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RESEARCH ARTICLE

Hawthorn Polysaccharide Alleviates Cyclophosphamide-Induced Immunosuppression and Intestinal Mucosal Injury in Mice

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ABSTRACT

Immunosuppression and intestinal mucosal injury are relevant concerns in animal health. Plant-derived polysaccharides have attracted interest as potential immunomodulators, although evidence from experimental models requires further evaluation before veterinary application. This study evaluated the effects of hawthorn polysaccharide (HP) on cyclophosphamide (Cy)-induced immunosuppression and intestinal mucosal injury in mice. The results showed that HP alleviated Cy-induced systemic immunosuppression and intestinal mucosal injury, as reflected by improvements in immune organ indices, hematological parameters, mucosal morphology, goblet cell abundance, inflammatory and mucosal immune parameters, and barrier-related proteins. High-dose HP was also associated with changes in Cy-related intestinal transcriptional profiles, particularly those involving cell-cycle- and proliferation-related processes, together with alterations in gut microbial composition. The associations among bacterial taxa, representative genes, and barrier indicators suggest that these responses occurred in parallel during HP intervention. Although the underlying causal relationships require further verification, the findings support further study of HP as a potential immunomodulator, with its relevance to veterinary use requiring evaluation in the intended animal species.

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INTRODUCTION

Immunosuppression is an important health concern in animals because persistent impairment of immune competence can increase susceptibility to infectious and environmental challenges and compromise disease resistance. In livestock and poultry, immune dysfunction may arise from infection, nutritional imbalance, environmental stress and intensive production conditions, ultimately affecting animal health and productive performance (Niu *et al.*, 2022; Zheng *et al.*, 2022; Wang *et al.*, 2023). Moreover, prolonged stress and immune disturbance can adversely affect gastrointestinal integrity, further weakening the defensive capacity of the host (Sundaram *et al.*, 2022). Although vaccination, antimicrobial therapy and nutritional intervention remain important components of disease prevention and control, these approaches do not necessarily restore an already compromised immune system. Consequently, increasing attention has been directed toward natural immunomodulatory substances that may support immune recovery and maintain animal health.

Among the natural compounds investigated for immune regulation, plant-derived polysaccharides have

received increasing attention because of their broad immunomodulatory activities. Their biological effects are not determined by botanical origin alone, as chain conformation and higher-order structural organization can substantially influence the physicochemical properties and bioactivities of polysaccharides (Meng *et al*., 2026). Evidence from animal studies indicates that these polysaccharides can regulate immune organ development, immune cell activity and cytokine responses. Dietary glycans have also been associated with changes in gut microbial composition, intestinal barrier integrity and immune regulation, further indicating that their biological effects extend beyond systemic immune responses (Chinta *et al.*, 2025). These properties have prompted their investigation as potential natural immunomodulators for supporting animal health (Zhao *et al.*, 2023a). In experimental immunosuppression models, Astragalus polysaccharides improved immune organ indices and immune responses in cyclophosphamide (Cy)-treated mice, accompanied by marked modulation of the gut microbiota (Li *et al.*, 2023). Similar improvements in immune function and microbial composition have been reported for polysaccharides derived from *Holothuria leucospilota* (Zhao *et al.*, 2023a) and *Paecilomyces cicadae* (Tian *et al.*, 2023b). Beyond Cy-induced immunosuppression, orally administered plant polysaccharides have also shown protective effects in other chemically induced tissue-injury models in mice (Zohaib *et al.*, 2026). Collectively, these findings support plant-derived polysaccharides as promising candidates for the regulation of impaired immune function and provide a rationale for exploring additional polysaccharide sources with immunomodulatory potential.

Hawthorn (*Crataegus pinnatifida* Bunge) is a medicinal and edible plant rich in structurally diverse bioactive compounds. Among them, hawthorn polysaccharides (HP) have received increasing attention because of their immunomodulatory, anti-inflammatory and gut microbiota-regulatory activities (Jing *et al.*, 2023a; Sun *et al.*, 2024). Experimental evidence has further demonstrated that a heteropolysaccharide isolated from *C. pinnatifida* fruit restored body weight and immune organ indices and regulated cytokine secretion in immunocompromised mice, accompanied by changes in gut microbial diversity and composition (Jing *et al.*, 2023b). The intestinal regulatory potential of HP has also been demonstrated in experimental intestinal injury, where HP alleviated colonic pathological damage and inflammatory responses while reshaping the gut microbial community and its metabolic products (Guo *et al.*, 2021). More recent evidence further indicates that *C. pinnatifida* polysaccharides can preserve mucus and epithelial barrier integrity and regulate intestinal microbial composition (Wei *et al.*, 2025) Collectively, these findings suggest that HP may exert biological effects through coordinated regulation of immune responses and the intestinal environment. However, the relationship between HP-mediated immune recovery and intestinal mucosal homeostasis under Cy-induced immunosuppression remains insufficiently understood.

Increasing evidence indicates that intestinal dysfunction is an important component of Cy-induced immunosuppression. In addition to systemic immune impairment, Cy exposure can disrupt intestinal morphology, mucosal immune responses and epithelial barrier integrity, thereby weakening intestinal defense. Notably, recovery of immune function following polysaccharide intervention is frequently accompanied by improvement of the intestinal mucosal environment (Tian *et al.*, 2023a; Huang *et al.*, 2025). *Rehmannia glutinosa* polysaccharides alleviated immune organ atrophy and hematological abnormalities while improving goblet cell abundance, tight-junction protein expression, mucosal immune responses and gut microbial homeostasis in Cy-treated mice (Yu *et al.*, 2025). Similarly, Jiawei Yupingfeng polysaccharides enhanced intestinal immune responses and barrier-associated protein expression and regulated the intestinal microbiota (Wu *et al.*, 2025). These findings suggest that intestinal mucosal homeostasis is closely associated with immune recovery under immunosuppressive conditions. However, the coordinated relationships among HP-mediated immune recovery, intestinal mucosal improvement, host transcriptional responses and microbial alterations remain poorly defined.

Therefore, the present study investigated the protective effects of HP against Cy-induced immunosuppression in mice, with particular emphasis on the relationship between immune recovery and intestinal mucosal homeostasis. Systemic immune status was evaluated together with intestinal morphology, mucosal immune responses and barrier-related protein expression. Transcriptomic and 16S rRNA gene sequencing were further employed to characterize intestinal host responses and gut microbial alterations, respectively. Correlation analyses were subsequently performed to explore the associations among representative microbial taxa, key intestinal genes and barrier-related indices. By integrating systemic immune parameters with intestinal phenotypes and multi-omics analyses, this study aimed to provide a more comprehensive understanding of the immunomodulatory effects of HP and experimental evidence for its potential application as a natural immunomodulator in animal health.

MATERIALS AND METHODS

Hawthorn (*Crataegus pinnatifida* Bunge) fruits were purchased from Beijing Tongrentang, China. Cy and astragalus polysaccharide (APS) were obtained from Beijing Solarbio, China. Other reagents were of analytical grade.

