Probiotic \textit{Lactiplantibacillus plantarum} N-1 could prevent ethylene glycol-induced kidney stones by regulating gut microbiota and enhancing intestinal barrier function

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Abstract
Defective permeability barrier is considered to be an incentive of hyperuricemia, however, the link between them has not been proven. Here, we evaluated the potential preventive effects of \textit{Lactiplantibacillus plantarum} N-1 (LPN1) on gut microbiota and intestinal barrier function in rats with hyperoxaluria-induced kidney stones. Male rats were supplied with 1% ethylene glycol (EG) dissolved in drinking water for 4 weeks to develop hyperoxaluria, and some of them were administered with LPN1 for 4 weeks before EG treatment as a preventive intervention. We found that EG not only resulted hyperoxaluria and kidney stone formation, but also promoted the intestinal inflammation, elevated intestinal permeability, and gut microbiota disorders. Supplementation of LPN1 inhibited the renal crystalline deposits through reducing urinary oxalic acid and renal osteopontin and CD44 expression and improved EG-induced intestinal inflammation and barrier function by decreasing the serum LPS and TLR4/NF-κB signaling and up-regulating tight junction Claudin-2 in the colon, as well as increasing the production of short-chain fatty acid (SCFAs) and the abundance of beneficial SCFAs-producing bacteria, mainly from the families of \textit{Lachnospiraceae} and \textit{Ruminococcaceae}. Probiotic LPN1 could prevent EG-induced hyperoxaluria by regulating gut microbiota and enhancing intestinal barrier function.

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Abbreviations: BUN, blood urea nitrogen; CaOx, calcium oxalate; EG, ethylene glycol; GC, gas chromatography; IL-1β, interleukin-1β; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; LPN1, \textit{Lactiplantibacillus plantarum} N-1; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-B; OPN, osteopontin; OUT, operational taxonomic unit; PCoA, principal coordinates analysis; SCFAs, short-chain fatty acids; TLR4, toll-like receptor 4; UPGMA, unweighted pair-group method with arithmetic means.

Zhitao Wei and Yaqian Cui contributed equally to this work.

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1 | INTRODUCTION

Excessive exogenous intake and endogenous synthesis of oxalic acid in the human body are the leading cause of hyperoxaluria, which can further lead to a series of kidney diseases, such as systemic oxalosis, nephrocalcinosis, renal failure, and specially Calcium oxalate (CaOx) stones.1 Oxalate is the terminal metabolite by the human liver, which usually sourced from the dietary,2 and ultimately precipitated as CaOx crystals in the kidney, which is a major constituent of the most kidney stones.3,4 Hyperoxaluria may be caused by ileal or colonic oxalate hyperabsorption or oxalate overproduction, and the increases in urinary oxalate and calcium enhance the chances of stone formation.5 Enteric hyperoxaluria and oxalate-uro lithiasis have been reported to be the common complications in patients with inflammatory bowel disease, particularly in patients harboring Crohn’s disease.6 Exposure to oxalate ingestion may cause severe hypercalcemia and kidney stones, even have severe toxicity to gastrointestinal tract (GIT) and induce intestinal dysfunction, including diarrhea, intestinal inflammation, gastric hemorrhage, and deterioration of epithelium in GIT.7,8 Defective permeability barrier due to inflamed, malfunctioning mucosa, and diarrhea seems to promote enhanced oxalate absorption.6 However, this association between intestinal permeability and increased oxalate absorption has not been completely investigated in these studies.

For mammals, the metabolism of oxalic acid depends on processes such as its limited absorption, excretion process, and microbial degradation due to the lack of enzymes for the biotransformation of oxalate.9 Oxalate microbial biodegradation in the GIT overcomes the circulation of oxalate in blood, hence it has been suggested as a negative risk factor for diseases related to kidneys. Within this field of research, the main focus of the previous studies were on few oxalate-degrading bacteria, including Bifidobacterium spp., Lactobacillus spp., and Oxalobacter formigenes,9,10 thus the supplements of oxalate-degrading probiotic to prevent stone formation is highly recommended.11 There are some accumulating evidences for the role of gut microbial metabolism involved in the formation kidney stones,12 but results from studies carried out to identify a linkage of fecal oxalate-metabolizing bacterial species to the excretion of urinary oxalate, remain inconclusive.13

Endogenous metabolic processes are the main cause of urinary oxalate formation. In addition to that, the consumption of foods containing oxalic acid also contributed to oxalate formation in urine.14 Dietary composition is also considered as one of the vital factors that determines the diversity of gut microbiota and its metabolic capabilities.15 In a normal healthy person, 2% ~ 10% of oxalate in food is absorbed in blood while 90% ~ 98% is utilized by the gut microbiome as an energy source for growth.16 Certainly, the ability to obtain energy from oxalate seems to be common among gut bacteria, and the bacterial consortia of the gut may possibly develop a competitive advantage to utilize oxalate for their growth due to this ability, thereby shaping different gut ecosystem.17 The link of hyperoxaluria and gut microbiota disorder has been confirmed and focused on the microbial bio-degradation of oxalate.18 Also, the gut microbiota being a key multifaceted component of inflammation, appeared to play a potential role in balancing the system inflammation and preserving the intestinal epithelial barrier, which has been widely investigated in intestinal dysfunction.19 The relationship between gut microbiota disorder and intestinal dysfunction remains unexplained in hyperoxaluria. An adjunct approach to lower urinary oxalate level is to consume probiotic with the potential to degrade dietary oxalate.9,14 In contrast, the functions of probiotic in enhancement of immunomodulation, barrier function, and competitive adherence to the mucus and epithelium seems more worthy of attention in the prevention of hyperoxaluria. Whether resulting from dietary habits, transit-related nutrient depletion, or host-microbe interactions, it is crucial to identify the importance of oxalate in the development of gut ecosystem when it comes to developing microbial strategies to overcome host risk of stone formation.

