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Phallus impudicus polysaccharide protects against acute lung injury via inhibition of inflammatory/antioxidant pathways and regulation of gut microbiota and lung tissue metabolites

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ABSTRACT: *Phallus impudicus* L. (*P. impudicus* L.) is an edible fungus with a delicious taste and considerable medicinal-edible value. Preliminary studies have confirmed that its refined polysaccharide (PIRP) exhibit significant anti-inflammatory and antioxidant effects. Acute lung injury (ALI) is a severe disease characterized by severe inflammation and oxidative stress, with high incidence and mortality, yet lacks effective clinical treatments. This study aimed to evaluate protective effect of PIRP against lipopolysaccharide-induced ALI in mice and comprehensively clarify its therapeutic mechanisms. The therapeutic efficacy of PIRP was assessed by testing the degree of pulmonary edema, antioxidant enzymes/peroxidases and inflammatory factors, as well as by histopathological analysis of the lungs. Furthermore, we investigated the mechanism of PIRP action using immunofluorescence, protein immunoblotting, transmission electron microscopy, 16S-rRNA gene sequencing, and metabolomics experiments. The results showed that PIRP significantly ameliorated histopathological lung damage in ALI mice, reduced bronchoalveolar lavage fluid (BALF) protein concentrations and inflammatory cytokine levels, and increased the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). PIRP also improved mitochondrial structural integrity and effectively mitigated reactive oxygen species (ROS) accumulation caused by mitochondrial damage. Mechanistically, PIRP suppressed the expression of downstream proteins in the MAPK and NF- κ B signaling pathways, thereby inhibiting the production of inflammatory factors and alleviating inflammation. Additionally, PIRP upregulated the expression of Nrf2 and HO-1 and downregulated Keap1 expression, stimulating antioxidant enzyme activity and ultimately mitigating LPS-induced oxidative stress imbalance. Furthermore, PIRP modulated the composition and structure of intestinal flora, decreasing the abundance of harmful bacteria while increasing beneficial bacterial genera, thereby ameliorating intestinal flora dysbiosis in ALI mice. Metabolomic analysis identified differentially abundant metabolites between LPS- and PIRP-treated groups; PIRP reversed LPS-induced dysregulation of 4-hydroxybutanoic acid, (R)-3-hydroxybutanoic acid, and L-aspartic acid. These findings demonstrate the multifaceted protective effects of PIRP against ALI and contribute to a deeper understanding of its anti-inflammatory, antioxidant, and microbiome-modulating functions.

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1. Introduction

Mushroom *Phallus impudicus* (*P. impudicus*) is a kind of rare edible fungus with a delicious taste and is rich in protein, polysaccharides, dietary fibre, vitamins, minerals, and other bioactive ingredients^[1]. It is still a natural medicine and has the functions of an antitumour agent, antidepressant, and thromboembolic prophylaxis^[2-4]. Polysaccharides are among the primary bioactive components of edible mushrooms. Extensive research has demonstrated that mushroom polysaccharides possess a wide range of biological activities, including antioxidant, antitumor, anti-inflammatory, immunomodulatory, hypoglycemic, gut microbiota-regulating, and antiviral effects, which are highly beneficial to human health^[5-8]. Nevertheless, research on the biological activity of polysaccharides from *P. impudicus* is very limited^[9]. Preliminary research by our group revealed that *P. impudicus* refined polysaccharide (PIRP) exhibit significant anti-inflammatory, antioxidant, and immunomodulatory effects^[10,11].

Acute lung injury (ALI) is a clinical illness characterized by increased hypoxemia, significant edema of the lung parenchyma and alveoli, and the infiltration of inflammatory cells into the lungs. It is a serious illness with high rates of occurrence and death^[12]. The pathogenesis of ALI is complex and not yet fully elucidated. In the COVID-19 era, ALI may manifest as the earliest symptom of SARS-CoV-2 infection, and in more severe cases, it can progress to acute respiratory distress syndrome (ARDS)^[13]. The pathogenesis of ALI includes an intense inflammatory response due to the release of large amounts of proinflammatory cytokines, oxidative stress injury due to excess reactive oxygen species or reduced oxygen radical scavenging enzymes, and pulmonary edema due to damage to the alveolar-capillary barrier^[14-16].

