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Effects of *Opuntia ficus-indica* fruit juice-fermented by *Lactiplantibacillus plantarum* HNU082 on kidney injury, gut microbiota and metabolites in hyperuricemia mice based on multi-omics techniques

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ABSTRACT: Hyperuricemia (HUA), as a disease of purine metabolic disorder, seriously affects human health, mainly manifested in kidney injury, gut microbiota dysbiosis, and metabolic disorders. In this study, C57BL/6J mice were used to establish a HUA model by a combination of potassium oxonate and adenine to investigate the effect of fermented *Opuntia ficus-indica* juice-based *Lactiplantibacillus plantarum* HNU082 (OFIBP) on HUA. The results showed that OFIBP alleviated kidney inflammation and gut damage in HUA mice and regulate uric acid (UA) homeostasis in HUA mice through modulating UA production and excretion, which was evidenced by enhancing the expression of UA excretory protein and gene and decreasing the activity of key enzymes of UA synthesis. In addition, OFIBP supplementation restored HUA-induced gut microbiota disturbance, as well as increased metabolites of short-chain fatty acids (SCFAs) levels. Importantly, OFIBP supplementation modulated intestinal metabolic dysbiosis and mediated amino acid metabolism to ameliorate HUA in mice. Therefore, probiotic fermented juice could play a positive role in improving HUA as a novel and efficient functional food.

Keywords: Hyperuricemia, *Lactiplantibacillus plantarum* HNU082, Kidney injury, Gut microbiota, Amino acid metabolism

1. Introduction

Hyperuricemia (HUA) is a highly prevalent metabolic disorder, which results in chronic inflammation via monosodium urate crystal deposition in tissues and may cause chronic kidney disease and gout ^[1]. In addition, HUA has a close relation with multiple comorbidities, such as obesity, hypertension, diabetes, and atherosclerosis ^[2-3]. High purine foods including seafood, animal viscera, and beer, make a very positive contribution to increase HUA. The uric acid (UA) is eliminated by the kidney (two-thirds) and intestinal tract (one-third) to maintain purine metabolism homeostasis ^[4]. Therefore, gut microbiota dysbiosis is involved in the etiopathogenesis of HUA.

The profound impact of the gut microbiota on the onset and progression of many diseases is of great attention. Studies have reported gut microbiota dysbiosis is inextricably related to the occurrence and

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development of metabolic, neurological, and immune diseases [5-6]. The mechanism by which gut microbiota may contribute to HUA could involve the alteration of gut microbiota structure and gut microbiota-derived metabolites. The imbalance of gut microbiota leads to the obstruction of UA metabolism in the intestine, leading to an increase in blood UA levels. On the other hand, the imbalance of gut microbiota aggravates the inflammation response caused by monosodium urate crystals [7]. The inflammation caused by increased blood UA levels and accumulation of urate salts will further affect the structure and composition of gut microbiota. Evidence from studies suggest *Lactobacillus* and *Pseudomonas* participate in the synthesis of UA-degrading enzyme. Furthermore, the gut microbiota metabolites, such as trimethylamine-N-oxide, short-chain fatty acids (SCFAs), and amino acids were altered in HUA mice [8-10].

Since Tissier discovered the first probiotic strain in 1899, scientists around the world have not stopped exploring the relationship between probiotics and human health [11]. In recent years, scientific research on probiotics has continued to deepen, and numerous results have been achieved in identifying probiotic species, functional mechanisms, and probiotic properties, with *Bifidobacterium* and *Lactobacillus* studies in particular. The expansion of livestock farming has led to a high production of greenhouse gases. Against this background, more and more people are focusing on veganism. As a result, plant-based products have become a new trend. Due to the nutritional properties of plant-based products, probiotics show great adaptability and they have been used as an ideal substrate for probiotics in the development of plant-based beverages [12]. The multifunctional metabolic activity of probiotics is capable of altering the chemical composition of the plant substrate and converting it into metabolites with higher bioactivity, increasing the bioavailability of phytochemicals and adding to the functional advantages of fermented foods [13]. The metabolites of the fermentation process of probiotics also have a variety of health functions, including antioxidant, anti-inflammatory, and antibacterial effects, such as indole-3-lactic acid and threonyl-tRNA synthetase were responsible for host health [14-15]. Therefore, fermentation using preferred probiotics and plant substrates can result in functional foods that are rich in nutrients and flavor.