Preparation and characterization of hawthorn polysaccharide: The HP used in this study was the preparation previously characterized by Zhang *et al*. (2025). Dried fruits were powdered and extracted with hot distilled water. The extract was concentrated and precipitated with ethanol. The precipitate was redissolved, deproteinized by the Sevag method, dialyzed to remove low-molecular-weight components, and purified chromatographically before being concentrated, freeze-dried, and stored at −20°C. The previously reported weight-average molecular weight was 168.47 kDa, with galacturonic acid (85.10%), mannose (4.506%), and

galactose (3.983%) as the predominant monosaccharides. Methylation and NMR analyses indicated mainly homogalacturonan regions with minor rhamnogalacturonan-I regions.

Animals and experimental design: Male C57BL/6 mice (6-8 weeks, 18-22 g) were obtained from SPF (Beijing) Biotechnology Co., Ltd and acclimated for one week under specific pathogen-free conditions. Mice were randomly divided into six groups (n=12/group): control (Con), cyclophosphamide (Cy), positive control (PC), low-dose HP (HPL), medium-dose HP (HPM), and high-dose HP (HPH).

For 14 days, mice in the PC group received APS (400 mg/kg), while the HPL, HPM, and HPH groups received HP at 100, 200, and 400 mg/kg/day, respectively, by oral gavage. Con and Cy mice received an equal volume of distilled water. Immunosuppression was subsequently induced by Cy (80 mg/kg/day, i.p.) for three consecutive days in all groups except Con, which received sterile saline. Oral treatment was discontinued during Cy administration. Mice were euthanized 24 h after the final injection following overnight fasting, and blood, spleen, thymus, colon, ileum and fecal samples were collected. Body weight was recorded for all 12 mice per group. Other analyses used randomly selected, overlapping subsets of animals, with assay-specific sample sizes indicated in the figure legends. The protocol was approved by the Institutional Animal Care and Use Committee of Yunnan Agricultural University.

Immune organ indices and hematological analysis: Spleen and thymus indices were calculated as organ weight (mg)/body weight (g). WBC, LYM, RBC, HGB, and PLT were determined using an automated hematology analyzer.

Determination of intestinal immune and inflammatory parameters： IL-1β, IL-6, TNF-α, and IL-10 were measured in serum and ileal tissue homogenates. Secretory immunoglobulin A (sIgA) was determined in ileal lavage fluid, while calprotectin was measured in fecal samples. Commercial ELISA kits were used according to the manufacturers' instructions.

Histopathological examination: Colon tissues were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E). Histopathological injury was scored based on epithelial damage, crypt alterations, and inflammatory cell infiltration. Alcian blue-periodic acid-Schiff (AB-PAS) staining was used to visualize mucus-producing goblet cells, and PAS-positive goblet cells were quantified in randomly selected colonic crypts.

Immunohistochemical analysis: Colon sections were immunoassayed for MUC2, ZO-1, Claudin-1, and Occludin. Staining was quantified using ImageJ software.

16S rRNA gene sequencing and gut microbiota analysis: Fecal samples from the Con, Cy, and HPH groups were used for microbial DNA extraction and V3–V4 16S rRNA gene sequencing. After quality control and taxonomic annotation, sequencing coverage, Chao1, Shannon and Simpson indices, principal coordinate analysis (PCoA) and bacterial composition at the phylum and genus levels were analyzed.

RNA sequencing and bioinformatics analysis: Ileal tissues from the Con, Cy, and HPH groups were subjected to RNA sequencing. After quality control and gene expression quantification, principal component analysis (PCA) was performed. Differentially expressed genes (DEGs) were identified for Cy versus Con and HPH versus Cy using thresholds of adjusted $P<0.05$ and |log2 fold change|>1.

Statistical analysis: Data are presented as mean ± SD. Differences among groups were analyzed by one-way ANOVA followed by Tukey's multiple-comparison test. Correlations with $|r| >0.6$ and $P <0.05$ were considered significant. Statistical analyses and data visualization were performed using GraphPad Prism 10.0 and R software (version 4.3.2). The value of $P<0.05$ was considered statistically significant.

RESULTS

HP alleviates Cy-induced systemic immunosuppression in mice: The experimental design is shown in Fig. 1A. Cy administration caused a marked loss of body weight compared with the Con group ($P<0.05$; Fig. 1B). HP supplementation attenuated this reduction to varying degrees, with greater improvement observed in the HPM and HPH groups. Cy treatment also significantly reduced the thymus and spleen indices compared with the Con group ($P<0.05$; Fig. 1C and D). These reductions were partially reversed by HP supplementation, and the HPH group showed the greatest improvement among the three HP-treated groups.

Consistent with the changes in immune organs, Cy markedly decreased WBC, LYM, RBC, HGB, and PLT levels compared with the Con group ($P<0.05$; Fig. 1E–I). HP administration improved these hematological parameters to different extents. WBC and LYM showed a more evident increase in the HPH group, while RBC, HGB and PLT were also increased following HP supplementation (Fig. 1E–I). Overall, these changes showed that HP attenuated the systemic immunosuppressive phenotype induced by Cy.

HP attenuates Cy-induced colonic mucosal injury: Histological examination revealed marked colonic mucosal injury following Cy administration (Fig. 2A). Compared with the Con group, Cy-treated mice showed disruption of mucosal architecture, crypt abnormalities, and increased inflammatory cell infiltration, accompanied by a higher histopathological injury score ($P<0.05$; Fig. 2C). These alterations were alleviated after HP supplementation, as reflected by better preservation of mucosal and crypt structure and reduced inflammatory infiltration. Histopathological scores were correspondingly decreased in the HP-treated groups, with the HPH group showing the greatest improvement.

