Received: 20 April 2024 | Revised: 5 August 2024 | Accepted: 8 August 2024

DOI: 10.1002/efd2.70002

RESEARCH ARTICLE

eFood WILEY

A novel arabinogalactan extracted from *Epiphyllum oxypetalum (DC.) Haw* improves the immunity and gut microbiota in cyclophosphamide-induced immunosuppressed mice

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Funding information

Fundamental Research Funds for the Central Universities, Grant/Award Number: 2021CDLZ-18; Key R&D projects of the Science and Technology Department of Sichuan Province, Grant/Award Number: 2021YFN0092; Sichuan Science and Technology Program, Grant/Award Numbers: 2021ZHFP0045, 2022YFQ0100; National Key Research and Development Projects, Grant/Award Number: 2019YFE0103800; Hungarian National Research, Development, and Innovation Fund for projects, Grant/Award Number: 2020-1.1.5-GYORSÍTÓSÁV.

Abstract

A novel type I arabinogalactan (AG-I) polysaccharide (EPS) from *Epiphyllum oxypetalum (DC.) Haw*'s flowers is hypothesized to possess immunomodulatory activity. This study investigated EPS's effects on immune functions and its potential mechanism for enhancing intestinal health in immunosuppressed mice. The results showed that supplementing EPS significantly alleviated immune organ damage, increased the thymus index ($p < 0.01$), and regulated the key immune factors, including the tumor necrosis factor-alpha (TNF-α), immunoglobulin A (IgA), and complement 3 (C3) in the liver ($p < 0.05$). EPS promoted the expression of intestinal immune barrier and chemical barrier proteins such as interferon-γ (IFN-γ) and mucin 2 (MUC2) ($p < 0.05$), effectively repairing intestinal damage. EPS improved the diversity and structure of intestinal microbiota in immunosuppressed mice ($p < 0.05$) and significantly altered the abundance of intestinal immune-related microbial taxa, including *Lactobacillaceae* and *Lactobacillus* ($p < 0.01$). Furthermore, EPS supplementation altered intestinal lactic acid metabolism, significantly increasing lactic acid levels by up to 3.4-fold ($p < 0.01$), and enhanced the expression of Gpr81, Wnt3a, and β-catenin proteins at the bottom of the colonic crypts, which may repair the intestinal physical barrier. Overall, EPS represents a novel AG-I immunomodulatory dietary polysaccharide that enhances immunity and improves gut health.

KEYWORDS

Epiphyllum oxypetalum (DC.) Haw, gut barrier function, gut microbiota, immunity, lactic acid, type I arabinogalactan polysaccharide

Jin Dai and Zhiwei Zhou contributed equally to this work.

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1 | INTRODUCTION

The *Epiphyllum oxypetalum (DC.) Haw* (EOH), belonging to the Cactaceae family, is celebrated as the "Queen of the Night" for its unique characteristic of blooming at night (Thiago et al., 2014). This perennial succulent plant holds an irreplaceable ornamental value in the gardening community due to the rarity and brief blooming period of its flowers (Prajitha et al., 2019). In traditional Chinese culinary culture, the flower of the EOH is not only regarded as a delicacy but also recorded in the Chinese Pharmacopoeia "*Chinese Materia Medica Dictionary*" for its significant pharmacological activity in treating gastrointestinal diseases and tuberculosis. Moreover, in modern applications, extracts from the EOH are widely used in popular cosmetics, providing moisturizing, whitening, and antioxidant functions (Baek et al., 2019; Feng et al., 2024). However, despite the diverse health benefits presented by the EOH, the scarcity of its flowers due to the lack of breakthroughs in long-term cultivation techniques limits its application on a large scale (Lesmana et al., 2022; Thiago et al., 2014; Upendra & Khandelwal, 2012), and the specific roles that the components of the EOH, especially its active ingredients, play in the body remain unclear.

Through over a decade of cultivation technology research and development, a large-scale EOH planting farm spanning 500 acres has been established in Zizhong County, Sichuan Province, China (Ma et al., 2023). This farm has increased the yield of EOH per acre from approximately 5000 flowers to about 50,000 flowers, marking a tenfold increase. The new cultivation techniques have also extended the blooming period from the original 4 months of the year to 8 months, with concentrated flowering occurring approximately every 20 days. These technological breakthroughs have not only promoted the commercial cultivation of EOH but also opened up possibilities for further exploration of its nutritional and medicinal values.

The EOH is rich in a variety of beneficial components, including proteins (14 mg/g), fatty acids (4.6 mg/g), and vitamins (0.18 mg/g) (Upendra & Khandelwal, 2012). Compared to these nutrients, mucus polysaccharides are one of the most abundant carbohydrates in Cactaceae plants, accounting for more than 18% of the dry weight of flowers and stems (Gheribi & Khwaldia, 2019; Sepúlveda et al., 2007). These polysaccharides typically have high molecular weights and branched structures (Saeidy et al., 2021), which is also the case with the EOH. Natural polysaccharides, as a class of biologically active macromolecules with potent functionalities, offer diverse health benefits, such as immunomodulation (Guo et al., 2023), antitumor (Li et al., 2023), antidiabetic (Wu et al., 2024), and liver-protective activities (Wang et al., 2023; Yuan et al., 2022). The biological functions of different polysaccharides are closely related to their structural variations, including monosaccharide composition, glycosidic bond types, molecular size, and the degree of branching. These structural characteristics determine their specific roles in biological processes such as cell recognition, signal transduction, and immune response (Mohammed et al., 2021). Currently, the biological activities of polysaccharide derived from EOH remain unknown.

In the preliminary phase of this study, a novel water-soluble polysaccharide (EPS) was isolated from EOH, with a maximum solubility of 2.75 mg/mL. Preliminary results indicated that the polysaccharide is composed of arabinose, galactose, glucose, xylose, mannose, fructose, and galacturonic acid. Among these, the molar ratios of arabinose and galactose monosaccharides are respectively 11.480% and 53.791%. The weight-average molecular weight of the EPS reached 5.577×106 Da, and its main chain structure is β-Galp linked by (1→4) bonds (Supporting Information S1: Figure S1) (Ma et al., 2023). Compared with type II arabinogalactan (AG-II), which has a backbone structure linked by (1→6) and/or (1→3)-Galp, these results indicate that the EPS is a typical type I arabinogalactan (AG-I) (Ferreira et al., 2015; Saeidy et al., 2021). The functional activities of natural polysaccharides are closely related to their specific molecular structures. Compared with other natural polysaccharides, EPS has notable characteristics, with its molecular weight being about 8–600 times that of these polysaccharides (Wang et al., 2023). Related studies show that high molecular weight polysaccharides often act as dietary fibers/prebiotics interacting with the body, such molecules are difficult to digest in the small intestine and enter the colon, providing substrates for the complex bacterial ecosystem there, thereby regulating the body's intestinal flora imbalance and metabolic disorder (Bamigbade et al., 2024; Rastall et al., 2022). Moreover, the total molar ratio of arabinose and galactose in the EPS is higher than that of most other AG-I polysaccharides (Saeidy et al., 2021), which helps the polysaccharide to form specific conformations, achieving a good response in stimulating the immune system (Chen et al., 2022). Overall, EPS may exert a beneficial effect on the host's immunity through the gut microbiota.

Despite the EOH being an important plant for both medicinal and dietary uses as well as for ornamental purposes, its flowering period is short and raw materials are hard to obtain, leading to limited research on the immunomodulatory effects of its main component—EPS. Furthermore, whether these effects are related to gut microbiota remains unknown.

Therefore, in this study, we used a cyclophosphamide (CTX)-induced immunosuppression model in mice to explore the potential immunoenhancing activity of AG-l type polysaccharides derived from EOH. Lentinan, with strong immunomodulatory activity and widely used in clinical settings (Vijayaram et al., 2022), was applied as the positive control. Through 16 s high-throughput sequencing, we investigated the possible immunomodulatory mechanisms of EPS from the perspective of gut microbiota. This research provides the first report on the immune effects and preliminary mechanisms of polysaccharides derived from

EOH based on gut microbiota, offering new insights into the breeding of precious ornamental plants like EOH and expanding the application potential of AG-I type polysaccharides such as EPS.

2 | MATERIALS AND METHODS

2.1 | Materials and reagents

EOH was collected from the EOH planting demonstration base of Sichuan Yuanlan Agricultural Development Co., Ltd. The extraction process of EPS is as follows, the petals of EOH were dried and subsequently pulverized into a powder. The polysaccharide was then extracted from the powder using the hydroalcoholic precipitation, resulting in a purity level of 95% and an average molecular weight of 5.577×10^6 Da.

CTX (C849559) was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. ELISA kits for cytokines and antibodies were purchased from Quanzhou Ruixin Biotechnology Co., Ltd. and Jiangsu Meimian Industrial Co., Ltd. Four percent paraformaldehyde tissue fixation (BL539A), RIPA lysate (BL504A), protease inhibitor (BL612A), and BCA protein content assay kits (BL521A) were purchased from Biosharp Biotechnology Co., Ltd. Lentinan polysaccharide (H42022727) was purchased from Hubei Guangren Pharmaceutical Co., Ltd. (Jiang, Wang, et al., 2021). Lactic acid assay kit (A019-2-1) was purchased from Nanjing Jiancheng Bioengineering Institute. Hematoxylin and eosin (H&E) staining kits (G1120) were purchased from Solarbio Science & Technology Co., Ltd. and periodic-acid-schiff staining (PAS) dye (G1008) was purchased from Servicebio Technology Co., Ltd. All other chemical reagents were of analytical grade.