Opuntia ficus-indica fruit is a plant rich in bioactivities and its fruit contains a wide range of healthful compounds such as antioxidants, including vitamin C, polyphenols, flavonoids and anthocyanins [16]. These components may help reduce oxidative stress, indirectly affecting UA levels. In a randomized, double-blind study, Nopal fruit (*Opuntia* genus) juice was shown to reduce inflammatory response and maintain UA in a healthy range [17]. *Lactiplantibacillus plantarum* HNU082 (Lp HNU082) was isolated from the traditional fermented food of Hainan Province in China. *In vivo* experiments, Lp HNU082 has been confirmed to exhibit anti-inflammatory, antibacterial, and maintain the ecological and genetic stability of gut microbiota [18-19]. Notwithstanding, whether the anti-trioxypurine effect of *Opuntia ficus-indica* juice (OFIJ)-based fermented by *Lactiplantibacillus plantarum* HNU082 (OFIBP) has not been investigated. In our previous studies, we have demonstrated that HNU082-fermented OFI not only has great antioxidant properties and flavor characteristics, but also has a well inhibiting effect on xanthine oxidase. Also, the metabolic profile of OFI was altered by HNU082 fermentation. Some volatile constituents (neryl butyrate and carvacrol) and

non-volatile constituents (pyrazofurin) levels were significantly up-regulated in the HNU082-fermented OFIJ, and these constituents have been reported to have favorable biological activities [20]. Here, in this study, the animal model of HUA induced by potassium oxonate and adenine was established, and the impact of OFIBP supplementation on HUA kidney injury, gut microbiota composition, and related metabolites were evaluated. The findings will provide a theoretical basis and data support for the development of plant-based fermentation products to improve HUA.

2. Materials and methods

2.1 Materials

Opuntia ficus-indica fruit are grown in Sanya, Hainan. *Opuntia ficus-indica* fruit juice was obtained from Hainan Xianyou Food Co., Ltd. (Haikou, China). Lp HNU082 was isolated from Hainan traditional fermented food and preserved in the Guangdong Microbial Culture Collection Center (GDMCC, Guangdong, China) with the preservation numbers of GDMCC 61552. To minimize the number of animals and reduce animal pain, a sample size of 8 animals per group was chosen for the test. Laboratory animal standard diet was obtained from Jiangsu Xietong Pharmaceutical Bioengineering Co., Ltd. (Jiangsu, China). Potassium oxonate, adenine, carboxymethylcellulose sodium (CMC-Na), and allopurinol were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China).

2.2 Preparation of *Opuntia ficus-indica* juice-based *Lactiplantibacillus plantarum* HNU082 (OFIBP)

Lp HNU082 was restored to the exponential phase after activation, and 4% (v/v) of the bacterial suspension was inoculated into the pasteurized *Opuntia ficus-indica* fruit juice in a sterile environment, and cultured for 48 h in a 37°C incubator shaker. The total number of colonies at the end of fermentation was (8.71 ± 0.14) log CFU/mL. Also, the pH and soluble solids content were (3.37 ± 0.03) (8.2 ± 0.10) °Brix, respectively. The prepared fermented juice was subjected to instantaneous high-temperature sterilization to obtain OFIBP.

2.3 Animal experiment design

The experiments were performed in accordance with the provisions of the Experimental Animal Research Center of Hainan University. All experimental protocols were approved by the Ethics Committee of Hainan University (Approval No: HNUAUCC-2023-00176). C57BL/6 mice (6-8 weeks, male) were purchased from Hunan SJA Laboratory Animal Co., Ltd. (Hunan, China). All animals were kept under specific pathogen-free conditions. The mice were fed with laboratory standard diet, free access to water, and artificial light for 12 h per day (12 h of darkness and 12 h of light alternation). The environmental conditions were controlled at $(24 \pm 1)^{\circ}\text{C}$, $55\% \pm 5\%$ (relative humidity). After one week of adaptive feeding, 40 mice were randomly divided into five groups ($n=8/\text{group}$): control (CON), model (MOD), OFI, OFIBP, and allopurinol (ALP) group. All mice were given the laboratory standard maintenance diet (the energy supply ratio of protein: fat: carbohydrate in the laboratory standard maintenance diet was 21.50%: 11.10%: 67.40%). Dietary composition was controlled consistently across groups except for the oral intervention drug and water.

CON group: 0.2 mL/20 g 0.5% CMC-Na; after 1 h, 0.2 mL/20 g double-distilled water. MOD group: potassium oxonate (300 mg/kg·BW)+BW adenine (100 mg/kg); after 1 h, 0.2 mL/20 g double-distilled water. OFIBP group: potassium oxonate+adenine; after 1 h, 0.2 mL/20 g OFIBP. OFI group: potassium oxonate+adenine; after 1 h, 0.2 mL/20 g OFI. ALP group: potassium oxonate+adenine; after 1 h, allopurinol (10 mg/kg·BW). The mice in each group were intervened continuously for 4 weeks. After 12 h of fasting, the mice were euthanized, the serum, cecal contents, feces, liver, colon, and kidney tissues were collected, while the weight of the tissue samples were recorded to estimate the organ indexes: organ index=tissue weight (g)/body weight (g) × 100%.

2.4 Measurement of biochemical indexes

The serum uric acid (SUA, C012-2-1), serum creatinine (SCr, C011-2-1), serum urea nitrogen (SUN, C013-2-1), kidney interleukin-6 (IL-6, H007-1), tumor necrosis factor-alpha (TNF- α , H052-1), and lipopolysaccharide (LPS, A054-2-1) concentrations, serum adenosine deaminase (ADA, A048-2-1) and liver xanthine oxidase (XOD, A002-1-1) activities were measured using commercial kits and obtained from Nanjing Jian Cheng Bioengineering Institute (Nanjing, China).