The current interventional treatments for ALI primarily focus on lung-protective ventilation strategies and glucocorticoid-based pharmacotherapies. However, commonly used glucocorticoids are often accompanied by adverse reactions such as coagulation disorders and gastric ulcers; thus, they cannot be used as routine therapeutic agents^[17]. In this context, abundant natural medicinal and edible resources in traditional Chinese medicine (TCM) have garnered significant attention due to their multitarget pleiotropic effects and potential in the development of functional foods for therapeutic applications^[18]. This is particularly evident in the context of ALI, where bioactive components derived from natural medicines and edible sources offer promising intervention avenues. The role of TCM-based functional foods has been further emphasized by their notable ability to alleviate COVID-19-associated symptoms^[19-21]. Active compounds extracted from these natural, food-grade resources—such as polysaccharides, polyphenols, alkaloids, and flavonoids—not only exhibit therapeutic potential but also align with the growing demand for safe, edible interventions in managing respiratory and inflammatory conditions. These compounds possess anti-inflammatory, antibacterial, antioxidant, and lung-protective properties, and have also shown promise for protecting against ALI.

In this study, we assessed the protective benefits of PIRP in a mouse model of ALI. By analysing the effects of PIRP on inflammatory/oxidative signalling pathway, as well as on the gut microbiota and lung tissue metabolomics, we aimed to elucidate the mechanisms underlying the alleviation of ALI by PIRP. These findings establish the theoretical foundation for PIRP's clinical development and future applications of PIRP in pulmonary health management.

2. Materials and methods

2.1 Antibodies and reagents

Dexamethasone (Dex) acetate tablets were obtained from Xianju Pharmaceutical Co. (Zhejiang, China). Lipopolysaccharide (LPS) (*Escherichia coli* serotype 055:B5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). SOD, GSH-Px, NO detection kits, protease inhibitors, RIPA lysis buffer, and phosphatase inhibitors were provided by Beyotime Biotechnology Co. (Shanghai, China). The myeloperoxidase (MPO) detection kit was provided by Jiancheng Institute of Bioengineering (Nanjing, China). A bicinchoninic acid (BCA) protein assay reagent kit, and skim milk powder were obtained from Solarbio Co. (Beijing, China). An enzyme-linked immunosorbent assay (ELISA) kit for IL-1 β was purchased from Ruifan Biotechnology Co. (Shanghai, China). See Supporting Information 1 for information on the antibodies and monosaccharide standards.

2.2 Preparation, molecular weight and monosaccharide composition analysis of PIRP

The dried *P. impudicus* was sheared and extracted with 80% ethanol, then the residue was extracted with deionized water. The aqueous extract was subsequently subjected to concentration, alcoholic precipitation, deproteinization ^[22], and dialysis to obtain a *P. impudicus* refined polysaccharide (PIRP). The total sugar content of PIRP was determined by the phenol sulfate method ^[23], and its homogeneity and molecular weight were determined using high-performance gel permeation chromatography (HPGPC) ^[24]. The monosaccharide composition of PIRP was determined via gas chromatography-mass spectrometry (GC-MS) ^[25]. See Supporting Information 2 for the detailed steps.

2.3 Animals and experimental design

Six-week-old male Balb/c mice (20 $\pm$ 2 g) were provided by Speiford Biotechnology Co., Ltd. (Beijing, China). The mice were kept in climate-controlled settings between 22°C and 26°C with a 12 h light/dark cycle. Their access to food and drink was unrestricted. The ethical guidelines of the Institutional Animal Care and Use Committee (IACUC) were strictly followed during all experimental procedures, and on March 1, 2023, the Guizhou Medical University Animal Experiment Ethics Committee authorized the protocols (permission number No. 2304035). Following a week of flexible feeding, the mice were split into six groups at random: normal control group (Control), LPS control group (LPS, 8mg/kg) ^[26,27], dexamethasone acetate positive control group (Dex, 5 mg/kg) ^[28], PIRP low-dose (PIRP-L, 100 mg/kg), PIRP medium-dose (PIRP-M, 200 mg/kg) and PIRP high-dose (PIRP-H, 400 mg/kg) group, with 12 mice per group. An ALI model was

established by nebulizing LPS via inhalation ^[29]. As shown in Fig. 1, different drug treatments were administered to each group of mice ^[30].