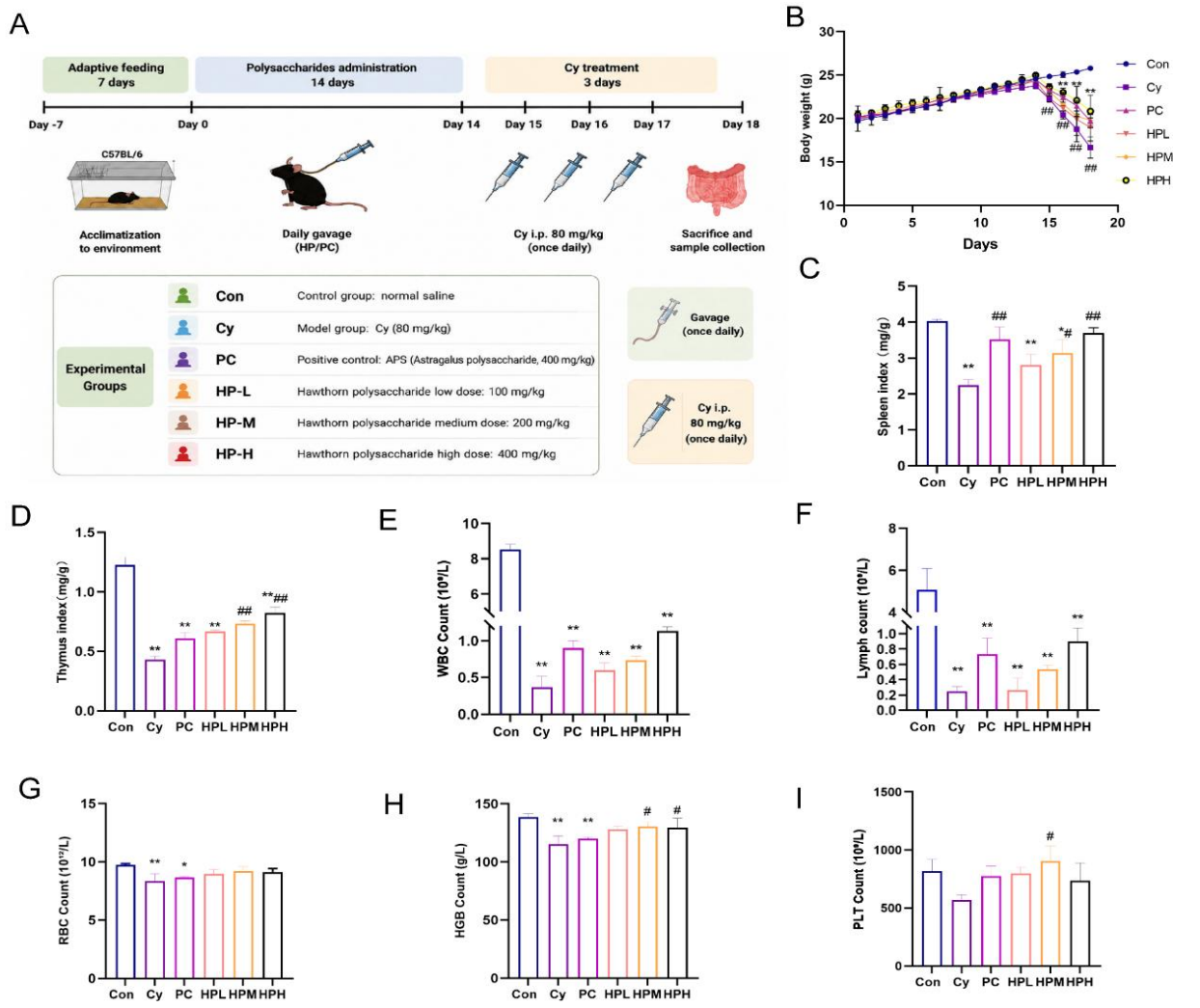


Fig. 1: HP alleviates Cy-induced immunosuppression by improving physiological and hematological parameters. (A) Experimental design of Cy-induced immunosuppressive mouse model and HP intervention. (B) Body weight changes. (C) Spleen index. (D)Thymus index. (B-D, n= 12 per group). (E–I) Hematological parameters, including WBC, LYM, RBC, HGB, and PLT (n=3 per group). Note: Data are presented as mean ± SD. **P*<0.05, ***P*<0.01 compared with the Con group; #*P*<0.05, ##*P*<0.01 compared with the Cy group.

AB-PAS staining further showed a reduction in PAS-positive goblet cells in the Cy group compared with the Con group (Fig. 2B&D). HP supplementation partially restored goblet cell abundance, and the increase was more evident in the HPH group. Together, the histological and AB-PAS findings showed that HP attenuated Cy-induced colonic mucosal damage and loss of goblet cells.

HP improves Cy-induced intestinal barrier-related protein alterations in mice: Immunohistochemical analysis showed reduced MUC2, ZO-1, Claudin-1, and Occludin expression in the colon following Cy treatment (Fig. 3A–E). HP supplementation partially counteracted these changes, although the response varied among the four proteins. MUC2 expression increased in the HPM and HPH groups, with the HPH group showing values closer to those of the Con group (Fig. 3B). ZO-1 showed a modest increase after HP treatment but remained below the Con level (Fig. 3C).

A clearer response was observed for Claudin-1 and Occludin. Claudin-1 expression was higher in the PC, HPM, and HPH groups than in the Cy group (*P*<0.05 or *P*<0.01; Fig. 3D), while Occludin was significantly increased in the HPH group (*P*<0.05; Fig. 3E). Thus, the changes in barrier-related proteins were not uniform across HP doses, but the overall response was more evident in the HPH group.

HP modulates inflammatory responses and mucosal immune parameters in Cy-treated mice: Cy treatment altered the inflammatory profile in both serum and ileal tissues, with increased IL-1β, IL-6, and TNF-α and reduced IL-10 compared with the Con group (Fig. 4A-H). HP supplementation moderated these changes to varying degrees. The three pro-inflammatory cytokines declined after HP treatment, whereas IL-10 showed an opposite pattern. Among the HP groups, the changes were generally more evident in HPH.

Mucosal immune-related parameters showed a similar response. Cy exposure reduced sIgA in ileal lavage fluid and increased fecal calprotectin (*P*<0.05; Fig. 4I and J). HP treatment increased sIgA while lowering calprotectin relative to the Cy group, with the HPH group showing the greatest overall change. These results showed that the improvement in intestinal morphology and barrier-related proteins was accompanied by changes in local inflammatory and mucosal immune parameters.