Ethylene glycol(EG)-induced hyperoxaluria of rat model has been used to understand the pathogenesis of hyperoxaluria and stone formation.20 However, the effect of EG intake on the microbial biodegradation of oxalate and intestinal barrier function are unclear. In the current study, we examined the effects of gut microbiota and intestinal barrier function on oxalate excretion in EG-induced hyperoxaluria rats, and the probiotic Lactiplantibacillus plantarum N-1 (LPN1) was supplemented to rats before EG intake, in order to investigate the potential beneficial effects on gut microbiota and intestinal barrier function.
2 | MATERIALS AND METHODS

2.1 | LPN1 and experimental animals

The strain of LPN1 (CGMCC NO.15463) was isolated from traditional cheese in Daocsheng County, Sichuan Province by our laboratory. In our previous study, we demonstrated that the biological properties of LPN1 in vitro, including acid and bile salt tolerance, ability to adhere to the intestinal tract, and oxidation resistance, as well as the abilities to reduce cholesterol and promote short-chain fatty acids (SCFAs) in the gut in vivo, which make it valuable as a probiotic.

In our another previous study, we found that the direct addition of SCFAs could prevent the formation of renal calcium oxalate crystals. Since LPN1 produced SCFAs in relatively high amount and had antioxidant effect, we applied this strain to test its potential inhibition effect on the formation of calcium oxalate crystals in rat kidney by interrupting the oxalic acid metabolism.

The fresh culture of LPN1 was applied in this animal study for the highest effect. The LPN1 strain was subcultured to the logarithmic growth phase, centrifuged at 3000 g and 4°C for 5 min, washed with sterile 0.9% normal saline for twice, centrifuged again, and then resuspended in sterile saline for fed within 48 h. The concentration was adjusted to $1 \times 10^8$ CFU/ml for subsequent use. In our preliminary experiment, EG-induced kidney stones rats were administered intragastrically with three doses of LPN1 at $10^7$, $10^8$, $10^9$ CFU/day, respectively, for 14 consecutive days, and it was found that the numbers of crystallization and stone formation markedly decreased with increasing of LPN1 concentrations, and a significantly drop of 50% was observed for the treatment of $10^8$ CFU/day to $10^7$ CFU/day (OD $3.63 \pm 1.60$ vs. OD $6.63 \pm 2.92$), while only 16% for $10^9$ CFU/day to $10^8$ CFU/day (OD $3.00 \pm 2.88$ vs. OD $3.63 \pm 1.60$). Therefore, we chose the relatively low dosage of $10^8$ CFU/day in this animal study for its satisfactory effect and lower cost, although the higher concentrations like $10^9$ CFU/day and even $10^{10}$ CFU/day may bring greater benefit.

SD rats of 6 weeks (male) and weighting 200–230 g were housed by following standard laboratory conditions of dark/light cycle for 12 h at 27 ± 1°C, and provided sterile water ad libitum. A total of 18 male rats were randomly distributed into three groups as follow: control group, model group (received 1% EG in drinking water for 4 weeks), and prevention group (was administered with LPN1 [1 × $10^8$ CFU/rats/day] for 4 weeks before given EG added into drinking water). As a control treatment, the control and model groups were treated with saline by intragastric administration at 9:00 AM daily for 4 weeks together with the prevention group. All animal maintenance and experiments were approved by the ethical guidelines recommended by the Institutional Animal Ethics Committee of Sichuan University with a permit number (2017063A).

2.2 | Urine biochemical analyses

For the evaluation of urinary parameters determining kidney stone formation, rat was housed in a metabolic cage to collect urine sample under acidified conditions at the end of 4 weeks treatments. The supernatant was separated from collected urine samples after 5 min centrifugation at 2500 rpm for further application for estimating the amount of calcium, uric acid, oxalic acid, and creatinine using colorimetric assay kits (Weibochem, Guangzhou, China).

2.3 | Serum analyses

Blood samples were drawn from the aorta artery of the rat's abdomen after anesthetized by 3% isoflurane inhalation, and samples were then transferred separately to serum separator and anticoagulant EDTA tubes. Analysis of creatinine and blood urea nitrogen (BUN) was carried out through an enzymatic method with the help of an automatic analyzer (Chiron Diagnostics Corporation, OH, USA). Furthermore, an assay named as EndoZyme II Recombinant Factor C endotoxin kit (Hyglos GmbH, Germany), was used for the evaluation of serum LPS level. All designed protocols for experiments were performed according to the instructions given by the manufacturer.