2.2 | Animals and treatment

A total of 35 male specific pathogen-free (SPF) Kunming mice, aged 4 weeks and weighing between 18 and 22 grams, were purchased (HE-12-54-04) from Dashuo Laboratory Animal Co., Ltd. The schematic diagram of the experiment is shown in Figure 1 A. They were housed in standard indoor conditions (20–26°C, 40%–70% humidity) with a 12-h light/12-h dark cycle and had ad libitum access to standard maintenance feed and water. After 3 days of acclimatization feeding in SPF-level experimental animals at Chengdu Medical College, the animals were randomly divided into five groups of seven animals each, namely, blank control group (CON), model control group (MDC), lentinan polysaccharide positive control group (LNP), EPS low dose group (EPL), and EPS high dose group (EPH). CTX is a frontline chemotherapy drug used to treat various cancers and autoimmune diseases (Hao et al., 2019; Madondo et al., 2016; Manente et al., 2018). Nonetheless, the long-term use of CTX can also have harmful effects on immune organs such as the thymus and spleen, disrupt the gastrointestinal mucosal barrier, and lead to an imbalance in the gut microbiota (Iqubal et al., 2019). Therefore, CTX is often used to construct immune-suppressed animal models, especially in mouse models with anatomical structures, including the gastrointestinal tract, and immune systems similar to humans, rather than in zebrafish (Kamareddine et al., 2020). Referring to the method of Zhou et al. for immunosuppression modelling in mice (Zhou et al., 2018), normal saline was injected intraperitoneally into the CON group and the CTX (70 mg/kg bw per day) was injected into the other groups during Days 1–5 of the experiment. Due to the biting of mice from one another and the immunosuppressive toxicity of the drug leading to a high risk of mortality, the final number of successfully modelled mice was determined to be 5 per group. On Days 6–19, the CON and MDC groups received normal saline via gavage, while the LNP group was administered lentinan polysaccharide (5 mg/kg/d bw per day) by gavage (converted based on the manufacturer's recommended human dose) (Wei et al., 2010), and the corresponding doses of EPS were gavaged in the EPL (25 mg/kg/d bw per day) and EPH (35 mg/kg/d bw per day) groups. On Day 19, faeces were collected from each group. On Day 20, after anesthetizing the mice by isoflurane, the mice were humanely killed by cervical dislocation and other tissues were collected. During the experimental period, the mice were observed daily and their body weight and diet were recorded.

2.3 | Blood and organ collection

After anesthetizing the mice with isoflurane, the eyes were removed and blood was collected, left at room temperature for 20 min, then centrifuged at 3500 r/min for 20 min, and the supernatant was taken to obtain serum, which was frozen at −80°C in the refrigerator for later indexing. Then the mice were humanely killed by cervical dislocation, and the organs including liver, thymus, spleen, kidney and colon were removed and weighed. The organ index was calculated as organ weight (mg)/body weight (g) ×10.

2.4 | H&E staining of the spleen, thymus and colon

Spleen, thymus and colon tissues were fixed in 4% paraformaldehyde tissue fixative for 48 h, dehydrated in alcohol, and then processed into paraffin-embedded blocks. Sections (3–5 μm thick) were stained with the H&E staining solution to observe histological changes.

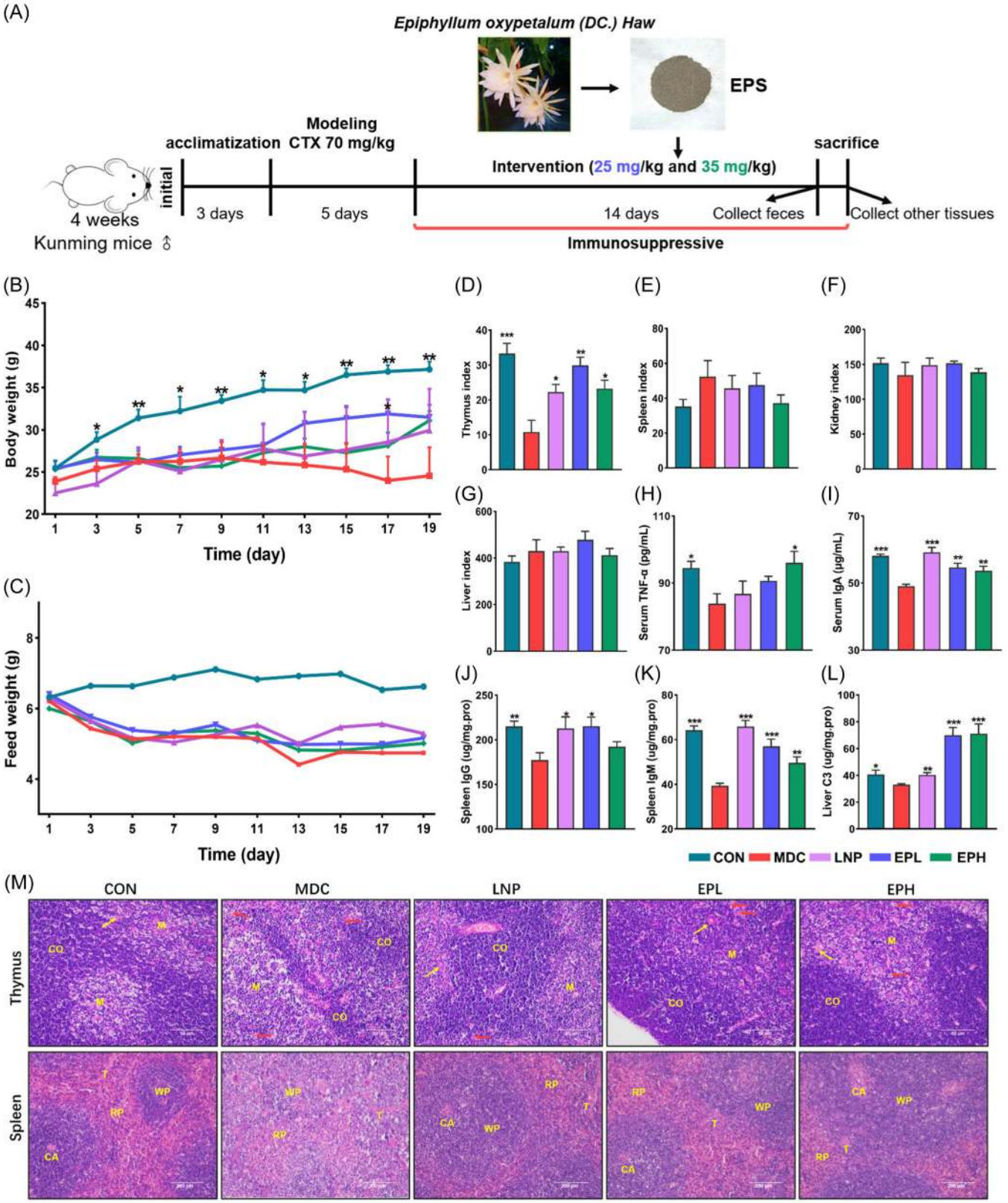

FIGURE 1 Effects of EPS on apparent parameters and cytokine levels in immunocompromised mice. The schematic diagram of the experiment (A). Changes in body weight (B) and diet of mice (C). Differences between groups in (D) thymus index, (E) spleen index, (F) kidney index, and (G) liver index of mice. Expression levels of mice (H) serum TNF-α, (I) serum IgA, (J) spleen IgG, (K) spleen IgM, and (L) liver C3. H&E-stained histological sections of mice (M) thymus (100×) and spleen (40×). CO represents cortex, M represents medulla, yellow arrows indicate thymic corpuscle, red arrows indicate necrotic cells; WP represents white pulpa, RP represents red pulpa, CA represents central artery, and T represents spleen trabeculae. Data are expressed as mean ± SEM, $n = 5$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, vs. the MDC group.

Images were acquired using the CI-L plus light microscope (Nikon) (Spleen: 40× magnification, thymus: 100× magnification, and colon: 200× magnification). The depth of the five intact colonic crypts in each colonic tissue section was measured using Nis-elements analysis software (version: 5.42.00, Japan) and the mean value was calculated. The description of pathological sections was based on the relevant literature method (Jiang, Hou, et al., 2021; Zhang et al., 2019).

2.5 | PAS staining of the colon

The number of stainable goblet cells in the tissue (3–5 μm thick) of colon sections was determined by PAS method. After staining the colon tissue, then full-field images were taken using a light microscope (40× magnification) and the stainable goblet cells in the field of view were counted using Image-J (version:1.8.0.172).