2.5 Histopathological analysis

The colon and kidney tissues were collected and fixed with 4% paraformaldehyde solution [21]. Subsequently, gradient ethanol was used for dehydration, and the wax-impregnated tissue was embedded and made paraffin sections at 4 μ m thickness, which were stained with hematoxylin and eosin (H&E) or Masson's trichrome. The morphology of tissue sections was observed under a Nikon Eclipse E100 microscope (NIKON Co., Japan). The Masson's trichrome-stained renal sections were semi-quantitatively scored using Image J software to estimate the degree of tubulointerstitial fibrosis.

2.6 Immunofluorescence analysis of colon tissue

The colon tissues were subjected to paraffin section and dewaxing. After antigen repair and washing with PBS (pH 7.4), primary antibodies (claudin-1, ZO-1, and occludin) were added and incubated at 4°C overnight. Then the corresponding horseradish peroxidase-labeled secondary antibodies were added to cover the tissue and incubated at 25°C. Subsequently, the nuclei were re-stained with 4', 6-diamidino-2-phenylindole. Fluorescence microscopy (Nikon Eclipse E100, NIKON Co., Japan) was used to observe images and collect pictures. Image J software was used to measure fluorescence intensity.

2.7 Real-time quantitative PCR (RT-PCR)

Total RNA was extracted from kidney tissue using Trizol reagent (G3013, Servicebi, Wuhan, China), the RNA concentration and purity were detected. The total RNA was reverse-transcribed into cDNA using Servicebio ® RT First Strand cDNA Synthesis Kit (G3330, Servicebi, Wuhan, China). The RT-PCR was performed by the SYBR Green method, and the specificity of PCR products was confirmed by melting curve analysis and sequencing. The expression of mRNA was calculated using the $2^{-\Delta\Delta CT}$ method with β -actin as the internal reference gene. The primer sequences are described in Table S1. The BLAST analysis was performed

on the NCBI website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the sequences were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China)

2.8 Western blot analysis

Kidney tissue was thoroughly ground by adding 10 times the tissue weight of the lysate. The total protein solution of the samples was diluted with buffer (v/v=4:1) and denatured in a metal bath at 40°C for 40 min. Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Subsequently, the membranes were incubated with primary antibodies against ABCG2 and β -actin (dilution ratio 1:1000) at 4°C overnight. Incubation with secondary antibody was followed by visualization with an ECL system and quantified by ImageJ software [22].

2.8 Measurement of SCFAs concentrations

The levels of SCFAs in fecal samples and colon contents were detected by gas chromatography-mass spectrometry [23]. Briefly, the colon contents and feces were freeze-dried, and added with saturated sodium chloride for homogenization. After homogenization, 10% sulfuric acid (v/v) was added for acidification. Subsequently, 800 μ L n-hexane was added to samples to extract the SCFAs. After standing for 30 min, the mixture was centrifuged at 12,000 r/min for 15 min, and anhydrous sodium sulfate was added to remove residual water. SCFAs concentrations were analyzed by Agilent 8890 gas chromatography system (Agilent, Santa Clara, CA, USA) and flame ionization detector (Agilent, Santa Clara, CA, USA) equipped with HP-INNOWAX column (30 m $\times$ 0.320 mm $\times$ 0.50 μ m, Agilent, Santa Clara, CA, USA).

2.9 16S rRNA sequencing analysis

The DNA of calculate in cecal contents were extracted using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). A NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1.0% agarose gel electrophoresis were used to determine DNA quality and concentration. The bacterial 16S rRNA gene's hypervariable region V3-V4 was amplified using primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTA AT-3') by T100 Thermal Cycler PCR thermocycler. Pooled equimolar amounts of purified amplicons were paired-end sequenced on an Illumina platform (Illumina, San Diego, CA, USA) following standard protocols. The V3-V4 primer set was chosen due to its broad taxonomic coverage (94.5% of bacterial taxa) and optimal amplicon length for sequencing. The α -diversity (Kruskal-wallis rank sum test) and β -diversity (bray-curtis distance algorithm) were used to analyze the inter-group differences and similarity of microbial community structure, respectively. The differential genera at the genus level and biochemical parameters were screened by Linear discriminant analysis (LDA) Effect Size (LEfSe, LDA score > 4, P < 0.05) and Variance Inflation Factor (VIF<10), respectively.

2.10 Non-targeted metabolomics analysis

The cecal contents were homogenized and centrifuged to collect the supernatant. The analysis was done using an ultra-high performance liquid chromatography (UHPLC, detection of lipid and water-soluble metabolites) system (Thermo Fisher Scientific, Waltham, MA, USA) that was connected to an Orbitrap Q Exactive TM HF-X mass spectrometer. The metabolite was predicted by molecular ion peaks and fragment ions. And then the metabolite was matched with databases (including mzVault, and MassList) to receive precise qualitative result. The metabolites were annotated by comparison with the Kyoto Encyclopedia of Genes and Genomes (KEGG) compound database to obtain the KEGG compound ID numbers. Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal projections to latent structures discriminant analysis (OPLS-DA) analysis were performed. Differential metabolites were screened by 7-fold cross validation and variable influence on projection (VIP)>1 using OPLS-DA as a supervised model. Based on the KEGG database, significant differential metabolites were performed to metabolic pathway analysis. False discovery rate (FDR)-adjusted P -values < 0.05 showed statistical significance.