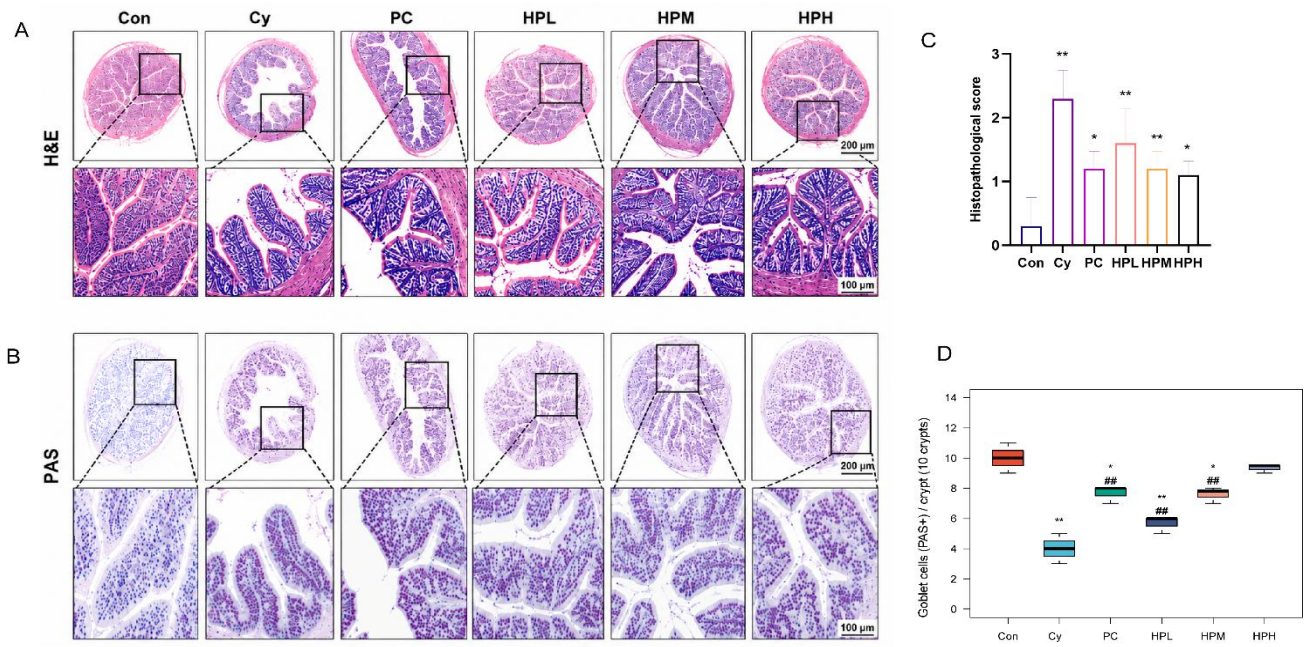


Fig. 2: HP attenuates Cy-induced colonic mucosal injury and preserves goblet cell function in mice. (A) Representative images of H&E-stained colon sections showing mucosal architecture, inflammatory cell infiltration, and crypt morphology (scale bars=200μm and 100μm). (B) Representative images of AB-PAS-stained colon sections showing mucus-producing and PAS-positive goblet cells (scale bars=200μm and 100μm). (C) Histopathological injury scores of colon tissues (n=3 per group). (D) Quantification of PAS-positive goblet cells per crypt (n=5 per group). Data are presented as mean ± SD.*P<0.05, **P<0.01 compared with the Con group; #P<0.05, ##P<0.01 compared with the Cy group.

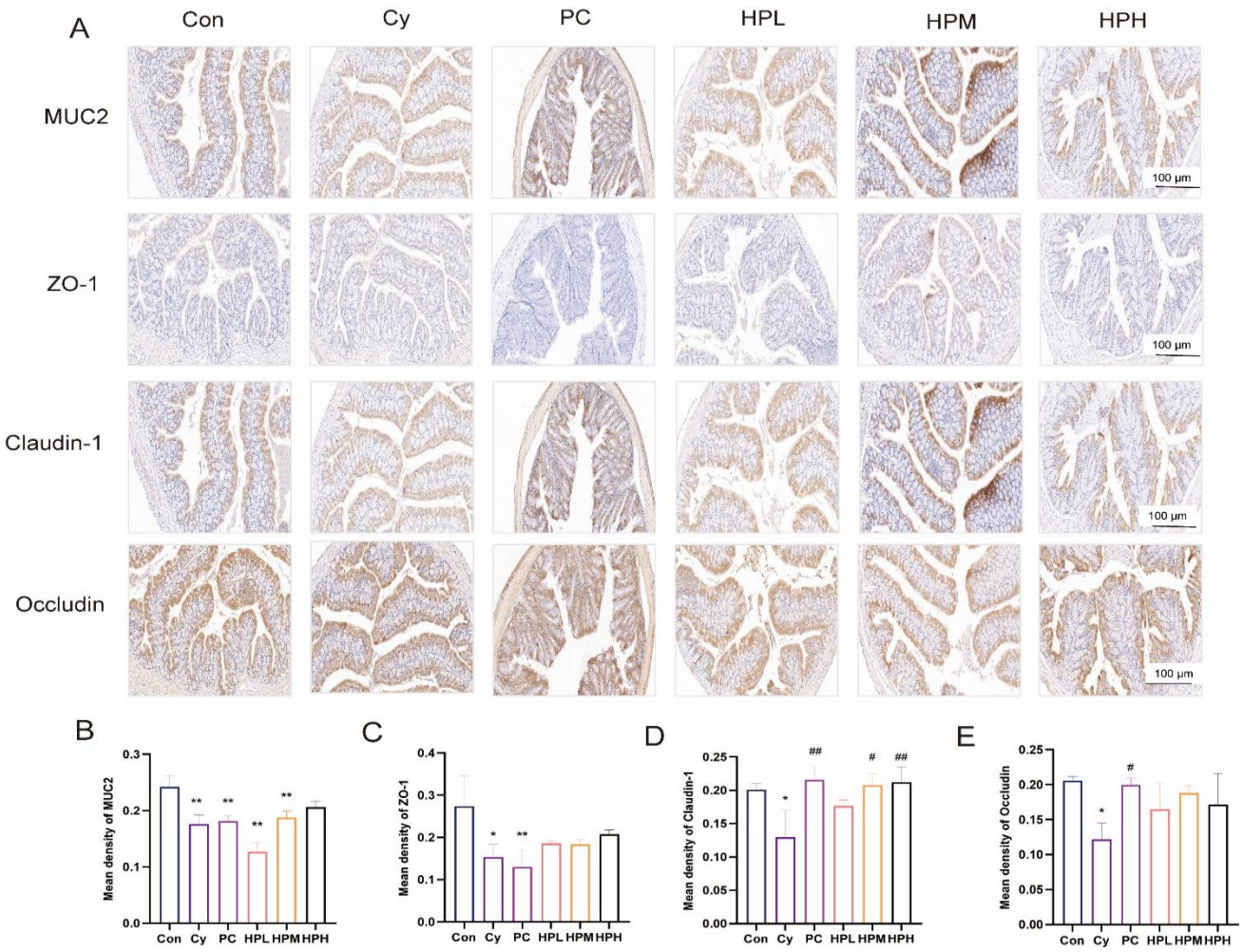


Fig. 3: HP improves Cy-induced intestinal barrier-related protein alterations in mice. (A) Representative immunohistochemical staining images of MUC2, ZO-1, Claudin-1, and Occludin in colon tissues (scale bar=100μm). (B–E) Quantitative analysis of MUC2, ZO-1, Claudin-1, and Occludin expression levels based on mean density analysis (n=3 per group). Data are presented as mean ± SD.*P<0.05, **P<0.01 compared with the Con group; #P<0.05, ##P<0.01 compared with the Cy group.