2.6 | Determination of immunoglobulins and cytokines in serum, colon and liver

Blood samples were collected and serum was separated, and Tumor necrosis factor-alpha (TNF-α) and immunoglobulin A (IgA) levels were measured with ELISA kits. One hundred mg of spleen, liver and colon tissues, respectively, were mixed with 900 μL of phosphate-buffered saline (PBS) and homogenized thoroughly, centrifuged for 20 min (8500 r/min, 4°C), and the supernatants were collected to measure protein concentrations for immunoglobulins (IgM, IgG), complement 3 (C3), Secretory IgA (sIgA), interleukins (IL-10, IL-17), interferon-γ (IFN-γ), transfer growth factor beta3 (TGF-β3), tight junction-associated proteins (tricellulin, occludin), and mucoprotein 2 (MUC2) expression levels. The assay was performed according to the instructions provided by the ELISA manufacturer.

2.7 | Determination of lactic acid content in colon and faeces

Colon tissue samples were added to nine times the volume of PBS at a ratio of 100 mg to 900 μL, and faeces samples were added to three times the volume of PBS at a ratio of 100 mg to 300 μL, centrifuged for 20 min (3500 r/min, 4°C), and the supernatant was collected and then the lactic acid concentration was determined by enzymatic colorimetric assay (Lin et al., 2022).

2.8 | Immunohistochemistry

Colon tissue sections were treated with xylene and gradient ethanol, incubated with 0.1% Tritonx-100 and rinsed with PBS. Sections were serially sealed with BSA and serum. Sections were incubated with primary antibodies GPR81, Wnt3a, and Ctnnb1 (β-catenin) at 4°C overnight, followed by incubation with secondary antibodies at room temperature, dropwise addition of horseradish peroxidase-labelled streptomycin protein followed by development with DAB developer. During microscopic observation, five random fields of view were obtained and photographed (200× magnification). The quantitative automatic measurement of the positive area is defined by the ImageJ software (NIH) and expressed as Average optical density (AOD) values.

2.9 | 16S rRNA sequencing of gut microbiota

Total faecal DNA was extracted according to the instructions of the E.Z.N.A®soil DNA kit (Omega Bio-Tek) and detected by 1% agarose gel electrophoresis. The V3-V4 hypervariable region of the bacterial 16S rRNA gene was amplified using universal primers 338 F (5′-ACTC CTACGGGAGGCAGCAG-3′) and 806 R (5′-GGACTAC HVGGGTWTCTAAT-3′). The PCR products were recovered using 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences), and quantified using QuantiFluor™-ST (Promega). PE libraries were created using TruSeqTM DNA sample preparation kit and sequenced on the Illumina Miseq PE300 platform (Illumina) according to the standard protocol of Majorbio, Inc. PacBio raw reads were processed using SMRTLink (version 8.0) to obtain demultiplexed circular consensus sequence (CCS) reads with at least three full passes and 99% accuracy. CCS reads were barcode-identified and length-filtered, removing sequences <1000 bp or >1800 bp. Optimized CCS reads were clustered into operational taxonomic units (OTUs) using UPARSE 7.1 at 97% sequence similarity. The most abundant sequence for each OTU was chosen as the representative sequence. Chloroplast sequences were manually removed from the OTU table. Paired-end forward and reverse sequences were merged using FLASH software (version 1.2.11). The Silva database was used for sequence alignment, and BLAST was used to annotate species information for each OTU. Alpha-diversity and beta-diversity were analyzed based on the abundance of OTUs using the R package. The linear discriminant analysis (LDA) and LDA effect size (LEfse) were used to analyze the dominant bacterial communities between groups. The functional composition of microbial communities from amplicon sequencing results was predicted using phylogenetic investigation of communities by reconstruction of unobserved states 2 (Picrust2). One-way correlation networks and correlation heatmap were drawn using Spearman rank correlation coefficient analysis.

2.10 | Data analysis

Each experiment was repeated at least three times and statistical analysis was performed using GraphPad Prism

(version: 8.0, GraphPad Software, Inc.) and data were expressed as mean and standard error (mean ± SEM). The Shapiro–Wilk test was used to assess the normality of the data. Comparisons of biochemical index data between groups with MDC group were performed using the Student's *t*-test, and the nonparametric Mann–Whitney test was used to determine the statistical significance of alpha-diversity and Picrust2 measurements. Differences were considered statistically significant when $p < 0.05$.

3 | RESULTS

3.1 | Effects of EPS on epistatic parameters and cytokine levels

After 2 weeks of polysaccharide intervention, compared with the MDC group, the immunosuppressed mice intervened with EPS and lentinan polysaccharide did not significantly improve the body weight, diet, spleen index, kidney index and liver index (Figure 1B,C,E–G) ($p > 0.05$), but showed a certain trend of improvement for the above indices, and the improvement trend was similar to that of the CON group. Notably, mice treated with EPS significantly altered the thymus index compared to MDC group and significantly increased the levels of serum TNF-α, serum IgA, spleen IgG and IgM (Figure 1D,H–K; $p < 0.05$). Interestingly, EPS exhibited extremely strong activity in promoting C3 synthesis in the liver (Figure 1L; $p < 0.05$), which we speculate that this may be related to the monosaccharide components of AG-I itself, which are rich in galactose and arabinose (Togola et al., 2008).

The histopathological condition of the thymus and spleen was visualized and evaluated through H&E staining (Figure 1M). There was no obvious boundary between the cortex and medulla in the thymus of MDC group mice, few thymus corpuscles and more necrotic cells. The spleen of MDC group mice showed a lighter color and less obvious boundary between red and white pulpa, indicating a significant decrease in the number of lymphocytes. Spleen trabeculae were visible, while the central artery was difficult to see. These results demonstrated the toxic damaging effect of CTX on thymus and spleen. Compared with the MDC group, the mice in all three polysaccharide intervention groups showed improvement in the above parameters, with the high dose of EPS showing a slightly better improvement than the low dose of EPS. In conclusion, EPS has the potential to alleviate CTX-induced immune damage.

3.2 | EPS intervention repaired damaged intestinal barrier

The intestine is a vital immunological organ equipped with mechanical, chemical, immune, and biological barriers that defend against pathogens and maintain internal equilibrium. When the body's immune system is compromised, intestinal mucosal immunity, a crucial component of the immune defense, is also adversely affected. The integrity of intestinal barrier function is essential for maintaining healthy homeostasis in the organism. MUC2, secreted by goblet cells, plays a central role in constituting the chemical barrier of the intestinal mucosa, which lubricates the intestine and antagonizes pathogenic bacteria (Birchenough et al., 2015). After EPS intervention, especially at high doses, the number of colonic stainable goblet cells and the expression level of MUC2 were significantly increased in immunocompromised mice (Figure 2A–C; $p < 0.05$). The depth of intestinal crypt can represent the integrity of intestinal morphology (Bao et al., 2022), and both EPS and lentinan polysaccharide significantly increased the length of colonic crypt compared with the MDC group (Figure 2A,D; $p < 0.05$). In addition, mice in the MDC group had loose intestinal mucosal and muscular connections and showed lymphocytic infiltration. The mice in the three polysaccharide intervention groups improved in the injury mentioned above, the changes in the EPL group were slightly weaker but still better than those in the MDC group (Figure 2A).

CTX causes disruption of the mucosal mechanical barrier in mice, leading to increased intestinal permeability. Compared to the MDC group, both lentinan polysaccharide and EPS significantly increased the expression level of intestinal tricellulin in immunosuppressed mice (Figure 2E; $p < 0.05$), but not that of occludin (Figure 2F; $p > 0.05$). SIgA limits the growth of bacterial pathogens by shaping resident microbial communities and by immune rejection through enhancing host protective immunity and playing an important role in intestinal barrier protection (Doron et al., 2021). The results showed that sIgA expression levels were significantly lower in the colon of mice in the MDC group compared to the CON group (Figure 2G; $p < 0.05$), whereas high doses of EPS improved intestinal sIgA expression levels in immunocompromised mice. IFN-γ, IL-10, TGF-β3, and IL-17 are cytokines secreted by T-helper cells Th1, Th2, Treg, and Th17, respectively. Compared with the CON group, the expression levels of IFN-γ, IL-10, TGF-β3, and IL-17 in the colon of MDC mice were significantly reduced, indicating that the intestinal immune barrier of immunosuppressed mice was damaged, and the EPS intervention reversed this immune damage and showed a dose-dependent improvement (Figure 2H–K; $p < 0.05$).

In conclusion, the above results suggest that EPS can restore CTX-induced damage to the intestinal chemical, mechanical and immune barriers. This includes increasing the number of colonic stainable goblet cells, improving intestinal morphological disorders and crypt depth, promoting the expression levels of MUC2 and tricellulin, and promoting the secretion of sIgA and T-helper cell-associated cytokines.