2.4 | Histopathological examinations

Kidneys, colonic tissue were carefully removed and followed by phosphate buffered saline (PBS) wash. The kidneys and colon were fixed in 4% paraformldehyde for 24 h and then embedded in paraffin, sectioned 4 µm intervals by Leica RM 2126 microtome and mounted on slides. The slides were dewaxed, hydrated and then processed by Hematoxylin and Eosin (H&E) staining for histopathological analyses. A microscope (Olympus BX51; Olympus optical, Tokyo, Japan) was employed to capture images to observe the tubular damage and crystal deposition in kidneys and the colon mucosal morphology.

2.5 | Real-time quantitative PCR

To extract total RNA from colon tissue, the RNA extraction kit (TianGen Biotech, Beijing, China) was used, following
the manufacturer’s instructions. To measure the RNA concentrations, a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) was employed. The PrimeScript cDNA synthesis Kit (TaKaRa, Japan) was utilized to reverse-transcribe one microgram of total RNA to synthesize cDNA. SYBR Green Supermix (Bio-Rad) was used to perform qPCR on a CFX96 real-time system (Bio-Rad) and the procedures were performed with regard to the manufacturer’s protocol. The sequences of the designed primers used in this study have been listed in Table 1, and the primers were synthesized by Invitrogen (Invitrogen, Shanghai, China). The mRNA expression was designed primers used in this study have been listed in gard to the manufacturer’s protocol. The sequences of the considering the 2−ΔΔCt method, the relative gene expression was evaluated.

2.6 Western blotting

After kidney collection from rats, the crushing of kidney was done by a pestle and mortar in liquid nitrogen, and then lysed with RIPA lysis buffer having 1% of protease inhibitor cocktail (Roche, Basel, Switzerland). Subsequently, centrifugation was carried out at a speed of 12 000 g at 4°C for 30 min and supernatant was then collected. The concentration of protein was measured using BCA assay Kit (Beyotime, Shanghai, China). After denaturation in boiling SDS sample buffer for 5 min, an amount of 50 μg of total protein was separated on 12% SDS-PAGE gels, blotted onto a PVDF membrane, and then probed with antibodies such as anti-OPN antibody (1:1000), anti-CD44 antibody (1:1000), anti-TLR4 antibody (1:1000), anti-NF-κB p50 antibody (1:1000), anti-IL-1p antibody (1:1000), anti-Claudin 2 antibody (1:1000), and monoclonal anti-GAPDH antibody (1:4000; Sigma, USA), followed by horseradish peroxidase-conjugated antibody (1:4000). Bands of protein were visualized by incubating for 1 min with BeyoECL Plus kit (Beyotime, P0018, China) and images were digitized by a Gel Image System (Bio-Rad). Densitometry measurement was carried out with an enhanced chemiluminescence (ECL) Detection System (Millipore, USA).

2.7 16S rRNA analysis of fecal samples

The fresh fecal samples were collected from rats followed by immediate freezing at −80°C. Analysis of the 16S rRNA from the fecal microbiota was carried out through Shanghai Majorbio Biotechnology Co., Ltd (Shanghai, China). The extraction of fecal DNA was accomplished with the help of aZR Fecal DNA Extraction Kit (Zymo Research, CA, USA). Amplification of Genomic DNA was conducted by using Phusion High-Fidelity polymerase (Thermo Fisher Scientific) and bacterial 16S rRNA gene (V3-V4 region)-specific primers: 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-ACTCCTACGGGAGGCAGCAG-3′). After the purification and uniformity, PCR amplicons were submitted to sequencing on an Illumina Miseq PE300 system. Reads of the original sequencing from the sample were arranged by unique barcodes, followed by the removal of the barcodes, linkers, and PCR primer sequences. After quality filters, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at a sequence similarity threshold of 97% through a reference-based USEARCH (version 5.2) pipeline, considering the Greengenes (Release 13.8) and SILVA databases as a closed reference. Distance calculation, rarefaction analysis, OTUs cluster, α-diversity, and β-diversity were carried out by the Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0) program.

2.8 Determination of SCFAs in fecal samples

The extraction of SCFAs of feces was conducted by considering the method of De Baere et al.24 with slight modifications. An amount of 0.3 g of fecal was diluted in sterile distilled water at a ratio of 1:4 to 1:8 (w/v). After mixing by vortex for 1 min, samples were centrifuged for 10 min at a speed of 3500 g, the supernatant containing SCFAs was collected and then filtered with the help of cellulose acetate membrane (0.22 μm pore size). Analyses of SCFAs

| TABLE 1 | Sequences of the primers used in this study |
| --- | --- |
| Gene | Primer sequence (5′-3′) |
| ZO-1 | F: ACCCCGAAACTGATGCTATGGA |
| | R: GCAGCCCTTGGGAAATGTATGGT |
| Claudin-2 | F: CCTCCTGGCTGAGACTCCATCACCTT |
| | R: GTGTGAGACGATAGCCGATCATCATA |
| TLR4 | F: CATTGGTCCCAACATCATCACA |
| | R: CCAGACGCGCTACTCAGAAACT |
| P50 | F: GCAAGCTCCATTGGAAATTCTCGAT |
| | R: CCCAGAGACTCATAGTTGTCCATA |
| P65 | F: ACCTGGAGACGACCATGACC |
| | R: CGCAGTCTGCACCTGGAAAGCA |
| IL-β | F: ATCTCCTCAGCTCAGGCTCTTTGCTG |
| | R: AGCTTGGCTGAGATGCTGCTG |
| IL-6 | F: GTGGCTTCTTTGGGACTGATGT |
| | R: TCTGTGTTGAGGTGTATATCCTG |
| β-actin | F: AGATCAAGATGACTTGCTCTT |
| | R: TACTTCTGCTTGCTGATCCA |