2.11 Targeted metabolomics analysis of amino acids

The 20 mg sample, 141 μ L water and 100 μ L 0.15% ursodeoxycholic acid were thoroughly mixed, and ultrasonicated for 10 min at 5°C under 40 KHz. After adding 5 μ L 10 M trichloroacetic acid, the sample was frozen and settled for 10 min, then centrifuged for 10 min at 14000 r/min. The 25 μ L supernatant was adequately blended with 375 μ L water and then passed through 0.2 μ m polytetrafluoroethylene filter membrane. UHPLC Qtrap MS/MS (Thermo Fisher Scientific, Waltham, MA, USA) was used for the qualitative and quantitative determination of amino acids in samples. The chromatographic column was dvanceBio MS Spent Media (2.1 mm $\times$ 50 mm, 2.7 μ m), the column temperature and injection volume were 40°C and 1 μ L, respectively. The mobile phase A (0.1% formic acid, 10 mmol/L ammonium formate, 95% aqueous solution) and mobile phase B (0.1% formic acid, 10 mmol/L ammonium formate, 95% acetonitrile solution) were used as the mobile phase. The elution gradient was as follows: 0-3 min, 10% A; (3-4) min, 40% A; (4-6) min, 10% A. The flow rate was 5 min/mL.

2.12 Statistical Analysis

Origin 2021 (OriginLab Corporation, Northampton, MA, USA) and SPSS 26.0 statistical software (SPSS Inc., Chicago, IL, USA) were used for drawing figures and data analysis, respectively. Statistical analyses of non-omics data were performed using mean $\pm$ standard (SD) deviation, and differences between groups were analyzed by one-way ANOVA. A statistically significant difference was considered at P < 0.05.

3. Result

3.1 Effects of OFIBP intervention on physiological and serum indexes

During the experiment, body weight and food intake of the experimental mice were recorded (Table S2). Four weeks after intervention with potassium oxonate and adenine, in comparison with CON group, body weight and organ indexes of the MOD group significantly decreased and elevated, respectively (P <

0.05). However, the organ indexes of liver and kidney recovered in OFIBP and ALP groups, indicating that OFIBP and allopurinol intervention reduced liver and kidney damage. The SUA level in CON group (51.88 ± 8.90) $\mu\text{mol/L}$ was significantly less than the MOD group (106.02 ± 22.02) $\mu\text{mol/L}$, revealing that the HUA model was successfully established (Figure 1A, $P < 0.05$). Importantly, OFIBP and OFI intervention significantly reduced the SUA level in HUA mice (Figure 1A-C, $P < 0.05$). The SUA level was almost restored to normal levels upon OFIBP and allopurinol intervention. Moreover, compared with the MOD group, OFIBP (decreased by 41.91 and 43.06%), OFI (decreased by 45.78 and 35.24%), and allopurinol (decreased by 71.14 and 54.61%) intake decreased the SCr and SUN levels, respectively. The findings showed that OFIBP administration could alleviate HUA induced by potassium oxonate and adenine.

3.2 Influences of OFIBP intervention on UA synthesis-related enzymes activities

As displayed in Figure 1C-D, in comparison with the CON group, the serum ADA and liver XOD activities in MOD group were significantly elevated ($P < 0.05$), and the enzymes activities were remarkably decreased after OFIBP intake. Significantly, in comparison with the OFI group, OFIBP group had a more remarkable result in suppressing ADA and XOD activities ($P < 0.05$). Allopurinol, as an inhibitor of XOD, significantly inhibited ADA and XOD activities and restored them to the CON group levels ($P < 0.05$). The findings indicated that OFIBP intake was able to inhibit UA production by suppressing ADA and XOD activities.

3.3 Impacts of OFIBP intervention on UA excretion in kidney

The RT-PCR results showed that the mRNA expression levels of ATP-binding cassette subfamily G member 2 (ABCG2) in the MOD group was significantly down-regulated in comparison with the CON group (Figure 1F, $P < 0.05$). Compared with the MOD group, the ABCG2 mRNA expression levels in the OFIBP, OFI, and ALP groups were increased by 49.80, 40.94, and 102.23%, respectively. Therefore, western blotting was used to evaluate ABCG2 expression in the kidney. As expected, ABCG2 protein levels were down-regulated in the MOD group but increased in the OFIBP and ALP groups (Figure 1G-H). The outcomes suggested that OFIBP intervention could reduce the mRNA expression of UA transporter proteins to maintain UA homeostasis.