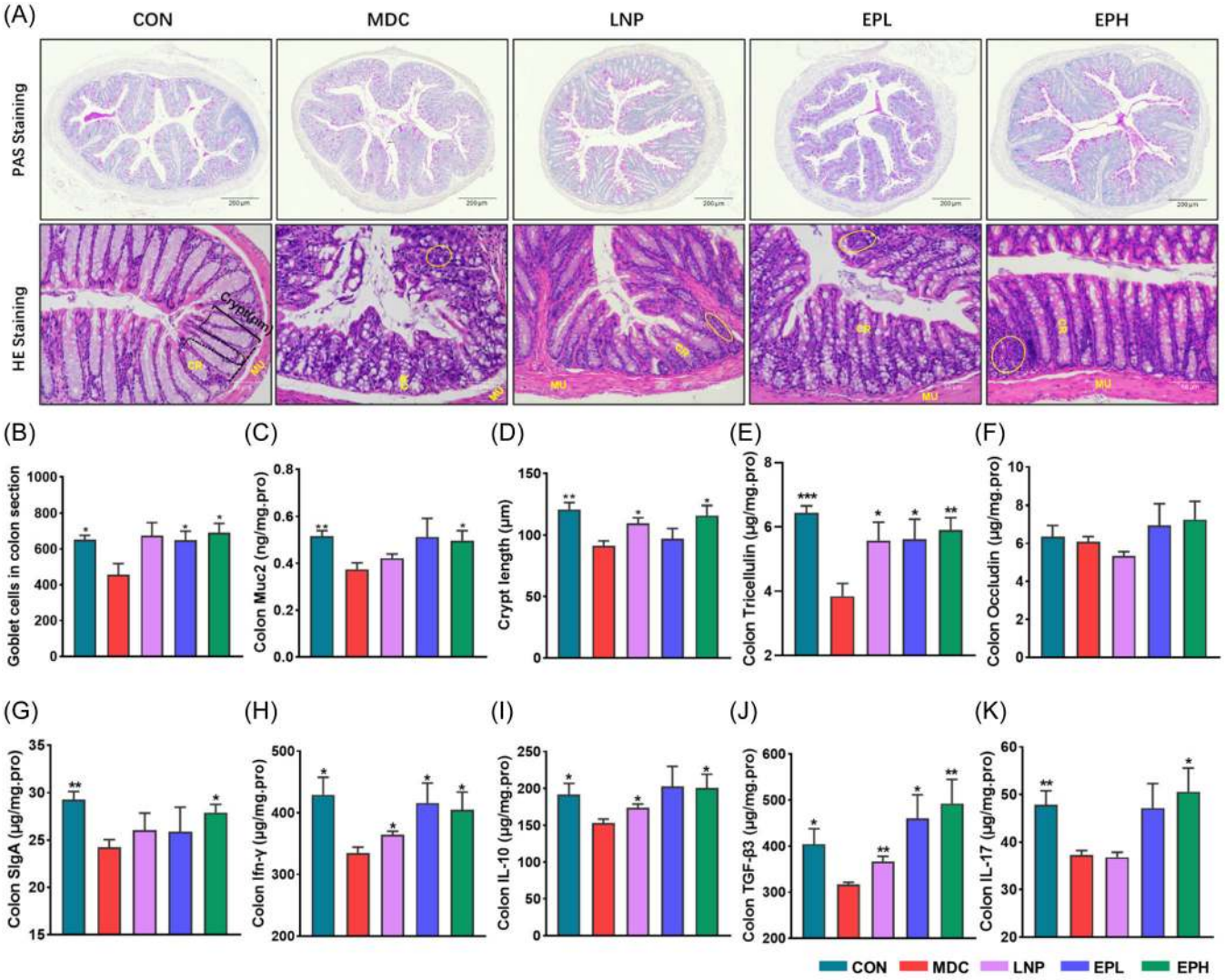

FIGURE 2 Effect of EPS on the intestinal barrier of immunosuppressed mice. Colon tissue section staining (A) PAS staining (40×) and H&E staining (200×), (B) number of goblet cells, (D) crypt length. Colonic barrier-associated proteins (C) MUC2, (E) Tricellulin, (F) Occludin, (G) sIgA, (H) IFN-γ, (I) IL-10, (J) TGF-β3, (K) IL-17. Data are expressed as mean ± SEM, $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$, vs the MDC group. The purple granules in the colon tissue section represent the stained goblet cells, CR indicates crypt, MU indicates the muscle layer, and yellow circle indicates lymphocyte infiltration.

3.3 | EPS changed the structure and diversity of gut microbiota

To investigate the impact of EPS on gut microbiota, fecal samples from each group of mice were subjected to 16S rRNA sequencing analysis. The dilution curve analysis indicated that the curves of all samples exhibited a flattened trend (Supporting Information S1: Figure S2, A,B), suggesting that the sequencing data reached saturation and effectively captured the majority of species within the gut microbiome community.

The structure of the gut microbiota of mice in the MDC and CON groups was found to be significantly different by principal component analysis (PCA) (Figure 3A; $p < 0.05$). After EPS intervention, the gut microbiota of mice in the EPL and EPH groups were significantly altered compared with those in the MDC group (Figure 3C,D; $p < 0.05$), and the ability of EPS to alter the structure of gut microbiota showed a certain dose-dependence, while the effect of lentinan polysaccharide was not significant (Figure 3B; $p > 0.05$). Partial least squares discriminant analysis (PLS-DA) was used to classify the gut microbiota structure of all groups of mice (Figure 3E), and the results showed that the gut microbiota of mice in the three polysaccharide intervention groups clustered together, while the gut microbiota of mice in the CON and MDC groups clustered into one category each.

The results of diversity difference analysis on the OTU level showed that the OTU number, chao1 index and ace index of gut microbiota of mice in the MDC

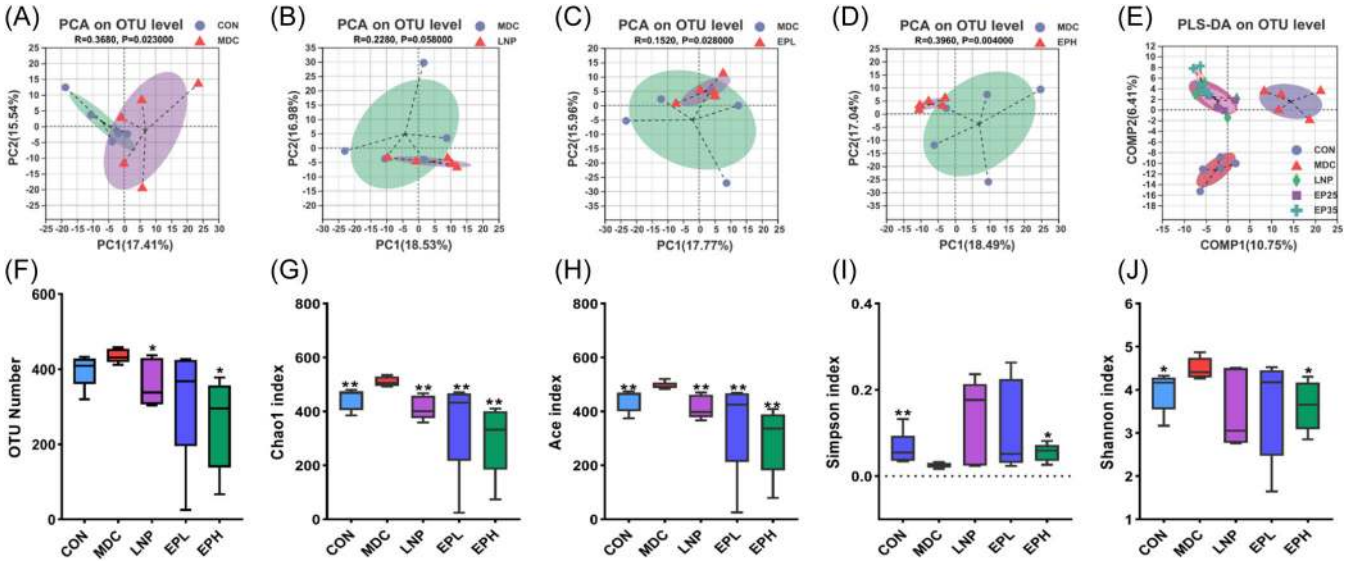

FIGURE 3 Effect of EPS on intestinal microbial diversity in immunosuppressed mice. PCA on OTU level (A) MDC vs CON, (B) MDC vs LNP, (C) MDC vs EPL and (D) MDC vs EPH. All groups (E) PLS-DA analysis. Alpha diversity analysis at OTU level for (F) OTU number, (G) Chao1 index, (H) Ace index, (I) Simpson index and (J) Shannon index. $N = 5$. $*p < 0.05$, $**p < 0.01$, vs the MDC group. In the PCA analysis, different colors represent different groups, and within the same group, individuals are depicted in circular plots based on a 95% confidence interval.