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| TLR4 | F: CATTGGTCCCAACATCATCACA |
| | R: CCAGACGCGCTACTCAGAAACT |
| P50 | F: GCAAGCTCCATTGGAAATTCTCGAT |
| | R: CCCAGAGACTCATAGTTGTCCATA |
| P65 | F: ACCTGGAGACGACCATGACC |
| | R: CGCAGTCTGCACCTGGAAAGCA |
| IL-β | F: ATCTCCTCAGCTCAGGCTCTTTGCTG |
| | R: AGCTTGGCTGAGATGCTGCTG |
| IL-6 | F: GTGGCTTCTTTGGGACTGATGT |
| | R: TCTGTGTTGAGGTGTATATCCTG |
| β-actin | F: AGATCAAGATGACTTGCTCTT |
| | R: TACTTCTGCTTGCTGATCCA |
were assessed by following the GC method as described previously Liu J et al.\textsuperscript{25}

\section*{2.9 Statistical analysis}

All values presented in the current study are expressed as a mean ± SD. SPSS 20.0 software (SPSS Inc, New York, USA) was applied to establish statistical analyses of experiments. After verifying a normal or non-normal distribution, one-way analysis of variance (ANOVA) followed by a Tukey’s multiple comparisons post-test was done. A $p$-value of less than .05 was considered as significant value. The R packages (v3.2.0) and QIIME (v1.8.0) were used to analyze the sequence data of fecal samples.

\section*{3 RESULTS}

\subsection*{3.1 LPNI prevented EG-induced kidney stone formation in kidney}

In the current study, we analyzed the urinary and serum parameters after supplemental LPNI for 4 weeks to investigate the development of hyperoxaluric state with clear crystal deposition and also to explore the preventive effect of LPNI (Table 2). The animals in EG-induced model group showed a major increase in urinary calcium, creatinine excretion, oxalic acid, and uric acid as compared to control rats, whereas the urinary calcium, creatinine uric acid, and oxalic acid were significantly found to be reduced in hyperoxaluric rats supplemented with LPNI. Furthermore, the increased BUN level in hyperoxaluric rats was also inhibited by LPNI supplement. H&E staining procedure was used to analyze the pathological status of urine tubules (Figure 1A). After 4 weeks of 1% EG feeding, significant deposition of crystals accompanied with tubular ectasia and light lymphocyte infiltration were found in the kidney sections of the model rats. The glomeruli of the rats from the preventive group were complete in morphology with clear contours and regular arrangement, where only slight tubular ectasia were observed. Furthermore, the expressions of OPN and its’ receptor CD44, related to kidney stone formation, were both examined at mRNA and protein levels. EG intake promoted the expression of OPN and CD44 in the kidney, while LPNI supplement inhibited the expressions of OPN and CD44 at mRNA (Figure 1B) and protein (Figure 1C) levels in EG-induced hyperoxaluric rats. Taken together, these data suggested that LPNI provided a protection against EG-induced kidney damage and inhibition of kidney stone formation.

\subsection*{3.2 LPNI prevented gut microbial dysbiosis of EG-induced hyperoxaluria rats}

Oxalate metabolism in mammals depends on the oxalate-metabolic enzymes secreted by gut microbiota, because themselves do not express endogenous oxalate-metabolizing enzymes. To investigate the relationship between gut microbiota and calcium oxalate stone formation, we analyzed the fecal microbiome based on Illumina sequencing of gut microbial 16S-rRNA genes.

Multiple alpha diversity metrics of diversity and richness explored the evident distinctions of microbial populations among control, model, and prevention groups. The alpha diversity indexes of Chao1, ACE, and Shannon in EG-induced hyperoxaluria rats were significantly lower than control rats, but LPNI supplementation could increase those alpha diversity indexes (Figure 2A). According to the PCoA on OUT level, an apparent separation of microbial population structures between EG-induced hyperoxaluria rats and control rats was illustrated (Figure 2B), demonstrating that the distinction between hyperoxaluria and control rats of major shifted in the composition of fecal microbiota. Furthermore, the result of samples from model groups were found to be clustered differently with those from controls groups, were further confirmed by a Hierarchical clustering of UPGMA.

\begin{table}[h]
\centering
\caption{Biochemical analysis of rats administered with LPNI for four weeks}
\begin{tabular}{|l|c|c|c|}
\hline
Parameter & Control ($n = 6$) & Model ($n = 6$) & Prevention ($n = 6$) \\
\hline
Body wt (g) & 376.3 ± 3.70 & 381.4 ± 5.30 & 375.8 ± 6.40 \\
\hline
Calcium (mol/L) & 0.430 ± 0.16 & 0.815 ± 0.11*** & 0.464 ± 0.12** \\
\hline
Uric acid (mol/L) & 8.857 ± 11.02 & 163.9 ± 61.95*** & 84.36 ± 46.87** \\
\hline
Oxalic acid (mg/L) & 51.99 ± 5.62 & 129.3 ± 6.71*** & 100.49 ± 11.77*** \\
\hline
Creatinine (μmol/L) & 1286 ± 561.2 & 4464 ± 1640*** & 1671 ± 853.8*** \\
\hline
BUN (mg/dl) & 356.2 ± 145.3 & 764.3 ± 170.9*** & 430.8 ± 154.9*** \\
\hline
\end{tabular}
\begin{flushright}
*** $p < .001$ versus the control group. \\
** $p < .01$ \\
* $p < .05$
\end{flushright}
\end{table}
using the unweighted UniFrac (Figure 2C). The differences observed among microbial communities by studying the hyperoxaluria rats and control rats proposed the presence of gut microbial dysbiosis in hyperoxaluria rats. However, LPN1 intake deflated EG-induced gap in PCoA and distance in cluster analysis (Figure 2B,C), suggested that LPN1 supplementation is beneficial to resistance to EG-induced gut microbial dysbiosis.