3.4 Effects of OFIBP intervention on kidney injury

The improvement effect of OFIBP on kidney injury in HUA mice was verified by histopathological analysis. The glomerular size and morphology of mice in the CON group were normal, the boundary was clear, and the brush border structure inside the kidney tubules was intact (Figure 2A-B). In the contrary, the kidney tissue of mice in MOD group was seriously damaged. Fortunately, the kidney injury in all treatment groups was improved, especially OFIBP intervention. However, OFI intervention did not completely ameliorate glomerular atrophy and proximal tubular brush structure of proximal tubules in kidney. Allopurinol treatment also did not fully recover the infiltration of inflammatory cells in mice. As expected, Masson's trichrome showed that severe kidney fibrosis was observed in the tubulointerstitial compartment of

the MOD mice. The supplementation of OFIBP and allopurinol alleviated the impairments. The quantification of kidney fibrotic area was consistent with Masson's trichrome results (Figure 2C, $P < 0.05$). The inflammatory factors levels in the kidney of HUA mice were depicted in Figure 2D-F. Compared with the CON group, the LPS, IL-6, and TNF- α levels in the MOD group were raised by 21.42, 14.86, and 31.34%, respectively ($P < 0.05$). Furthermore, in contrast with MOD group, the LPS, IL-6, and TNF- α levels in the OFIBP group were diminished by 22.77, 31.32 and 28.86%, respectively ($P < 0.05$). Of interest, the OFIBP group was more effective than the OFI group in relieving kidney injury. The findings showed that OFIBP could alleviate kidney inflammation and injury in HUA mice.

3.5 Influences of OFIBP intervention on SCFAs levels

The Figure 3A indicated that total SCFAs level of colon content in MOD group was obviously lower than the CON group ($P < 0.05$). As expected, the levels of total SCFAs in the OFIBP and ALP groups were significantly raised in comparison with the MOD group ($P < 0.05$). The levels of propionic, isobutyric, n-butyric, isovaleric, and caproic acids in OFIBP treatment were obviously higher than those in MOD group ($P < 0.05$). In addition, OFI intake did not significantly change SCFAs levels ($P > 0.05$). As reflected in Figure 3B, the alteration trend of SCFAs levels in feces were consistent with those in colon contents, but total SCFAs content decreased slightly. The results revealed that OFIBP intervention could stimulate the production of gut metabolites in HUA mice.

3.6 OFIBP intervention restored the gut barrier in HUA mice

It is well known that HUA causes intestinal barrier damage in mice. H&E staining of the colon tissue showed that crypt structural abnormalities, the intestinal cavity became larger, and the colon tissue was accompanied by inflammatory cells infiltration in the MOD group mice. Conversely, OFIBP and allopurinol intervention could significantly improve lesions and reduce inflammatory cells infiltration (Figure 4A). Nevertheless, large numbers of inflammatory cells were still observed in the OFI group. Moreover, immunofluorescence staining revealed the expression of Claudin-1 and ZO-1 in the MOD group were markedly smaller than those in CON group (Figure 4B, D). As anticipated, OFIBP intervention enhanced the Claudin-1 and ZO-1 expression in colon tissue. Unfortunately, allopurinol supplementation reduced the expression of Claudin-1, while OFI intervention decreased the expression level of ZO-1 (Figure 4F-G). Hence, OFIBP could reduce the intestinal structure damage caused by HUA, and maintain the intestinal barrier stability.

3.7 OFIBP intervention regulated the gut microbiota composition

More and more evidences show that gut microbiota disruption is a considerable factor affecting the occurrence and development of HUA. We evaluated the effect of OFIBP intervention on the diversity and composition of gut microbiota in mice by 16S rRNA sequencing. The alpha diversity (ACE and Chao1) of the gut microbiota did not differ significantly between the five groups ($P > 0.05$, Figure 5A-B). However, PCA and PCoA (principal co-ordinates analysis) showed the composition of gut microbiota among different

groups had significant differences (Figure 5C-D). The taxonomic composition of gut microbiota showed that Firmicutes, Verrucomicrobiota, Actinobacteriota, and Bacteroidota as dominant phylum of various groups at the phylum level (Figure 5E). Compared with the CON group, the abundance of Firmicutes and Bacteroidota increased, Verrucomicrobiota and Actinobacteriota abundance decreased in MOD group. In contrast, OFIBP, OFI, and allopurinol intervention upregulated Actinobacteriota abundance and downregulated Bacteroidota abundance. As reflected in Figure 5F-G, the abundance of *Akkermansia*, *Bifidobacterium*, *Lactobacillus*, and *Candidatus Saccharimonas* at the genus level elevated significantly after OFIBP intervention in comparison with the MOD group ($P < 0.05$). The *Turicibacter* abundance exhibited an absolute advantage in the OFIBP group when compared to other treatment groups. Unfortunately, there were no dramatic difference in *Faecalibaculum* and *Desulfovibrio* abundance between the MOD and OFIBP groups. However, compared with the OFIBP group, OFI intervention did not restore *Akkermansia* and increased *Desulfovibrio* abundance ($P < 0.05$). Interestingly, *Faecalibaculum* abundance in the ALP group was markedly higher than that in the other treatment groups ($P < 0.05$), but *Bifidobacterium*, *Lactobacillus*, and *Turicibacter* abundance were significantly lower than the OFIBP group ($P < 0.05$). The results of the LEfSe analysis are expressed as LDA (Figure 5H). Consistent with the above analysis of the results, the OFIBP group demonstrated remarkable enrichment of *Turicibacter*, whereas the ALP group was markedly enriched with *Faecalibaculum*.