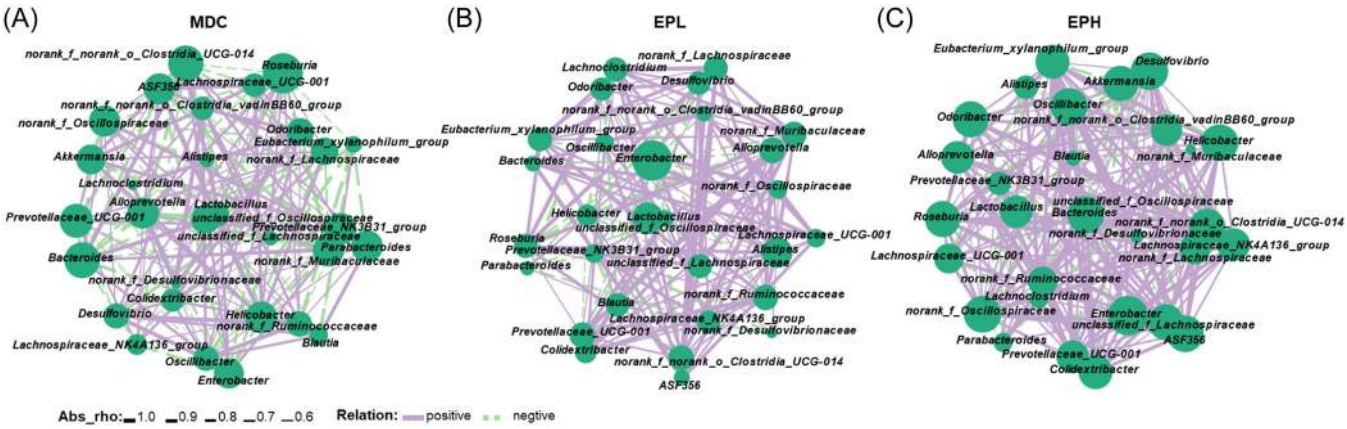

FIGURE 4 The impact of EPS on the gut microbiota relationship of immunosuppressed mice. One-way correlation networks of the top 30 abundance of gut microbiota genus levels in (A) MDC group, (B) EPL group and (C) EPH group. The purple solid line and the green dashed line indicate positive and negative correlations, respectively, and the width reflects the strength of the correlation.

group were significantly higher than those of other groups (Figure 3F–H; $p < 0.05$), indicating that the gut microbiota richness of mice in the MDC group was significantly higher than those of other groups. In addition, compared with the MDC group, the EPH and CON groups showed an increase in Simpson index and a decrease in Shannon index (Figure 3I,J; $p < 0.05$), indicating a significant decrease in gut microbiota diversity. Similar to the OTU level results in terms of species number performance at the genus level, mice in the three polysaccharide intervention groups had lower intestinal genus level species diversity than the MDC group (Supporting Information S1: Figure S2, C). In addition, the interactions between genus were visualized by one-way correlation networks. The results showed a higher degree of network complexity in the top 30 genera correlation networks in terms of abundance, especially for the positive relationships, in the EPL and EPH groups than in the MDC group (Figure 4; Supporting Information S1: Table S1).

In summary, the aforementioned results suggest that EPS has the ability to influence the diversity and abundance of gut microbiota in immunosuppressed mice. Additionally, EPS is capable of influencing the structure of the gut microbiota and impacting the interrelationships among intestinal microorganisms.

3.4 | EPS treatment affected the gut microbiota of immunocompromised mice

We conducted an analysis of the dominant phylum in the fecal samples of different groups of mice (Supporting Information S1: Figure S3.A). The results showed that the composition of dominant phylum was similar across all groups, with Firmicutes and Bacteroidota being the primary phylum, followed by Campilobacterota and Proteobacteria. Additionally, we analyzed the composition of the top 30 genera in the gut microbiota (Supporting Information S1: Figure S3.B). The results indicated that polysaccharide intervention led to changes in the abundance of certain genus among the groups, particularly *Lactobacillus*. Specifically, the abundance of *Lactobacillus* was significantly restored in the EPH group. Moreover, some genera (outside the top 30) with relatively low abundance exhibited significant differences between groups (Supporting Information S1: Figure S4), and previous studies have shown that these genera are related to gut immunity. The intervention of EPS had, to some extent, affected the abundance of these genera in the gut. *Tyzzerella* is found in higher abundance in the gut microbiota of Crohn's disease patients and is associated with intestinal inflammation (Olaisen et al., 2021). Supplementing gut butyrate leads to an increase in *Rikenella* abundance (Yu et al., 2019), reflecting a possible deficiency of butyrate in the gut of immune-deficient mouse models under CTX toxicity. The *Family_XIII_AD3011_group* and *Escherichia-Shigella* are negatively correlated with the concentration of acetate in the intestinal lumen (Yi et al., 2024). *Anaeroplasma* is positively correlated with the severity of experimental autoimmune encephalomyelitis (He et al., 2019). *Escherichia-Shigella* may be important indicators of gut sepsis (Zuo et al., 2023), while *Candidatus Arthromitus* is a recently discovered symbiotic bacterium associated with the maturation of gut immune function (Van Praet et al., 2015). Additionally, the abundances of low-prevalence bacterial genera such as *Defluviitaleaceae_UCG-011*, *unclassified_f_Anaerovoracaceae*, *Caldicoprobacter*, *Anaerovorax*, *Prevotellaceae_NK3B31_group*, *Butyricimonas*, *norank_f_UCG-010*, and *unclassified_f_Lachnospiraceae* changed following EPS intervention. These bacteria may serve as potential biomarkers for improving immunosuppressive conditions.

We additionally used LEfse measurement to determine the differential taxa between the polysaccharide intervention groups and the MDC group. Significant differences were found during the comparison. Compared with the two groups intervened by EPS (Figure 5C,D), *Caldicoprobacter*, *norank_f_norank_o_Rhodospirillales*, *Anaerovorax*, *Enterococcus*, *Tyzzerella* , *Butyricimonas*, etc. were more abundant in MDC group, while the abundance of *Erysipelotrichaceae* and *Lactobacillus* decreased significantly, and treatment with EPS could restore the levels of these microbes in the intestines of immunosuppressed mice to levels similar to those in normal mice (Figure 5A,B). Related study suggests that *Lactobacillus*, which uses carbohydrates for fermentation and produces lactic acid as the main end product, is able to create an acidic intestinal microenvironment, an environment that is not conducive to the survival of pathogens (Goldstein et al., 2015). It has also been demonstrated that *Lactobacillus* can promote the production of IgA, IL-6, IL-10, IFN-γ, and tumor necrosis factor by intestinal Peyer's patches cells as a way to regulate intestinal immunity (Kotani et al., 2014). In addition, *Erysipelotrichaceae*, which belongs to the same phylum as *Lactobacillus*, can produce adjuvant-like effects in the intestine, enhancing the response of T-helper 17 cells (Th17) and modulating immunity (Miyauchi et al., 2020). It is worth noting that a previous study has indicated that dietary fermentable fiber can enhance the metabolism of fiber by the gut microbiota, leading to alterations in the ratio of Firmicutes to Bacteroidota and an increase in the concentration of circulating short-chain fatty acids (SCFAs) (Trompette et al., 2014). However, our results did not demonstrate such effects, as there were no significant differences observed in either the individual abundance or the ratio of Firmicutes to Bacteroidota between groups (Figure 5E; Supporting Information S1: Figure S3.A). The above results indicated that EPS treatment affected the gut microbiota of immunocompromised mice, particularly contributing to the restoration of *Lactobacillus* abundance in the gut.

3.5 | Correlations of genera with immune parameters, functional changes in gut microbiota across groups

The correlation analysis between the top30 genera and metabolic parameters showed that *Lactobacillus* was positively correlated with most immune parameters, which indicated that *Lactobacillus* may play an important role in mediating the recovery of immune function in response to polysaccharide intervention. In addition, *Parabacteroides* was negatively correlated with some immune parameters (Figure 6A). To further reveal the correlation between functional changes in gut microflora and improved intestinal immunity in each group, we used Picrust2 to predict the functional composition of microbial communities from amplicon sequencing results (Douglas et al., 2020). Notably, the predicted abundance of ko02060: Phosphotransferase system (PTS) in the KEGG pathway level3 abundance statistics was significantly enriched in the three polysaccharide intervention groups ($p < 0.05$), but not in the CON group ($p > 0.05$), compared to the MDC group (Figure 6B). This difference may be due to polysaccharides intervention, where monosaccharides such as glucose and fructose enter the bacterium via PTS transport and phosphorylation when entering the homolactic fermentation pathway of lactic acid (Lauret et al., 1996). In addition, the KEGG module