### 3.3 | LPN1 could regulate the composition of gut microbiota in EG-induced hyperoxaluria rats

The phyla Bacteroidetes and Firmicutes were found to be dominant in the gut microbiota which were accounted for over 90% of the total microbial populations in all groups (Figure 3A). Despite, there was no difference in abundance of Firmicutes and Bacteroidetes among the three groups (data not shown), we found that the ration of Firmicutes to Bacteroidetes in fecal microbiota of hyperoxaluria rats was significantly higher than control rats (Figure 3B), suggesting that EG could induce an intestinal inflammation-associated alterations in fecal microbiota. Furthermore, the abundance of Proteobacteria in hyperoxaluria rats was also lower than control rats, but LPN1 supplementation could increase the abundance of Proteobacteria. Besides, we also observed that there was no colonization of Verrucomicrobia in fecal microbiota of hyperoxaluria rats, while LPN1 supplementation could promote the enrichment of Verrucomicrobia and Tenericutes in gut microbiota of hyperoxaluria rats.
which was found to be consistent with the alpha diversity among the three groups. Therefore, the decrease in alpha diversity of hyperoxaluria rats was related to the decrease of Proteobacteria, Verrucomicrobia, and Tenericutes, while LPN1 supplementation could increase the alpha diversity by increasing the abundance of Proteobacteria, Verrucomicrobia, and Tenericutes. Moreover, in accordance with the beta diversity, clustering analysis of the top 50 genera highlighted the distinctions of distributions among the three groups (Figure 3C).

Linear discriminant analysis (LDA) effect size (LEfSe) was carried out to further compare the composition of fecal microbiota among three groups (Figure 4). The main microbiota and structure of every single group were also displayed by a cladogram, and the maximum differences in taxa at family and genus level were found to be identified by linear discriminant analysis (LDA) scoring ($p < .05$, LDA score of at least 3). Although there was no difference in the abundance of Bacteroidetes and Firmicutes among the three groups, some fecal bacteria sharply varied from the family classification level. Compared with control rats (Figure 4A), EG-induced hyperoxaluria rats were enriched with genus Lactobacillus and Bifidobacterium, but absent from genus Eubacterium_nodatum_group in Family_XIII, and genus Lachnospiraceae_UCG_006, Roseburia, Tyzzerella and Tyzzerella_3 in family Lachnospiraceae, as well as genus Ruminoclostridium_5, Ruminoclostridium_9, Ruminococcaceae_UCG_005, Ruminococcaceae_UCG_002, Ruminococcaceae_UCG_013, Ruminococcaceae_UCG_007, and Ruminococcus_1 in family Ruminococcaceae. Compared with the baseline composition of gut microbiota in model group (Figure 4B), LPN1 supplementation had decreased the proportion of six genera, including Lactobacillus and Coriobacteriaceae_UCG_002, but increased that of the genus Eubacterium_nodatum_group and Family_XIII_AD3011_group in Family_XIII, the genus Eubacterium_ventriosum_group and Lachnospiraceae_UCG_006 in family Lachnospiraceae, the genus Papillibacter, Ruminococcaceae_UCG_002, Ruminococcaceae_UCG_005, Ruminococcaceae_UCG_009, Ruminococcaceae_UCG_014 and Ruminococcus_2 in family Ruminococcaceae. Furthermore, LPN1 supplementation also enriched the proportion of genus Akkermansia, Catabacter, Globicatella, Deflavulitaleaceae_UCG_011, and...
**FIGURE 3** *Lactiplantibacillus plantarum* N-1 altered the gut microbiota composition in EG-induced hyperoxaluria rats. (A) Percent of relative abundance on phylum level. (B) Comparison of the ratio of Firmicutes to Bacteroidetes and the relative abundance of Proteobacteria, Verrucomicrobia, and Tenericutes phyla within groups. Statistical tests were performed using post hoc ANOVA. (C) Heatmap analysis of gut microbiota on the top 50 genus based on the Bray–Curtis distance analysis.
FIGURE 4  Difference of abundances in taxa among three groups. (A) Linear discriminant analysis (LDA) Effect Size (LefSe) analysis between control and model groups. (B) Linear discriminant analysis (LDA) Effect Size (LefSe) analysis between model and prevention groups. Biomarker taxa are highlighted by colored circles and shaded areas in a cladogram. Each circle's diameter reflects the abundance of that taxa in the community. The cutoff value of $\geq 3.0$ used for the linear discriminant analysis (LDA) is shown
Candidatus Stoquefichus, and the genus Jeotgalicoccus and Staphylococcus in family Staphylococcaceae. Therefore, we speculate that the addition of LPN1 could effectively improve the intestinal flora diversity of rats in the prevention group.