To further explore the possible relationship between differential genera and biochemical parameters associated with HUA, Pearson correlations were estimated. The analysis revealed that *Akkermansia* and *Candidatus Saccharimonas* abundance were negatively related to Scr, SUA, and IL-6 levels, and positively correlated with SCFAs levels, especially the correlation between *Akkermansia* and n-butyric, isobutyric acid ($P < 0.05$, Figure 5I). Therefore, the results suggested OFIBP intervention ameliorates HUA-induced gut microbiota dysbiosis.

3.8 Effects of OFIBP intervention on the metabolites of cecal contents

To investigate whether OFIBP intervention influences the metabolic profile of HUA mice, the metabolites in cecal contents were measured. In ESI+ and ESI- ions, PCA and PLS-DA analysis found that there were significant differences of metabolites among five groups (Figure 6A-B). To further determine the overall metabolic variation between the MOD and OFIBP groups, OPLS-DA analysis performed that the two groups were significantly separated, suggesting that OFIBP intervention markedly changed the metabolic profile of HUA mice (Figure 6C). The corresponding S-plots was performed to identify differential metabolites in model and healthy mice, and each dot represents one metabolite (Figure 6D). Based on KEGG annotation and enrichment analysis, the differential metabolites were mainly concentrated in amino acid metabolism (Figure S1). The differential metabolites of amino acid metabolism between OFIBP and MOD groups were exhibited in Figure 6E ($VIP > 1.0$, and $P < 0.05$), and a total of 33 differential metabolites were displayed. Among them, 14 differential metabolites were up-regulated in MOD group, and 19 differential metabolites were up-regulated in OFIBP group. The enriched pathways of

displayed differential metabolites were further analyzed, Figure 6F exhibited the top 10 pathways of the topology analysis based on the impact score. More specifically, differential metabolites of amino acids were mainly involved in tyrosine metabolism, glycine, serine and threonine metabolism, valine, leucine, and isoleucine biosynthesis, tryptophan metabolism, etc.

To further investigate the effect of amino acid metabolism on HUA, 21 amino acids were used for quantitative analysis. As shown by OPLS-DA analysis, there was a clear separation between CON, MOD, and OFIBP groups (Figure 7A). Also, S-plot analysis demonstrated the differential expression of the 21 amino acids (Figure 7B). L-asparagine anhydrous was identified as the metabolite with the most significant difference among the three groups. The total amino acids content was elevated in the MOD group compared to CON group. Contrarily, the total amino acid content was decreased by 18.07% in HUA mice after OFIBP intake (Figure 7C). Among them, 18 amino acids were enriched in MOD mice except L-aspartic acid and were alleviated after OFIBP intervention (Figure 7D). The opinions suggested that OFIBP intervention could regulate disturbed metabolic profile and amino acid metabolic pathways in HUA mice.

4. Discussion

With the rapid economic development and improvement of living standards, HUA morbidity is on the rise globally. Although drugs for HUA are effective in reducing SUA level, the potential adverse effects often limit clinical investigation [24]. Recent reports have also revealed that probiotic fermented fruit and vegetable juice can reduce UA, however, the potential mechanisms in alleviating HUA remain to be enunciated. We successfully established the HUA model using potassium oxonate combined with adenine in this study. The results confirmed OFIBP intervention could alleviate HUA by suppressing XOD and ADA activities to inhibit UA production, and regulating the expression of UA transporters protein to promote UA excretion. Importantly, OFIBP intervention also regulated gut microbiota and amino acid metabolism to mitigate HUA of mice. As expected, OFIBP supplementation was more effective in alleviating HUA than OFI supplementation.

Previous research noted that HUA could increase reaction time and impulsivity of mice, which were contribute to weight loss [25]. The study also found OFIBP and allopurinol intervention attenuated weight loss in HUA mice. The excessive synthesis of UA is the main cause of HUA. XOD, as the key enzyme of purine metabolism, directly catalyzes the gradual oxidation of hypoxanthine and xanthine to form UA, as well as transform protein-rich foods to UA. Whereas, ADA catalyzes the conversion of adenosine to further generate hypoxanthine and xanthine, which indirectly catalyzes the formation of UA. The results confirmed that OFIBP intervention can reduce UA levels by decreasing XOD and ADA activities. Simultaneously, allopurinol, as an inhibitor of UA production, can inhibit XOD and ADA activities [26]. Significantly, the inhibitory effect of OFI intervention on XOD and ADA were weaker than that of OFIBP intervention. Thus, it can be demonstrated that OFIBP supplementation alleviated HUA by inhibiting UA synthesis.