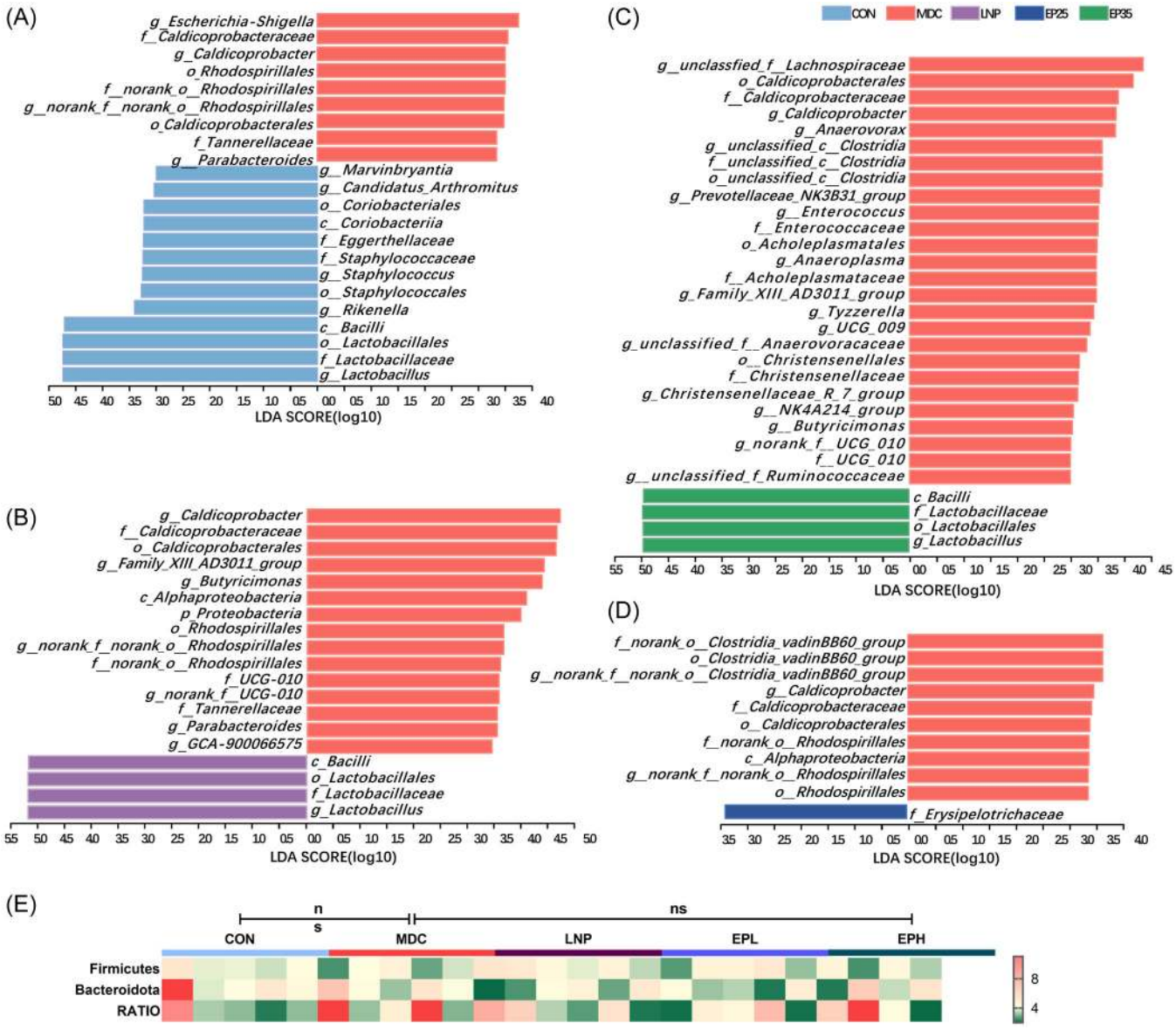

FIGURE 5 EPS alters the abundance of specific taxa in the intestine of immunosuppressed mice. LEfse analysis of gut microbiota in mice (A) MDC vs CON, (B) MDC vs LNP, (C) MDC vs EPL, (D) MDC vs EPH with LDA threshold = 3.0. (E) Abundance of Firmicutes and Bacteroidota between groups and their ratio. Data are expressed as Mean, $n = 5$. vs the MDC group.

abundance statistics of M00632 (Leloir pathway) was significantly enriched in the EPL group ($p < 0.05$) and showed some increasing trend in the EPH and LNP groups ($p > 0.05$), which is the metabolic pathway that degrades galactose to produce lactic acid (Figure 6C). In the abundance statistics of the Metacyc database, ANAEROFRUCAT-PWY (homolactic fermentation) and P122-PWY (heterolactic fermentation) showed a significant increase ($p < 0.05$) after the intervention of EPS compared to the MDC group, although this increase was not synchronized with the dose (Figure 6D). However, after combining the predicted abundance of the total lactic acid fermentation pathway in each group, the results showed that the abundance of lactic acid fermentation was significantly lower in the MDC group than in the other groups (Figure 6E; $p < 0.05$). Gut microbiota-derived SCFAs are known to play a crucial role in maintaining intestinal immune balance. Interestingly, in our study, the pathways related to gut microbial fatty acid metabolism did not exhibit significant differences between the groups (Figure 6F). This discovery is consistent with the absence of significant changes in the abundance of Firmicutes and Bacteroidota, as observed in previous results (Figure 5E).

The reduced lactic acid fermentation capacity of the gut microbiota might have been a functional manifestation of the gut microbiota in CTX-induced immunocompromised mice, whereas after the intervention of lentinan polysaccharide and EPS, the ability of the gut microbiota to ferment lactic acid converged with that of

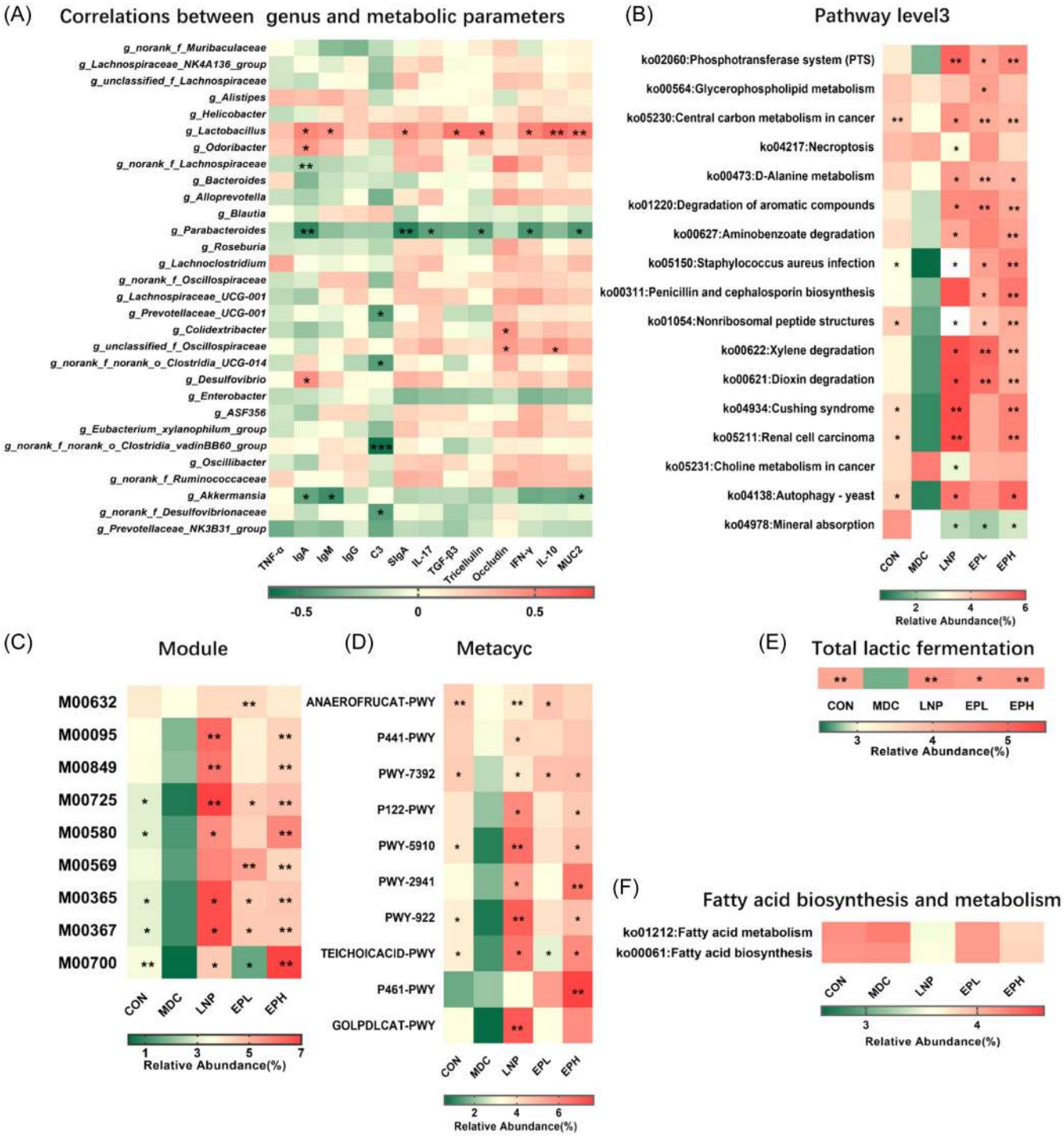

FIGURE 6 Correlations of genus with metabolic parameters, functional changes in gut microbiota across groups. (A) Correlations between genus and metabolic parameters, (B) KEGG Pathway Level3 level functionally predicted abundance, (C) KEGG Module functionally predicted abundance, (D) Metacyc functionally predicted abundance. (E) Total lactic acid fermentation abundance, (F) KEGG Pathway Level3 functional fatty acid metabolism predicts abundance. Data are expressed as Mean, $n = 5$. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, vs the MDC group.

the CON group mice. In addition to the inhibition of the growth of harmful intestinal bacteria by lactic acid as mentioned above, lactic acid also has a wide range of organismal immunomodulatory effects. Lactic acid can modulate the key functions of several key players of the immune system, such as macrophages and dendritic cells (Garrote et al., 2015). Furthermore, a study by Morita et al. revealed that lactic acid can activate the GPR31 receptor to enhance dendritic protrusion of small intestinal CX3CR1 cells and promote tubular antigen uptake (Morita et al., 2019). Therefore, combined with the results of LEfse analysis (Figure 5), EPS-induced increase

in intestinal *lactobacillus* abundance and lactic acid content in immunocompromised mice may be involved in the immune recovery process of immunosuppressed mice.