3.4 | LPN1 increased SCFAs

SCFAs perform a key role in balancing the inflammation and intestinal barrier. In this study, we have observed significantly lower level of butyric acid in EG-induced hyperoxaluria rats, and the level of butyric acid was significantly increased by LPN-1, while no difference was observed in the levels of acetic acid and propionic acid between hyperoxaluria rats and control rats (Figure 5).

3.5 | LPN1 attenuated intestinal barrier damage and inhibited local intestinal inflammation of EG-induced hyperoxaluria rats

Given that gut microbiota dysbiosis in hyperoxaluria rats may affect the permeability of the gut, consequently leads to bacterial LPS, going into the circulation and induce systemic low-grade inflammation. Here, we first performed a colonic histopathology analysis to observe the effect of EG and LPN1 on intestinal morphology. Necrosis of intestinal gland and local lymphocytic infiltration in laminae propria were observed in EG-induced hyperoxaluria rats, while the supplementation of LPN1 could reduce the necrosis of glandulae intestinales and lymphocytic infiltration of laminae propria (Figure 6A). Furthermore, we found that LPS level in the plasma was significantly increased in EG-induced hyperoxaluria rats (Figure 6B), and the expression of tight junction protein Claudin-2 was also decreased in hyperoxaluria rats (Figure 6E,F), while LPN1 supplementation significantly decreased LPS level and increased Claudin-2 expression. These data suggested that EG could induce intestinal barrier damage during the induction of hyperoxaluria rats, while LPN1 could protect hyperoxaluria rats from EG-induced intestinal barrier damage.

LPS-mediated inflammation is linked to the stimulation of TLR4/NF-κB signaling pathway. Consistent with increased LPS, the expression of TLR4, NF-κB p50, and pro-inflammatory IL-1β in colon was significantly increased in EG-induced hyperoxaluria rats, but there was no significantly difference in the expression of NF-κB p65 and IL-6. While, LPN1 supplementation overcome the expression of TLR4, NF-κB p50, and IL-1β in clone (Figure 6C,D). These data indicated that LPN1 could inhibit local intestinal inflammation of EG-induced hyperoxaluria rats through regulating TLR4/NF-κB signaling pathway.

4 | DISCUSSION

Previous researches have shown that defective permeability barrier may be an incentive of hyperuricemia in patients with inflammatory bowel disease. However, this association between intestinal permeability and hyperoxaluria has not been proven, and studies about it are scarce. In the current study, we assessed the effect of EG intake on gut microbiota and intestinal permeability of rats, and the protective mechanism of probiotic LPN1 supplementation on EG-induced hyperuricemia. Plainly, we found that the intake of EG disrupted the homeostasis of gut microbiota and induced damage to the intestinal barrier, whereas probiotic LPN1 supplementation before EG intake could resist gut microbiota dysbiosis and intestinal barrier damage in EG-induced hyperuricemia rats.

Gut microorganisms have developed an obligate or facultative relationship to perform a function in their host metabolism by the production of enzymes, while it can also result in the development of disorders when this relationship is disturbed. Some bacteria in the mammalian GIT have been identified as the specific oxalate-degrading bacteria, such as Lactobacillus spp., Bifidobacterium spp. and O. formigenes. Previous studies suggested that the reduction of the oxalate-degrading bacteria from the microbiota of the colon is supposed to increase absorption and reabsorption of large intestine, therefore facilitating the precipitation of calcium salt in the urinary microenvironment. However, no deficiency of those oxalate-degrading bacteria was observed in EG-induced hyperoxaluria rats. In contrast, we found that EG-induced hyperoxaluria rats were significantly enriched with genus Lactobacillus
**FIGURE 6**  *Lactiplantibacillus plantarum* N-1 attenuated intestinal barrier damage and inhibited local intestinal inflammation of EG-induced hyperoxaluria rats. (A) Hematoxylin and eosin (H&E) of the colon samples. Necrosis of intestinal gland is indicated by the red arrow. Proliferation of lymphocytes in laminae propria is indicated by the green arrow. (B) The plasma LPS concentration was measured with the ELISA kit. (C) The mRNA relative expressions of TLR4, NF-κB p50, NF-κB p65, IL-1β, and IL-6 in colon were assessed by qRT-PCR. (D) The protein expressions of TLR4, NF-κB p50, and IL-1β in colon assessed by western blot. (E) The mRNA relative expressions of ZO-1 and Claudin-2 in colon were assessed by qRT-PCR. (F) The protein expressions of Claudin-2 in colon assessed by western blot. Error bars indicate SD. *p < .05, **p < .01, ***p < .001 versus the control group. *p < .05, **p < .01, ***p < .001 versus the model group.
and *Bifidobacterium*, but absent from multitudinous genera in family *Lachnospiraceae* and *Ruminococcaceae* strains, especially the genus *Lachnospiraceae_UCG_006*, *Ruminiclostridium* and *Ruminococcaceae_UCG*. Although probiotic LPN1 supplementation resisted EG-induced microbiota disorders, it did not increase the abundance of oxalate-degrading bacteria. Both genus *lactobacillus* and *Bifidobacterium* belong to beneficial bacteria, but the characteristics of different *lactobacillus* are different, including LPN1 and other species. It is possible that LPN1 grew fast to produces enough acid that might have inhibited the growth of other acid intolerant bacteria. Moreover, high-throughput sequencing is only a qualitative technique, and cannot be accurately quantified.