Additionally, increasing UA excretion is also considered as one of the effective ways to treat HUA. The UA metabolism requires the involvement of UA transporter proteins in the kidney, which has emerged as a

prospective target spot for the treatment of HUA [27]. ABCG2 is an UA transporter that is highly expressed in proximal renal tubular epithelial cells and intestinal epithelial cells, and ABCG2 dysfunction reduces extrarenal UA excretion. Although UA levels in urine may provide additional evidence for the efficiency of kidney excretion, ABCG2 has been widely recognized as one of the key determinants of UA secretion in clinical studies [28–29]. Therefore, ABCG2 dysfunction directly affects UA excretion levels in HUA patients. Our results validated that OFIBP intervention could regulate the UA reabsorption and excretion in HUA mice by promoting ABCG2 expression. *Lactobacillus plantarum* LLY-606 treatment recovered mRNA expression levels of ABCG2 in HUA mice which were consistent with our findings [30].

UA is metabolized in the body through the kidney and intestine, and elevated concentration of UA can cause severe kidney damage and even lead to chronic kidney disease. The SCr and SUN are the most critical biochemical indexes in the evaluation of abnormal kidney function. As anticipated, all three treatment groups were effective in reducing SCr and SUN levels. Also, the present study demonstrated that OFIBP supplementation attenuated HUA-induced kidney injury, including morphologic changes and kidney fibrosis. It is generally known that high UA level can promote the expression of inflammatory cytokines and trigger a complex pro-inflammatory response, which in turn exacerbates kidney inflammation and injury [31]. High levels of pro-inflammatory mediators including TNF- α and IL-6 are expressed in patients and animal models and are strongly associated with HUA progression [32–33]. Overexpression of LPS level in kidney activates the LPS-mediated TLR4/NF- κ B pathway and promote the release of pro-inflammatory factors [34]. Fortunately, OFIBP intervention inhibited inflammatory cytokine levels to prevent potassium oxonate and adenine-induced kidney injury.

The consistent feature of HUA is increased intestinal permeability, which may be caused by impaired mucosal mechanical barrier integrity. Our findings indicated that the intestinal histopathological damage of HUA model mice was severe, including incomplete intestinal crypt structure and inflammatory cell infiltration. However, after OFIBP intervention, the colon damage was markedly attenuated. Furthermore, the OFI intervention did not fully recover intestinal tissue damage caused by HUA. Tight junction (TJ) proteins, including Occludin, Claudin-1, and ZO-1, are critical in regulating intestinal barrier function. Occludin provides structural integrity to TJ and is a major component in the barrier function [35]. Claudin can form TJ chains with paracellular barriers and channel functions, thereby regulating the microenvironment of biological systems [36]. ZO-1, a PDZ structural domain-containing protein, is crucial component for preserving the mechanical integrity and permeability of mucosal epithelium [37]. The Immunofluorescence staining results revealed that OFIBP intervention ameliorated the HUA-induced abnormalities of Claudin-1 and ZO-1 expression. These findings suggested that OFIBP supplementation contributed to the maintenance of intestinal integrity in HUA mice.

Gut microbiota is well-connected with the development of HUA may serve as a new target for early diagnosis and prevention of HUA [29,38]. HUA-induced kidney function impairment further increases the burden of UA excretion in the intestine. It may also lead to changes in the abundance and composition of the

gut microbiota. It has been reported that patients with HUA have decreased diversity and changed gut microbiota composition compared with healthy people [39]. Similarly, the study had shown that gut microbiota from HUA rats was transplanted to recipient rats, and resulted in an increase in UA levels of recipient rats [40]. The 16S rRNA sequencing analysis results observed that OFIBP intervention did not alter α -diversity in HUA mice, but changed β -diversity of gut microbiota. The α -diversity was not markedly impacted by the probiotics treatment, which is largely coincident with the previous literatures [41]. At the phylum level, both Firmicutes and Bacteroidota were increased in HUA mice compared to healthy mice, and OFIB intervention mitigated the phenomenon, which is similar to previous study, *Limosilactobacillus fermentum* JL-3 intake restored the abundance of Bacteroidota in HUA mice to normal level [42]. At the genus level, OFIBP intervention alleviated HUA-associated symptoms by modulating the gut microbiota of HUA mice, as evidenced mainly by an increased abundance of *Akkermansia*, *Bifidobacterium*, *Lactobacillus*, *Turicibacter*, and *Candidatus Saccharimonas*. *Akkermansia*, as a SCFAs-producing bacteria, exhibits excellent abilities on protecting gut barrier function, regulation of metabolic disorders, cancer immunity, and neurodegenerative diseases [43]. Simultaneously, the study revealed that *Akkermansia* abundance was positively correlated with n-butyric acid and isobutyric acid levels, while negatively correlated with the pro-inflammatory factor IL-6, which suggest that *Akkermansia* may protect the intestinal mucosal barrier by promoting SCFAs. As a kind of intestinal beneficial bacteria, *Lactobacillus* can enhance the intestinal barrier function and promote the production of SCFAs, thus regulate metabolic homeostasis and inflammatory response [44]. *Bifidobacterium* is recognized as a major colonizer of the intestinal epithelium and the producer of SCFAs [45]. Due to the health-promoting properties, *Bifidobacterium* has been incorporated as active ingredients in many functional foods. Moreover, *Turicibacter* and *Candidatus Saccharimonas* are also considered as beneficial bacteria with anti-inflammatory properties [46-47]. A study reported that the beneficial bacterium *Candidatus Saccharimonas* was confirmed to be potentially involved in immunomodulation and intestinal repair [48]. *Faecalibacterium* is well documented to have anti-inflammatory properties and is also thought to partially reflect intestinal health status [49]. *Faecalibacterium* was found to be significantly enriched by allopurinol treatment. Additionally, *Desulfovibrio* is a group of sulfate-reducing H₂S-producing anaerobes, and endogenous H₂S poisons intestinal epithelial cells and causes intestinal sensitization [50]. Several clinical studies have confirmed that increased populations of *Desulfovibrio* are an influential feature of diseases such as bacteremia and ulcerative colitis. Regretfully, OFIBP intervention did not alter the abundance of *Faecalibacterium* and *Desulfovibrio* in HUA mice. It is possible that the metabolites produced by probiotic fermentation promote changes in the gut microbiota in the host's gut. It is widely accepted that SCFAs such as acetic, propionic, and n-butyric acids are the main metabolites of gut microbiota and involved in regulation of immunity, apoptosis, inflammation, and lipid metabolism [51]. The results pointed out that SCFAs were decreased in colon contents and feces of HUA mice, and OFIBP intake up-regulated SCFAs levels, especially propionic, isobutyric, n-butyric, isovaleric, and caproic acids, which was similar to the research in Zhao et al [52]. OFIBP intervention alleviated gut flora disorders in HUA mice, increased the abundance of

