUN: Urine urea nitrogen (F). UCr: Urine creatinine (G). Data were expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All experiments were performed in triplicate.
Figure 2

The effect of *C. butyricum* treatment on pathological progress of PNS mice. Representative images of kidney pathological staining including HE: hematoxylin and eosin (A), Masson's trichrome stain (B), PAS: periodic acid Schiff (C), PASM: periodic acid-silver methenamine (D).
Figure 3

_C. butyricum_ treatment reduced lipopolysaccharide (LPS) level in mice with PNS. Gut LPS levels (A). Kidney LPS levels (B). Data were expressed as mean ± SD. **P < 0.01, ***P < 0.001, ****P < 0.0001. All experiments were performed in triplicate.
Figure 4

Regulation of Th17 / Tregs balance by dietary *C. butyricum* treatment in mice with PNS. Flow cytometry analysis was used to separately determine the proportions of splenic Th17 cells (A), splenic Treg cells (B), peripheral blood Treg cells (C) and colon Treg cells (D) in diverse groups. Data were expressed as mean ± SD. *P* < 0.05, **P** < 0.01. All experiments were performed in triplicate.
Figure 5

Suppression of the immune inflammatory reaction by dietary *C. butyricum* treatment in mice with PNS. Kidney tissue was elicited to determine the concentrations of IL-10 (A), IL-6 (B) and IL-17A (C). Data were expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All experiments were performed in triplicate.
C. butyricum alleviated the immune inflammatory response in PNS through HO-1 / STAT3 / RORγt signaling pathway. The mRNA levels of Keap1 (A), Nrf2 (B), HO-1 (C), JAK2 (D), STAT3 (E) and RORγt (F) in the kidney tissues. Representative western blot images and statistical results of HO-1 (G,H), JAK2 (G,I),STAT3(G,J) and RORγt (G,K) expressions of protein levels in the kidney tissues. HO-1 / STAT3 /
RORγt pathway diagram (L). Data were expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All experiments were performed in triplicate.

Figure 7

The modulation of gut microbiome by probiotic *C. butyricum* supplementation in mice with PNS. Dilution curve (A). Alpha diversity analysis included Observed species, Chao1 and Shannon (B). Beta diversity

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analysis includes principal coordinate (PcoA) analysis and non-metric multidimensional scaling (NMDS) analysis (C). Relative abundance of microbial species at the phylum level (D). Ratio of Firmicutes to Bacteroidetes (E). Relative abundance of microbial species at the genus level (F). Heatmap of species composition at the genus level of species clustering (G). LEfSe analysis displaying of inter-group differential taxa based on taxonomic tree (H). Bacteroides (I). Parabacteroides (J). Bacillus (K). Adlercreutzia (L). Venn diagram (M). Data were expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Figure 8

The changes of urinary microbiota after *C. butyricum* treatment in mice with PNS. Dilution curve (A). Alpha diversity analysis included Observed species, Chao1 and Shannon (B). PcoA analysis (C). NMDS analysis (D). Relative abundance of microbial species at the phylum level (E). Relative abundance of microbial species at the genus level (F). Heatmap of species composition at the genus level of species clustering (G). Venn diagram (H).
Figure 9

*C. butyricum* treatment increased the contents of short chain fatty acids (SCFAs) in feces of PNS mice. Chromatogram of mouse fecal samples (A). Relative standard deviation (B). Cluster heat map of whole metabolites of gut microbiota (C). Acetic acid (D). Propionic acid (E). Butyric acid (F). Caproic acid (G). Valeric acid (H). Isovaleric acid (I). Isobutyric acid (J). Data are expressed as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.}
Figure 10

Effect of *C. butyricum* treatment on the contents of SCFAs in urine of PNS mice. Chromatogram of mouse urine samples (A). Relative standard deviation (B). Cluster heat map of whole metabolites of urine microbiota (C). Acetic acid (D). Propionic acid (E). Butyric acid (F). Valeric acid (G). Caproic acid (H). Isobutyric acid (I). Isovaleric acid (J). Data were expressed as mean ± SD. *$P < 0.05$. 

Figure 11

Correlation analyses among relative abundance of gut/urine microbiota and other related indicators. Correlation of gut microbiota with inflammation, metabolic indicators and SCFAs in PNS (A). Correlation of urine microbiota with inflammation, metabolic indicators and SCFAs in PNS (B). *$P < 0.05$, **$P < 0.01$. 
Figure 12

Patterns of effectiveness of *Clostridium butyricum* for the treatment of PNS by regulating Th17 / Tregs axis via the HO-1 / STAT3 / RORγt signaling pathway and modulating gut microbiota in mice.