Actually, all the strains or species in a particular genera mentioned above could not reflect the oxalate-degrading activity, because the sequences were assigned to the closest genus but not to the species. Alternatively, the existence of genes encoding the enzymes for oxalate metabolism in these bacterial genome does not necessarily mean that the oxalate-metabolizing enzymes are functional or expressed in the intestinal tract. For example, neither *Lactobacillus* nor *Bifidobacterium* strains were able to utilize oxalate as a main source of carbon, and the oxalate could only be degraded when the growth medium consisting of oxalate along with glucose or lactose. Furthermore, some bacteria express enzymes for oxalate metabolism only under specific environmental conditions such as acidic pH. Therefore, increasing the oxalate-degradation activity of gut microbiota was not suitable for explaining the prevention effect of probiotic on CaOx kidney stone, and the relationship between gut microbiota and bio-degradation of oxalate by microbes in the GIT needs to be further study in EG-induced hyperoxaluria rats.

Ethylene glycol (EG) feeding resulted in a condition of hyperoxaluria as well as high renal excretion of oxalate and calcium. The oxalic acid and its crystals were toxic to renal cells, which could promote the growth of crystals through inducing the expression of macromolecular substances, such as osteopontin (OPN) and CD44. In this study, we found that LPN1 supplementation reduced the level of urinary calcium, uric acid, oxalic acid, and creatinine, as well as BUN level in serum. Moreover, OPN and CD44 expressions were both inhibited by LPN1, which suggested that LPN provided a protection against EG-induced kidney damage and inhibition of kidney stone formation. Therefore, reducing urinary oxalic acid concentration and crystal formation were the important strategies for LPN1 to prevent kidney stones. Except for kidney, intestinal tract is also an important pathway for oxalate excretion and absorption, which depending on the paracellular transport of tight junction in gastrointestinal epithelial cells, which is important for maintaining the dynamic balance of oxalate in vivo. Tight junctions in the intestinal epithelial cells are the most important skeletal structure composed of the epithelial barrier function, and has the function of regulating the transmembrane transport of water, ions and macromolecules via paracellular pathways. Despite there was no difference in ZO-1 expression of colon, we found that EG intake resulted in a decrease of Claudin-2 in colon of rats. Claudin-2 protein, as the constituent part of tight junction strands, is directly involved in the barrier formation of epithelia cells, but with different ion and size selectivity and tightness depending on the cell type and physiological requirements. Increased intestinal permeability can cause hyperabsorption of nutrients, which can lead to the passive diffusion of oxalate. Also, the frequent intake of oxalate through diet and incremental intestinal absorption, are considered the major causes of urine oxalate, which are risk factors for the development of kidney stone. Furthermore, Claudin-2 proteins are critical for the reabsorption of calcium in enterocytes through paracellular pathways. Urinary calcium is predominantly a dietary source, although bone calcium may be an important contributor to a low-calcium diet in individuals. Therefore, we speculated that defective barrier function disturbed the reabsorption and excretion of calcium and oxalate in intestinal tract, thereby increasing renal excretion of oxalate and calcium, which would aggravate CaOx stone risk solely from this observation.

The normal physiological function of intestine relies on the maintenance and establishment of the mucosal epithelial barrier, thereby preventing the resident bacterial invasion on host tissues. Some recent studies have been discussed that gut commensal microbiota participate in the maintenance of barrier function by inducing translocation of the proteins in tight junction and up-regulating the genes associated with desmosome maintenance to improve intestinal epithelial integrity. Enteral colonization with the normal commensal microbiota is crucial for the normal function of the intestinal barrier, but the deletion of gut commensal bacteria leads to a severe intestinal mucosal injury. Although the ration of *Firmicutes* to *Bacteroidetes* in fecal microbiota of hyperoxaluria rats was significantly higher than control rats, which suggested an inflammation-associated alterations in fecal microbiota, we did not observed the enrichment of pathogenic bacteria. In LeFSe analysis, we noted a reduction of certain commensal microbes in EG-induced hyperoxaluria rats, particularly SCFAs producers. Those included multitudinous genera especially in family *Lachnospiraceae* and *Ruminococcaceae*, such as *Lachnospiraceae_UCG_006* and *Roseburia* in family *Lachnospiraceae*, as well as genus *Ruminiclostridium_5*,...
Staphylococcus in EG-induced hyperoxaluria rats, which intestinal barrier damage by increasing intestinal colo-
munity disorders and kines
tibilities of intestinal barrier.47 When the intestinal integrity was disturbed, LPS, bacterial translocation, and production of other metabolites by the pathogenic gut microbiota went through intestinal epithelial to activate TLR4/NF-κB signaling, eventually induced pro-inflammatory cyto-
kines expressions.48 The increased the plasma LPS levels, the activation of LPS-mediated TLR4/NF-κB inflammatory signaling, and the increased pro-inflammatory IL-1β expression in colon both confirmed the defective permeability barrier and local intestinal inflammation in EG-induced hyperoxaluria rats. While supplementation of probiotic LPN1 significantly inhibited and LPS level and LPS-mediated TLR4/NF-κB inflammatory signaling, and increased Claudin-2 expression. Protecting intestinal barrier might be a mechanism of probiotic LPN1 prevent oxalate excretion disorder caused by defective barrier function.