SCFAs-producing bacteria in the gut, and in turn increased the content of SCFAs. Meanwhile, SCFAs can act as receptors for G protein-coupled receptor ligands, which can regulate the inflammatory response and enhance the intestinal barrier integrity by improving the function of TJ proteins ^[53]. Thus, OFIBP intervention modulated gut microbiota disruption and mitigated gut barrier damage by increasing SCFA-producing probiotics in HUA mice.

Metabolomics has been used to analyze the important role of HUA in metabolic disorders and can be an effective tool for biomarker screening. The non-target metabolomics of cecal contents showed that OFIBP intervention modulated metabolic disturbances in HUA mice and was mainly enriched in amino acid metabolism. Previous study also found isoleucine, tryptophan, valine, and various other amino acids were enriched in HUA mice ^[54]. Branched-chain amino acids (BCAAs, leucine, isoleucine, and valine), as essential amino acids, are important signaling molecules and substrates for protein synthesis. Previous research presented that BCAAs were increased in patients with HUA and gout, which attributes to decreased mitochondrial branched-chain aminotransferase expression and impairs mitochondrial capacity ^[55]. Excitingly, BCAAs were enriched in HUA mice and OFIBP supplementation reversed the increase in these amino acids. Among them, aromatic amino acid metabolism (tyrosine metabolism and tryptophan metabolism) and glycine, serine, and threonine metabolism also play a role in regulating UA metabolism. As an intermediate metabolite of tyrosine, 3,4-dihydroxyhydrocinnamic acid possesses antioxidant, anti-inflammatory, and cytoprotective abilities *in vitro* ^[56]. OFIBP intervention upregulated 3,4-dihydroxyhydrocinnamic acid level, and which may attenuate HUA via its antioxidant and anti-inflammatory activities. An interesting study reported that combined glycine and tryptophan supplementation can inhibit SUA level by increasing UA excretion ^[57]. Creatine, a metabolite of glycine, is an important antioxidant in the organism and OFIBP intervention upregulated creatine abundance ^[58]. Tryptophan metabolism is considered to be an important metabolic pathway to regulate the production and development of HUA. Interestingly, indole-3-acetaldehyde is the tryptophan metabolite of gut microbiota, and was significantly upregulated in the OFIBP group. The indole-3-acetaldehyde helps in maintaining intestinal homeostasis and influences the development and progression of obesity, tumors, metabolic syndrome, and inflammatory bowel disease ^[59]. Fisetin has also been reported to slow down the development of HUA through gut microbiota-mediated tryptophan metabolism ^[9]. Notably, 2-isopropylmalic acid was detected in HUA mice, and down-regulated after OFIBP intervention, which was consistent with Chen et al, who found 2-isopropylmalic acid level was modulated by kidney tea supplementation in HUA mice ^[60]. In summary, OFIBP supplementation may mitigate HUA by modulating amino acid metabolism.

5. Conclusion

HUA is not only directly related to gout, but also causes damage to multiple organs of the body. In conclusion, the study illustrated that probiotic fermented OFI can reduce UA level by inhibiting UA synthesis and enhancing urate-transport activity of ABCG2 protein in HUA mice. Also, OFIBP supplementation alleviated HUA by alleviating kidney inflammation and intestinal barrier damage, and regulating intestinal

microbiota and amino acid metabolic pathways. These results provided valuable information for the future application of plant-based fermentation products in HUA. However, more in-depth molecular mechanisms research for alleviating HUA by plant-based fermentation products still need to explored in future work.

Notes

The authors have no conflicts of interest.

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