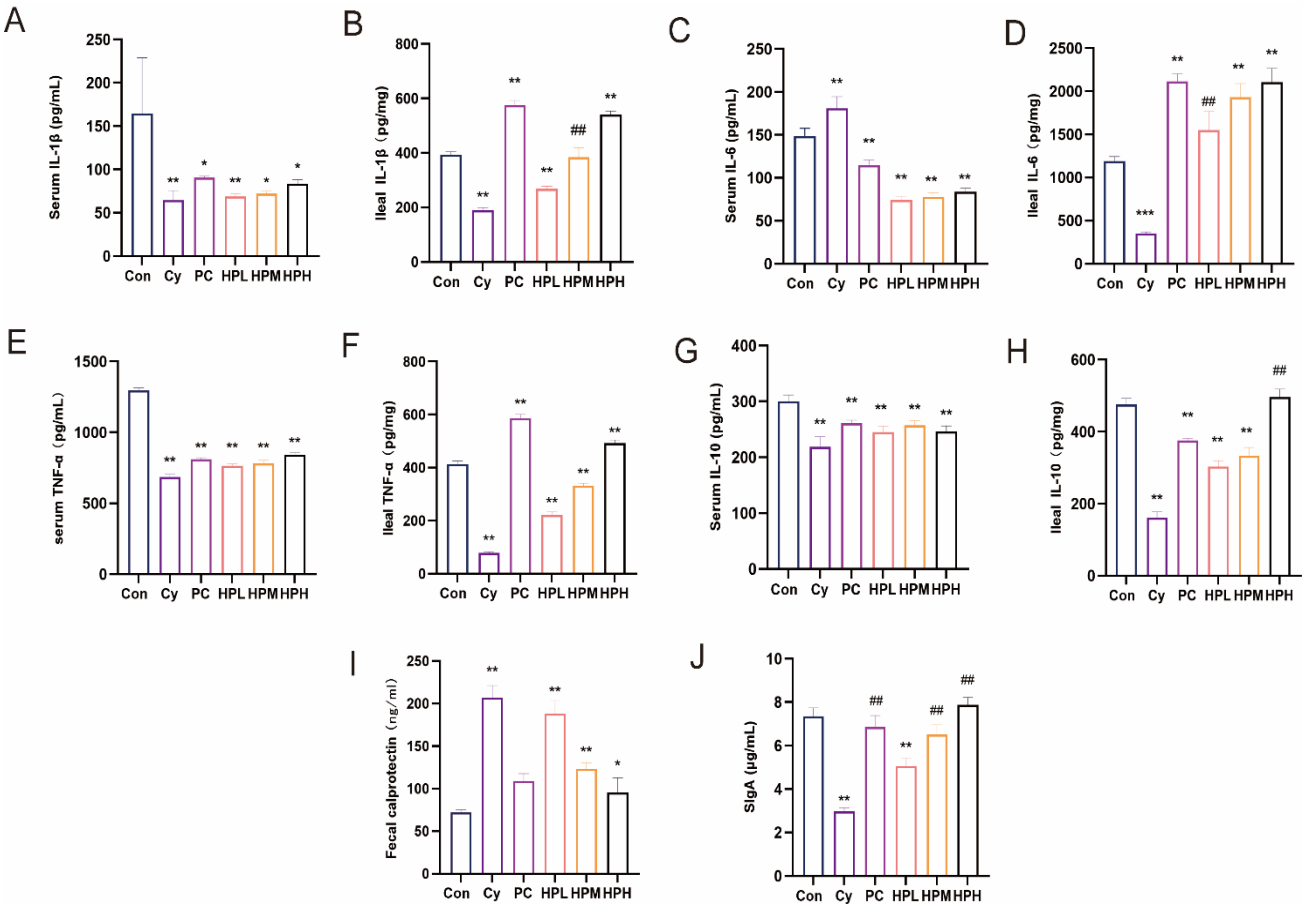


Fig. 4: HP alleviates Cy-induced intestinal inflammatory responses and improves mucosal immune status in mice. (A–H) Levels of IL-1β, IL-6, TNF-α, and IL-10 in serum and intestinal tissues determined by ELISA. (I) Calprotectin levels in fecal samples. (J) sIgA levels in intestinal tissues. Data are presented as mean ± SD.*P<0.05, **P<0.01 compared with the Con group; #P<0.05, ##P<0.01 compared with the Cy group (n=6 per group).

HP affected gut microbial richness and community composition: To characterize the gut microbial response to HP, fecal samples from the Con, Cy, and HPH groups were subjected to 16S rRNA gene sequencing. Venn analysis identified 1,767 OTUs across the three groups, of which 626 were shared. The Con, Cy, and HPH groups contained 191, 268 and 230 unique OTUs, respectively (Fig. 5A). The Chao1 index was higher in the Cy group than in the Con group ($P < 0.01$), whereas HPH treatment reduced this index relative to the Cy group ($P<0.05$; Fig. 5B). The Shannon index also increased following Cy exposure ($P<0.05$), but no significant difference was detected between the Cy and HPH groups (Fig. 5C). The Simpson index remained comparable among the three groups (Fig. 5D). Thus, HPH partially restored the Cy-associated alteration in microbial richness, while its effect on overall community diversity was less apparent.

PCoA showed partially distinguishable but overlapping distributions among the three groups. PC1 and PC2 explained 19.72 and 14.53% of the total variation, respectively (Fig. 5E). At the phylum level, *Bacillota* and *Bacteroidota* dominated the microbial communities across all groups. Cy treatment altered their relative proportions, whereas the phylum-level profile of the HPH group tended to shift toward that of the Con group (Fig. 5F). At the genus level, the Lachnospiraceae NK4A136 group and unclassified Muribaculaceae accounted for substantial proportions of the microbial community, and their relative distributions varied among the Con, Cy, and HPH groups (Fig. 5G). Taken together, these results indicate that HPH influenced microbial richness and community composition in Cy-treated mice, although the extent of recovery differed among individual diversity measures.

HPH reverses Cy-associated intestinal transcriptional alterations: RNA sequencing revealed distinct intestinal transcriptional profiles among the Con, Cy and HPH groups (Fig. 6). Differential expression analysis identified extensive transcriptional alterations in both the Cy versus Con and HPH versus Cy comparisons (Fig. 6A). PCA showed distinguishable but partially overlapping distributions among the three groups, indicating that Cy exposure and HPH supplementation were accompanied by broad changes in intestinal gene expression (Fig. 6B).

Further comparison identified 346 overlapping DEGs between the Cy versus Con and HPH versus Cy comparisons (Fig. 6C). GO enrichment analysis linked these genes mainly to cell-cycle regulation, cell proliferation, chromosome organization, and DNA replication (Fig. 6D). KEGG analysis further identified enrichment of pathways associated with cell-cycle and proliferation-related processes (Fig. 6E). PPI analysis identified several highly connected genes, including *Cdk1, Ccna2, Plk1, Bub1, Cdc20, Aurka, Cenpf*, and *Ccnb1* (Fig. 6F). Overall, the intestinal transcriptional response associated with HPH treatment was characterized predominantly by changes in genes involved in proliferative and cell-cycle-related processes.

Correlation analysis links gut microbial changes with host transcriptional and intestinal barrier responses: Spearman correlation analysis revealed significant associations between bacterial genera and representative intestinal genes, barrier-related proteins, and mucosal immune and inflammatory parameters (Fig. 7). Dubosiella was negatively correlated with *Mical2*, *Birc5*, and *Tspan8* expression and with MUC2 and Occludin measurements, whereas Escherichia–Shigella was positively correlated with ZO-1. Positive correlations were also observed between Ligilactobacillus and sIgA, IL-1β, and IL-6, and between Roseburia and sIgA. The direction of these associations varied among bacterial genera and host measurements. These findings indicate coordinated variation among gut microbial composition, intestinal transcriptional responses, and barrier-associated parameters following Cy exposure and HPH supplementation.