Probiotic supplementation appears to play a potential role in the prevention of gut microbiota disorders and intestinal barrier damage by increasing intestinal colon-
ization resistance of gut microbiota.49 In our study, EG intake caused a disorder of gut microbiota with abnormal structure and composition and decreased alpha diversity in the fecal microbiota of rats, while probiotic LPN1 sup-
plementation before EG intake attenuated the effect of EG on gut microbiota of rats. Supplementation of LPN1 increased the alpha diversity of fecal microbiota and the abundance of genus Eubacterium ventriosum_group and Lachnospiraceae_UCG_006 in family Lachnospiraceae, and the abundance of genus Papillibacter, Ruminococcus_2, Ruminococcus_UCG_002, Ruminococcus_UCG_005, and Ruminococcus_UCG_014 in family Ruminococcaceae.44 The depletion of Lachnospiraceae and Ruminococcaceae has often been reported in inflammatory bowel disease such as Crohn’s disease,45 and has been shown an anti-inflammatory properties and inversely correlated with intestinal permeability.46 Therefore, the depletion of those gut commensal bacteria might contribute to the intestinal barrier injury in EG-induced hyperoxaluria rats. In fact, we noticed a reduction of Jeotgalicoccus and Staphylococcus in EG-induced hyperoxaluria rats, which was contrary to the alternation of gut microbiota in EG-induced hyperoxaluria mice model (data were not shown). The results indicated that the impacts of these bacteria on the pathogenesis of kidney stones were different in rats and mice animal models. Moreover, high-throughput sequencing is only a qualitative technique, and cannot be accurately quantified, therefore, further investigations need to the extent of gut microbiota’s role in hyperoxaluria and kidney stones.

Furthermore, we examined LPS levels, which is considered as the plasma biomarker for increased permeability of intestinal barrier.47 When the intestinal integrity was disturbed, LPS, bacterial translocation, and production of other metabolites by the pathogenic gut microbiota went through intestinal epithelial to activate TLR4/NF-κB signaling, eventually induced pro-inflammatory cyto-
kines expressions.48 The increased the plasma LPS levels, the activation of LPS-mediated TLR4/NF-κB inflammatory signaling, and the increased pro-inflammatory IL-1β expression in colon both confirmed the defective permeability barrier and local intestinal inflammation in EG-induced hyperoxaluria rats. While supplementation of probiotic LPN1 significantly inhibited and LPS level and LPS-mediated TLR4/NF-κB inflammatory signaling, and increased Claudin-2 expression. Protecting intestinal barrier might be a mechanism of probiotic LPN1 prevent oxalate excretion disorder caused by defective barrier function.

In addition, probiotic LPN1 supplementation also promoted the production of butyric acid during regu-
ating the gut microbiota, thus protecting the intestinal barrier. Being an important end product of bacterial fermentation, SCFAs have been proposed to guard the intestinal mucosa by providing energy for colon cells and alleviate inflammation.51 Here in this study, significantly lower levels of butyric acid were observed in EG-induced hyperoxaluria rats, but there was no difference observed in the level of propionic acid and valeric acid by comparing hyperoxaluria rats and control rats. The decrease of butyric acid was consistent with the depletion of Lachnospiraceae and Ruminococcaceae, for those two species both producing butyrate.44 Propionate and butyrate are non-competitive type of inhibitors of histone deacetylases (HDACs) and particularly inhibit the activity of HDAC1 and HDAC3 enzymes, which in turn can inhibit NF-κB active and pro-inflammatory cytokine expression.52 Moreover, it is reported that butyric acid is mainly used by epithelial cells to promote the function of intestinal barrier by facilitating the assembly of tight junctions by activating AMPK.53 In other words, the gut microbiota disorder and the decrease of butyric acid in hyperoxaluria rats might be the incentive of intestinal barrier injury after EG intake. These results suggested that the improvement of intestinal permeability and local inflammation in colon may be due to the increased abundances of butyric acid and SCFAs producing bacteria after supplementation with probiotic LPN1.

Collectively, the interactions of gut microbiota disorder, intestinal barrier damage and local inflammation promoted the intestinal oxalate excretion and absorption disorder in EG-induced hyperoxaluria rats, which might increase the risk of the formation of kidney stone. Probiotic LPN1 could prevent EG-induced hyperoxaluria though resisting the interference of EG intake on gut microbiota and repairing the barrier function defect and local inflammation in colon.
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DISCLOSURES
No competing interest to declare.

AUTHOR CONTRIBUTIONS
Zhitao Wei, Yaqian Cui, Kunjie Wang, and Qun Sun conceived and designed the experiments. Zhitao Wei, Yaqian Cui, Lei Tian, Yu Liu, Yang Yu, and Xi Jin performed the experiments. Zhitao Wei, Yaqian Cui, and Kunjie Wang analyzed the data. Qun Sun, Yang Yu, and Hong Li contributed to the reagents and materials. Zhitao Wei, Yaqian Cui, and Qun Sun wrote the manuscript.

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