3.6 | Lactic acid promoted the expression of intestinal epithelial repair-related proteins at the bottom of colonic crypts

The intestinal epithelial tissue of the organism is highly sensitive to chemotherapeutic agents and intestinal damage is the main damage during chemotherapy. *Lactobacillus* is usually considered to contribute to the protection of the intestinal mucosa, and lactic acid as an end product of *Lactobacillus* fermentation plays an important role in the repair of the intestinal barrier. A study by Okada et al. demonstrated that lactic acid derived from flora enhances the proliferation of colonic epithelial cells and maintains the normal morphology and function of intestinal epithelial cells (Okada et al., 2013). Additionally, a study by Lee et al. revealed that lactic acid from the microbiota stimulates intestinal stem cell proliferation through Paneth cells and intestinal stromal cells via the Wnt/β-catenin signalling pathway, thereby repairing intestinal damage caused by chemotherapy and radiotherapy and maintaining the intestinal physical barrier (Lee et al., 2018). To verify the reparative effect of intestinal lactic acid on intestinal damage in mice with CTX-induced immunosuppression, we employed immunohistochemistry to visually confirm the expression sites and levels of factors related to intestinal barrier repair in the colons of mice from various groups (Figure 7A). Firstly, after testing the lactic acid content in the faeces and colon (Figure 7B,C; $p < 0.05$), the results were consistent with the results predicted by Picrust2, and the lactic acid content in the intestine and colon of mice in the three polysaccharide intervention groups was significantly higher than that in the MDC group. The content of lactic acid in intestinal feces of mice in EPH group was 3.4 times higher than that in MDC group. Lactic acid derived from the microbiota aids in repairing intestinal damage caused by chemotherapy drugs, primarily through a Gpr81-dependent mechanism by activating the expression of proteins such as wnt3a and β-catenin at the bottom of the colonic crypts to promote intestinal epithelial development (Lee et al., 2018). Quantitative results from ImageJ software showed that the expression of Gpr81, Wnt3a, and β-catenin was mainly concentrated at the bottom of the colonic crypts in the EPH group, and the expression levels were significantly higher than those in the MDC group (Figure 7D–F; $p < 0.05$). This helps to maintain the stemness of Lgr5+ intestinal stem cells within the intestine and repair the damaged intestinal physical barrier (Lee et al., 2018). The above evidence suggests that lactic acid from the intestine may play a key role in the recovery of the intestinal structural physical barrier during the process facilitated by EPS.

4 | DISCUSSION

Recently, many studies have demonstrated that immune activation plays a crucial role in the diverse pharmacological effects of herbal polysaccharides (Liu et al., 2022; Wang et al., 2021; Zeng et al., 2019). In our study, CTX caused damage to the immune organ thymus, spleen and intestine. However, histopathological results showed that EPS could repair structural damage in these immune organs and increased the number of colonic stainable goblet cells. In particular, thymic index was significantly restored in mice after EPS intervention. TNF-α is a pleiotropic pro-inflammatory cytokine involved in activating innate and adaptive immunity. C3 is synthesized mainly by liver and macrophages and is involved in various adaptive immune responses. The monosaccharide composition of EPS is dominated by galactose and arabinose, which together account for more than 60% of the monomer content. This type of polysaccharide is known as arabinogalactans, and is associated with good activity in the complement system (Togola et al., 2008). This property is not possessed by most lentinan polysaccharide (Wang et al., 2020). The results showed that EPS significantly increased serum TNF-α levels and liver C3 expression. IgA, IgG, and IgM can reflect the humoral immune function of the animal body, and the results indicated that EPS stimulated the secretion of the above antibodies in immunosuppressed mice.

EPS improved the intestinal chemical and immune barriers of immunosuppressed mice by increasing the secretion of MUC2 by intestinal goblet cells, improving the expression of tricellular tight junction proteins, and restoring the levels of sIgA, an important immune barrier component. IFN-γ, IL-10, TGF-β3, and IL-17 are cytokines secreted by T-helper cells Th1, Th2, Treg, and Th17, respectively, which help balance the immune response of the body. Under conditions of immunocompromise, the ability of helper T cells to proliferate, disseminate, and activate other immune cells responsible for direct immune responses is diminished (Duffy et al., 2011). The results showed that EPS effectively stimulated the release of immune factors, enhancing the intestinal immune barrier. Additionally, EPS showed better recovery in some indices compared to the reference dose of lentinan polysaccharide. Together, these results suggest that EPS has the activity to repair the intestinal barrier in immunosuppressed mice. It is necessary to mention that, being an AG-I, it can also demonstrate notable immunomodulatory effects by stimulating the proliferation of human peripheral blood mononuclear cells and enhancing their production of interleukin (Yin et al., 2012).

The immune system interacts extensively with the intestinal microbiota (Wastyk et al., 2021). Following immunocompromise, the body typically exhibits dysbiosis of the gut microbiota. A similar phenomenon was observed in this study following modeling with CTX (Li et al., 2021). However, EPS was able to improve the

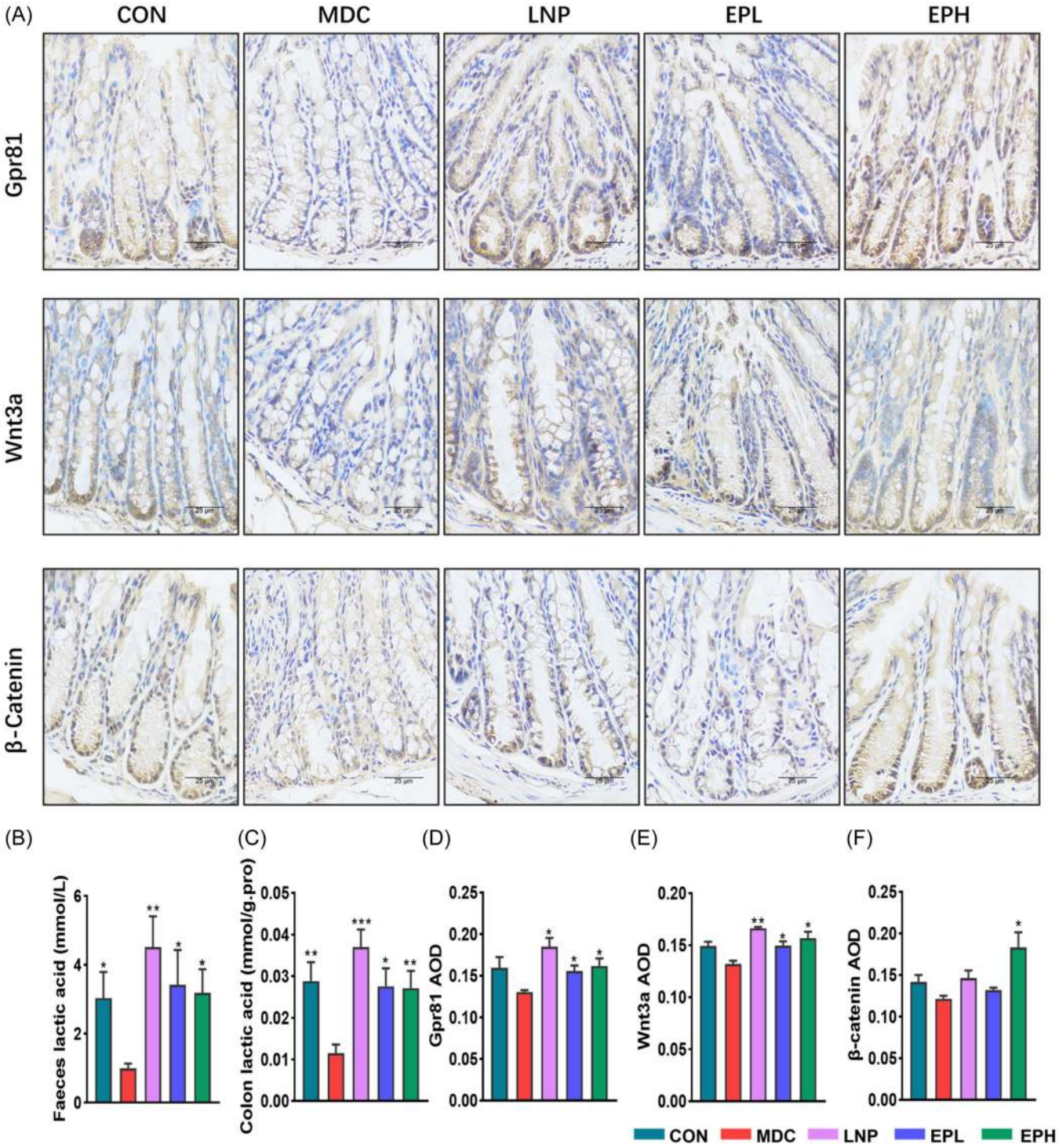

FIGURE 7 EPS intervention promoted the expression of Gpr81, Wnt3a and β-catenin at the bottom of colonic crypts. Immunohistochemical staining of (A) Gpr81, Wnt3a and β-catenin in colon tissue (200×). (B) Lactic acid content of faeces and (C) colon. Relative expression of (D) Gpr81, (E) Wnt3a and (F) β-catenin, expressed as AOD values. Data are expressed as mean ± SEM, $n = 5$. *$p < 0.05$, **$p < 0.01$, vs the MDC group.