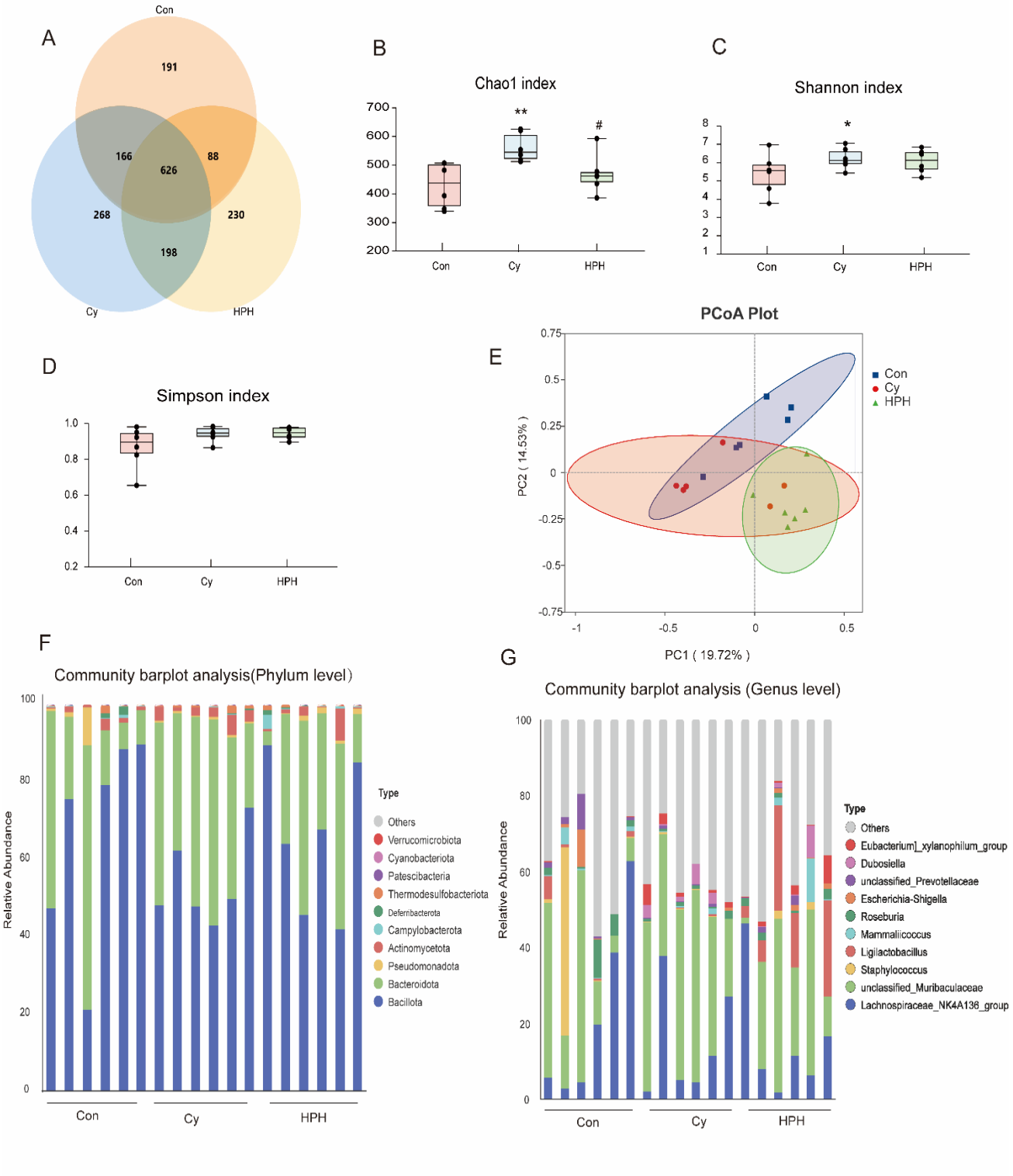


Fig. 5: HPH modulates Cy-induced gut microbiota dysbiosis in mice. (A) Venn diagram showing shared and unique OTUs among the Con, Cy, and HPH groups. (B–D) Alpha diversity analysis based on Chao1, Shannon, and Simpson indices. (E) Principal coordinate analysis (PCoA) of gut microbial community structures. (F) Relative abundance of gut microbiota at the phylum level. (G) Relative abundance of gut microbiota at the genus level. Data are presented as mean ± SD.*P<0.05, **P<0.01 compared with the Con group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared with the Cy group (n=6 per group).

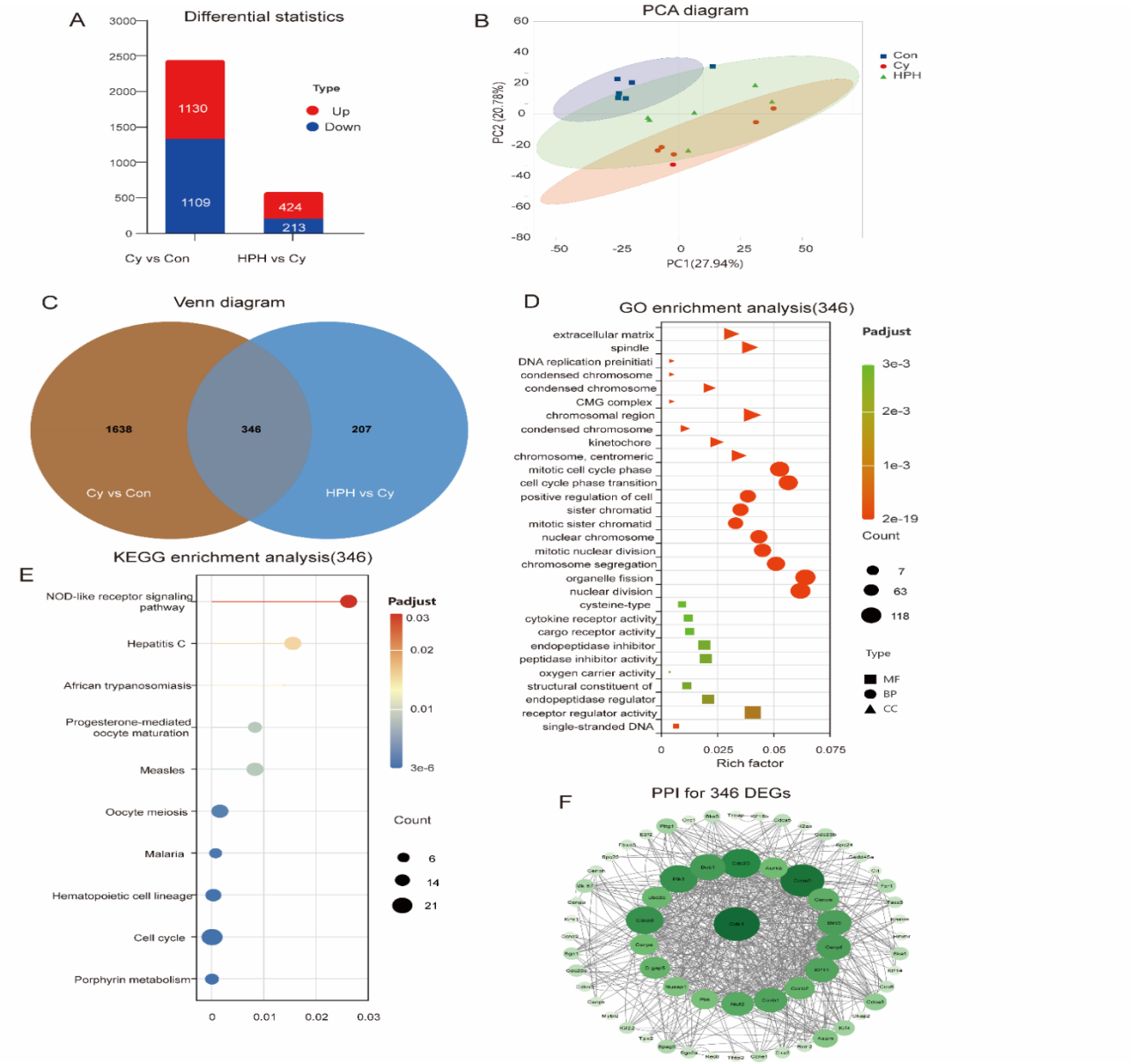


Fig. 6: Transcriptomic analysis reveals HPH-associated reversal of Cy-induced intestinal gene expression alterations. (A) Number of differentially expressed genes identified between the Cy vs. Con and HPH vs. Cy comparisons. (B) Principal component analysis (PCA) of intestinal transcriptomic profiles among the Con, Cy, and HPH groups. (C) Venn diagram showing overlapping reversal-associated differentially expressed genes between Cy-induced alterations and HPH-mediated responses. (D) Gene Ontology (GO) enrichment analysis of reversal-associated genes. (E) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of reversal-associated genes. (F) Protein–protein interaction (PPI) network analysis of reversal-associated differentially expressed genes and identification of hub genes.

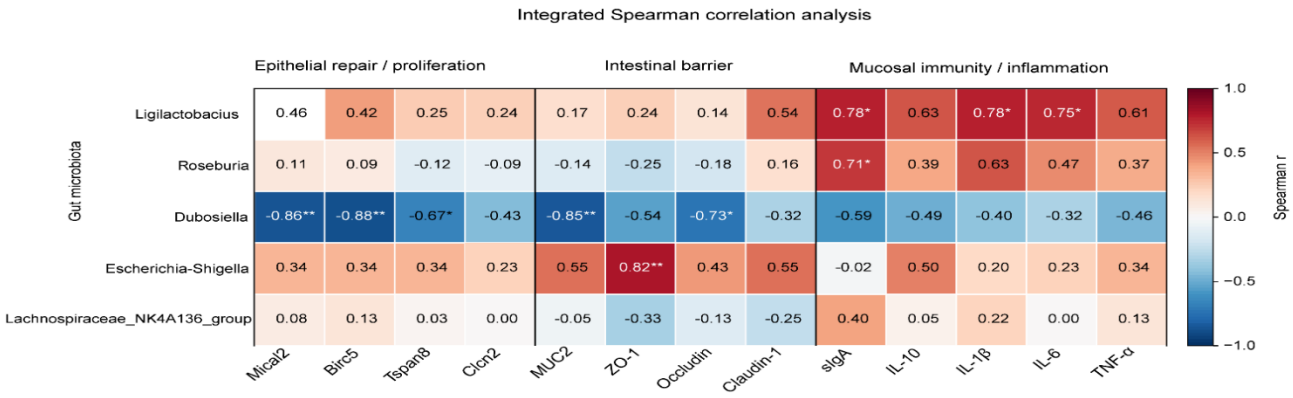


Fig. 7: Correlation analysis of gut microbiota, host transcriptional responses, and intestinal barrier-related protein expression following HPH intervention. Spearman correlation heatmap showing the relationships between differential bacterial genera, reversal-associated representative intestinal genes, and barrier-related protein measurements. The correlation coefficients (r) are represented by color intensity, with red indicating positive correlations and blue indicating negative correlations. Significant correlations were defined as $|r| > 0.6$ and $P<0.05$. Spearman correlations were calculated using pooled data from the Con, Cy and HPH groups (total n=9; 3 mice per group).

DISCUSSION

The results of this study found that cyclophosphamide-induced immunosuppression is not only manifested by impaired systemic immune function but is also accompanied by a significant disruption of intestinal homeostasis, primarily characterized by weight loss, atrophy of immune organs, abnormal hematological parameters, intestinal inflammation, mucosal damage, and alterations in barrier-related protein expression. HP intervention alleviated these abnormalities to varying degrees, with more pronounced improvements observed in the high-dose group. Furthermore, transcriptomic and gut microbiota analyses indicated that HPH treatment was associated with changes in Cy-related intestinal transcriptional profiles and gut microbial composition. The improvements in systemic immune indices and intestinal mucosal morphology were accompanied by these transcriptional and microbial changes. This combination of systemic and intestinal effects is relevant to veterinary health. In livestock and poultry, immune dysfunction may arise from infectious challenges, persistent stress, nutritional disturbances, and exposure to immunosuppressive substances, potentially weakening disease resistance and impairing productive performance (Zhao *et al.*, 2023b). Because the intestinal mucosa is continuously challenged by dietary components, microorganisms, and production-related stressors, disruption of this barrier may further intensify the consequences of impaired immune function (Yang *et al.*, 2024). The present findings therefore provide evidence from a controlled mouse model to guide further investigation of HP for immune and intestinal health in veterinary species.

Cy treatment resulted in substantial body weight loss, accompanied by decreases in thymus and spleen indices and marked alterations in hematological parameters. Among these changes, the pronounced reductions in WBC and lymphocyte counts were consistent with the hematological suppression commonly observed following Cy exposure (Xiang *et al.*, 2022). Previous studies using Cy-induced immunosuppressed mice have similarly shown that reductions in circulating immune cells and immune organ indices can be alleviated to varying degrees by bioactive polysaccharides (Fei *et al.*, 2022; Cheng *et al.*, 2021). In the present study, HP showed a similar pattern, with improvements in body weight, immune organ indices and hematological parameters, particularly at the higher dose. Despite these improvements, WBCs and lymphocyte counts did not return to the levels recorded in the Con group, showing that HP moderated, but did not fully offset, Cy-induced hematological suppression. Considered alongside the recovery of the thymus and spleen indices, these hematological changes indicate that the systemic response to HP involved more than one aspect of immune status. Studies conducted in poultry provide useful context for interpreting the veterinary relevance of these observations. *Caulis spatholobi* polysaccharide altered immune indices, mucosal barrier characteristics, and intestinal microbial composition in chickens subjected to Cy-induced immunosuppression (Cui *et al.*, 2022). A separate study in chicks found that mulberry leaf polysaccharide moderated Cy-related growth inhibition and intestinal injury while improving several immune and microbial measurements (Cheng *et al.*, 2024). The agreement between these poultry studies and the present mouse results supports further investigation of plant polysaccharides in production animals. It does not, however, establish equivalent efficacy for HP, because botanical sources, polysaccharide structure, dosage, and host species may all influence the response. Cy-induced immunosuppression is not limited to peripheral immune alterations but can also be accompanied by disruption of intestinal mucosal homeostasis (Huang *et al.*, 2021; Yu *et al.*, 2021), consistent with the simultaneous systemic and intestinal abnormalities observed in this study. Thus, the response to HP was not limited to systemic immune indices but also involved the intestinal mucosal compartment.