composition of the intestinal microbiota after EPS intervention, the α-diversity and β-diversity of gut microbiota in mice improved and were similar to the normal group, but different from the model group. The α-diversity of gut microbiota in mice after EPS intervention had a large variation within the group, but this was slightly improved in the group that received lentinan polysaccharide intervention. We think that the variation in the interaction between the intestines of different mice and EPS with large molecular weights was the reason for this difference. A diverse network of microbial members is a characteristic of a healthy gut microbiota (Van den

Abbeele et al., 2013). The EPS intervention increased the interaction of the main intestinal genera. At the family level, the effect of CTX led to a decrease in the abundance of *Erysipelotrichaceae* and *Lactobacillaceae* in mice. At the genus level, the action of CTX resulted in decreased abundance of mice *Lactobacillus*. *Erysipelotrichaceae* and *Lactobacillus* have been reported to regulate immunity in a specific manner (Kotani et al., 2014; Miyauchi et al., 2020), and EPS intervention resulted in increased abundance of both. In addition, EPS reduced specific genus, such as *Butyricimonas*, which is negatively associated with intestinal immune factors and antimicrobial peptides (Xia et al., 2021), *Tyzzerella*, which is negatively associated with IFN-γ, IL-4 and IgG (Huang et al., 2021), *Family_XIII_AD3011_group*, which is negatively associated with 5-hydroxytryptamine (Li et al., 2022), and *Enterococcus* involved in the impairment of epithelial barrier integrity (Steck et al., 2011). These results suggest that EPS helped to restore gut microbiota and enhance the intestinal biological barrier in immunosuppressed mice.

Further prediction of intestinal function showed that CTX-induced immunosuppressed mice had a reduced abundance of both homo- and hetero-lactic fermentation pathways. The total lactic fermentation abundance statistics revealed that the mice in the model group had a significantly lower abundance compared to those after lentinan polysaccharide and EPS intervention. This finding is consistent with a significant decrease in the abundance of *Lactobacillus* in the model group, which is similar to the discoveries of Florez and Ying et al. (Florez et al., 2016; Ying et al., 2020). Low doses of EPS significantly increased the abundance of intestinal Leloir pathway in mice, which was not observed in lentinan polysaccharide or high doses of EPS. This could be due to the fact that moderate amounts of galactose in EPS are more beneficial for gut microbiota to produce lactic acid via fermentation in the Leloir pathway. Mammalian digestive enzymes are unable to digest most complex carbohydrates and plant polysaccharides, which are metabolized by SCFAs-producing microorganisms, which have a wide range of immunomodulatory activities, but in our study, no significant changes in the predicted abundance of SCFAs production pathways and key enzymes or related enzymes were observed (Figure 6F) (Supporting Information S1: Table. S2). Furthermore, the role of lactic acid differs from one of the SCFAs, butyric acid (differentiated colon cells metabolize butyrate, possibly preventing butyrate from reaching stem cells in the crypts, and butyrate is an effective inhibitor of intestinal stem/progenitor cell proliferation at physiological concentrations) (Kaiko et al., 2016).

Based on the increase of specific microbes (*Lactobacillus*) and predictions from 16S gene functionality, we hypothesized that EPS might enhance the levels of lactic acid in the gut. Altering lactic acid abundance may represent one of the effective mechanisms by which EPS improves intestinal barrier function through modulating the gut microbiome. Consequently, we specifically measured the lactic acid content in intestinal feces and tissues to validate this hypothesis. In this regard, the activity exhibited by the lentinan polysaccharide as a positive control was indeed noteworthy. This might have been related to the monosaccharide composition of lentinan polysaccharide and their potent overall immune-regulating effects (Motta et al., 2021; Ren et al., 2018). However, it is worth noting that EPS also produced a similar effect. Lactic acid derived from the gut microbiome can not only enhance the intestinal immune barrier (Morita et al., 2019) but also activate the expression of proteins such as Wnt3a and β-catenin at the bottom of the colonic crypts through a Gpr81-dependent mechanism, promoting intestinal epithelial development to repair intestinal damage (Lee et al., 2018). This is important for various functions including early embryonic development, self-renewal of hematopoietic stem cells, and maintaining the stability of intestinal tissues. Our results indicated that EPS intervention significantly restored the levels of lactic acid in the feces and colon of mice compared to the model group. Moreover, the expression of key proteins such as Wnt3a and β-catenin, activated by the lactic acid receptor-dependent mechanism, significantly increased at the bottom of the colonic crypts. This plays a crucial role in maintaining the stemness of Lgr5+ intestinal stem cells in the gut (Wu et al., 2020). The above results suggest that EPS may enhance the gut immune response in mice induced by CTX through the modulation of the gut microbiome.

It is necessary to mention that several studies have shown that lentinan polysaccharide can significantly alter the composition of the intestinal microbial community and significantly affect the abundance of certain microorganisms, including lactic acid-producing bacteria of the genus *Lactobacillus* and *Bifidobacterium* (Wang, Chen et al., 2018; Wang et al., 2019), and promote an increase in intestinal fecal lactic acid content (Wang, Zhang, et al., 2018). These studies show that EPS exhibited similar activity to that of lentinan polysaccharides, but showed better improvement than the reference dose of lentinan polysaccharide in some other organismal immune indicators, such as C3, MUC2, and cytokines of colonic T-helper cells. The dose difference may be one of the reasons, and it may also be related to the large molecule polysaccharides to increase the volume and viscosity of intestinal contents and stimulate intestinal peristalsis, which can affect the activity and distribution of immune cells in the intestine and thus the function of the intestinal immune system. In the future, large polysaccharides, represented by EPS, might also be used due to other characteristics besides immunity such as food thickeners, drug carriers, humectants, gelling agents and adhesives, etc. Therefore, further research and development of EPS will help to realize its wider application prospects.

5 | CONCLUSION

The present study showed that EPS, extracted from EOH and belonging to AG-I, has a significant immune-enhancing effect by stimulating the expression of the immune factors while mitigating the damage of immune organs. The intestinal immune barrier was also repaired by EPS, not only by regulating the expression of key immune factors in the intestine but also by improving the diversity and overall structure of the intestinal microbiota. Moreover, EPS may also promote the repair of the intestinal physical barrier by activating the expression of proteins such as Wnt3a and β-catenin at the bottom of the colonic crypts through a Gpr81-dependent mechanism. In conclusion, this study highlights the potential of EPS as a novel ingredient for the development of immune-modulatory nutraceutical products.

AUTHOR CONTRIBUTIONS

Jin Dai: Data curation; Writing—original draft; Writing—review and editing. **Zhiwei Zhou**: Data curation; Formal analysis; Writing—original draft; Writing—review and editing. **Liangwei Chen**: Data curation; Writing—original draft. **Shujuan Cao**: Investigation. **Ke Luo**: Investigation. **Jinmei Zhang**: Investigation. **András Dinnyés**: Supervision. **Dan Wang**: Conceptualization; Project administration; Writing—original draft. **Qun Sun**: Conceptualization; Project administration; Writing—review and editing.

ACKNOWLEDGEMENTS

This research has received support from the National Key Research and Development Projects (2019YFE0103800), the Key R&D projects of the Science and Technology Department of Sichuan Province (2021YFN0092), the Sichuan Science and Technology Program (2021ZHFP0045, 2022YFQ0100), and the Fundamental Research Funds for the Central Universities (2021CDLZ-18). Additionally, András Dinnyés has been granted funding from the Hungarian National Research, Development, and Innovation Fund for project No.2020-1.1.5-GYORSÍTÓSÁV.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENT

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Chengdu Medical College, and the experiments were approved by the Animal Ethics Committee of Chengdu Medical College (CMC), with ethical approval number CMC Animal Ethics [2022] No.048.

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How to cite this article: Dai, J., Zhou, Z., Chen, L., Cao, S., Luo, K., Zhang, J., Dinnyés, A., Wang, D., & Sun, Q. (2024). A novel arabinogalactan extracted from *Epiphyllum Oxypetalum (DC.) Haw* improves the immunity and gut microbiota in cyclophosphamide-induced immunosuppressed mice. *eFood*, *5*(5), e70002. https://doi.org/10.1002/efd2.70002