Histological examination confirmed that Cy caused marked intestinal mucosal injury, including disruption of mucosal architecture, crypt abnormalities, and inflammatory infiltration, whereas HP alleviated these pathological alterations. This result agrees with previous evidence that intestinal epithelial injury is an important component of Cy-associated toxicity (Wei *et al.*, 2021). AB-PAS staining further showed that Cy reduced PAS-positive goblet cells, indicating that the mucus barrier was also affected. Goblet cells and their secreted mucins form a principal component of intestinal protection, and their depletion may increase epithelial exposure to luminal microorganisms and microbial products (Gustafsson and Johansson, 2022).Correspondingly, the reductions in MUC2, ZO-1, Occludin and Claudin-1 suggested disturbances in mucus-associated and epithelial tight-junction components. Similar changes have been reported in Cy-induced intestinal injury, while polysaccharide intervention can preserve these barrier components (Chen *et al.*, 2021; Wang *et al.*, 2024). Although the magnitude of recovery varied among proteins and HP doses, the overall improvement was more evident in the HPH group. The absence of a uniform response across all barrier-related proteins indicates partial preservation of barrier-associated features rather than complete normalization of epithelial integrity. Changes in mucosal structure were accompanied by a less pronounced inflammatory profile after HP intervention, including lower IL-1β, IL-6 and TNF-α levels and an increase in IL- 10 (Li *et al.*, 2021) .HP also increased sIgA, an antibody involved in immune exclusion and the regulation of host–microbial interactions at intestinal surfaces (Huus, Petersen, and Finlay, 2021),while reducing fecal calprotectin, a marker commonly used to assess intestinal mucosal inflammation (Jukic *et al.*, 2021).The response to HP therefore encompassed mucosal architecture, barrier-related proteins, inflammatory markers, and local immune indices. Whether these changes translate into greater resistance to enteric infection remains unknown because no pathogen-challenge experiment was performed.

Gut microbial alterations provided another dimension to the intestinal response to Cy and HPH treatment. Cy increased the Chao1 and Shannon indices relative to the Con group, whereas the Simpson index remained unchanged. HPH reduced the elevated Chao1 index toward the Con level but did not significantly alter the Shannon index relative to the Cy group. Within this dataset, the microbial response to HPH was therefore more evident in

richness than in indices reflecting broader aspects of community diversity. PCoA further showed partially distinguishable but overlapping microbial distributions among the three groups. Previous studies have reported that Cy-induced immunosuppression and intestinal injury are frequently accompanied by alterations in gut microbial communities, and polysaccharide intervention can partly reshape these disturbances (Chen *et al.*, 2024; Zhu *et al.*, 2024). At the taxonomic level, *Bacillota* and *Bacteroidota* predominated across the groups, while the Lachnospiraceae NK4A136 group and unclassified Muribaculaceae accounted for substantial proportions of the genus-level community. Their relative distributions varied following Cy and HPH treatments, with the HPH profile showing a general tendency to shift toward that of the Con group. These taxonomic variations occurred alongside changes in intestinal inflammatory and barrier-related parameters. Similar associations between altered microbial profiles and inflammatory activity have been reported in patients with Crohn's disease (Hu *et al.*, 2022). However, genus-level relative abundance alone is insufficient to determine the functional contribution of these taxa. Moreover, because the present 16S analysis was observational, it cannot establish whether the identified microbial changes directly contributed to the intestinal effects of HP. The microbiota results should therefore be interpreted as coordinated variation following HPH treatment rather than evidence of a confirmed microbiota-mediated mechanism. As the microbiota analysis included only Con, Cy, and HPH, dose-dependent microbial responses to HP remain to be assessed.

Transcriptomic analysis provided additional insight into the intestinal response associated with HPH intervention. Among the DEGs detected in the Cy versus Con and HPH versus Cy comparisons, 346 genes were shared between the two DEG sets. Functional enrichment of these overlapping genes was concentrated mainly in cell-cycle regulation, DNA replication, chromosome organization, and cell proliferation. This pattern is biologically relevant because intestinal epithelial renewal depends on tightly regulated proliferation of crypt epithelial cells, and reactivation of proliferative programs is an important component of epithelial regeneration after injury (Meyer *et al.*, 2022; Villablanca *et al*., 2022). The PPI network further identified several highly connected cell-cycle-associated genes, including *Cdk1, Ccna2, Plk1, Bub1, Cdc20, Aurka, Cenpf* and *Ccnb1*. Rather than representing specific molecular markers of mucosal repair, these genes collectively indicate altered regulation of proliferative and mitotic processes. When considered alongside the improvements in mucosal architecture, goblet cell abundance, and barrier-related proteins, the transcriptional changes are compatible with a broader epithelial recovery response following HPH treatment. The identified hub genes provide candidates for further investigation of this response, although their functional roles remain to be established.

The correlation analysis further identified associations among the microbial, transcriptional, and barrier-related changes observed following HPH intervention. Several bacterial genera were significantly associated with representative genes identified through transcriptomic and PPI analyses, while these genes were also correlated with MUC2 and the tight-junction-related parameters ZO-1, Claudin-1 and Occludin. Similar correlation-based approaches have been used to examine relationships between gut microbial composition and intestinal barrier characteristics in experimental models (Cheng *et al.*, 2023). Studies of polysaccharide intervention have likewise reported coordinated variation between specific microbial taxa and host mucosal or metabolic features (Yuan *et al.*, 2024). In the present study, these associations complemented the parallel changes in microbial composition, cell-cycle-related transcription, and barrier-related proteins. Together, these findings connect the microbial and host responses associated with HPH treatment and highlight candidate relationships for further investigation into intestinal mucosal protection. These relationships are correlative, however, and their direction and causal roles remain to be established through functional studies.

Taken together, HP alleviated several features of Cy-induced systemic immunosuppression and intestinal mucosal injury, with the high-dose group showing the clearest combined response. The intestinal improvement was accompanied by changes in barrier-related proteins, inflammatory and mucosal immune parameters, epithelial proliferation-related transcriptional responses, and gut microbial composition. Several limitations should nevertheless be acknowledged. The transcriptomic, microbiota, and correlation analyses provide associative rather than causal evidence, and the expression changes in the hub genes identified by RNA-seq and PPI analysis were not independently confirmed by qPCR or protein-level assays. Intestinal permeability was not directly assessed, so the improvements in mucosal morphology and barrier-related protein expression should be interpreted as indirect evidence of barrier protection. Further studies incorporating permeability measurements would help clarify the functional significance of these changes. Moreover, Cy produces an acute and experimentally controlled form of immunosuppression. Immune dysfunction encountered in livestock and poultry is commonly multifactorial and may simultaneously involve infection, nutritional status, housing conditions, and management-related stress. The mouse model used here cannot reproduce this complexity, and the present results do not demonstrate that HP can treat naturally occurring immunosuppressive diseases in animals. Subsequent studies should verify the molecular and barrier-related responses before evaluating HP in relevant animal species. Dose selection, repeated-use safety, productive performance, and compatibility with routine feeding and disease-control practices also require assessment. At this stage, HP is more appropriately considered a candidate dietary immunomodulator for further veterinary evaluation than an established therapeutic intervention.

Conclusions: HP partially alleviated Cy-induced systemic immunosuppression and intestinal mucosal injury in mice, as reflected by improvements in immune indices, mucosal morphology, and barrier-related protein expression. These improvements were accompanied by changes in inflammatory and mucosal immune parameters, intestinal transcriptional profiles, and gut microbial composition. The findings provide an experimental basis for further

investigation of HP in relation to immune function and intestinal mucosal protection.

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