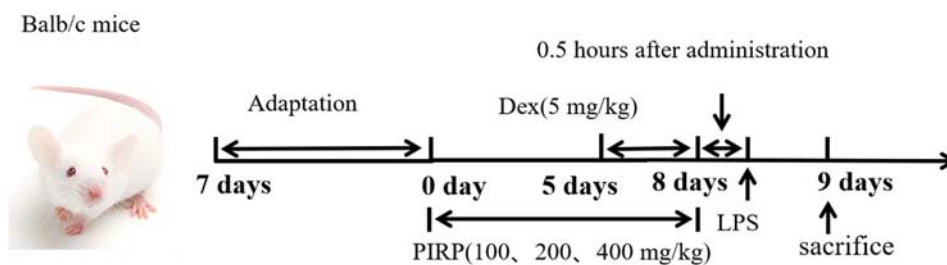


Fig. 1 Schematic of the timing of LPS induction modeling and drug therapy. LPS, lipopolysaccharide (8 mg/kg); Dex, dexamethasone acetate (5 mg/kg); PIRP, *P. impudicus* refined polysaccharide; PIRP low dose group (PIRP-L, 100 mg/kg), PIRP middle dose group (PIRP-M, 200 mg/kg) and PIRP high dose group (PIRP-H, 400 mg/kg), n=12.

2.4 Measurement of visceral index and lung Wet/Dry weight ratio

The thymus and spleen were collected to determine organ indices, calculated as organ weight (mg) / weight of mice (g). The lower lobe of the right lung (not operated on by alveolar lavage) was removed and weighed to obtain its wet weight. After that, the lung tissue was dried at 70°C for 48 hours in an oven to obtain its dry weight ^[31]. The wet weight to dry weight ratio (W/D ratio) was calculated by dividing the wet weight by the dry weight.

2.5 Cell number and protein concentration in bronchoalveolar lavage fluid (BALF)

After collecting BALF, the fluid was centrifuged at 1000 rpm for 5 minutes at 4 °C. The supernatant was refrigerated for further analysis ^[32]. Subsequently, after staining with Trypan blue, cell counts were performed. The total protein concentration in BALF was determined using the BCA Protein Concentration Assay Kit according to the manufacturer's instructions.

2.6 Antioxidant and inflammatory factor expression

The kits were used to measure the enzymatic activities of SOD and GSH-PX in the serum as well as the concentration of NO in the BALF. Enzyme activity was measured in 5% (w/v) lung tissue homogenates from mice using an MPO kit; IL-1 β expression levels in BALF were measured using an ELISA kit.

2.7 Histopathological examination and immunofluorescence study

The fresh upper lobe of the right lung was collected. It was preserved in 4% formaldehyde solution, embedded in paraffin, cut into 5 μ m sections, and stained. Subsequently, pathological changes were examined using a NIKON DS-U3 Imaging system (NIKON, Japan). A double-blind approach assessed ALI severity using a semi-quantitative histological score. The extent of interstitial and alveolar condensation under the microscope, as well as haemorrhage, alveolar wall thickening, destruction of the alveolar structure, inflammatory cell infiltration, and pulmonary interstitial swelling, determined the score ^[33].

For the immunofluorescence assay, paraffin sections were immunostained using with F4/80 and occludin primary antibodies. Antibody-antigen complexes were detected using HRP-conjugated goat anti-rabbit IgG and visualized. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI), after which the slides were

sealed. Staining was observed under an orthofluorescence microscope (NIKON, Japan) to evaluate the nucleation of lung tissue cells.

2.8 Transmission electron microscopy (TEM)

Fresh lung tissues harvested within 1–3 min were trimmed to 2 mm × 2 mm and fixed in electron microscope fixative at 4 °C. Tissues were rinsed thrice (15 min each) with 0.1 M PBS buffer, fixed in 1% osmium tetroxide at 25 °C (light-protected, 2 h). Subsequently, the tissue was subjected to gradient dehydration, osmotic embedding, polymerisation, localisation, staining of the sections (60–80 nm), and finally observation under a transmission electron microscope (HITACHI, Tokyo, Japan) to collect image information.

2.9 Western blotting

Lung tissues were homogenized and lysed on ice for 30 min. After centrifugation at 12 000 rpm for 10 min at 4°C, the supernatant was collected. Protein concentrations in the supernatant were determined using the BCA protein assay kit. Subsequently, the total protein concentration of lung tissue was adjusted to 50 µg/mL. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (0.45 µm). Furthermore, 5% skimmed milk was blocked for 2 h and conjugated with specific antibodies, incubated overnight at 4 °C. Samples were then incubated at room temperature for 1 h with anti-rabbit IgG conjugated to HRP. Finally, protein signals were detected using enhanced chemiluminescence (ECL) select kit solution. Images were obtained using the Chin-Shiang system, and grayscale values were calculated using Image J software.

2.10 Intestinal microbiota analysis

A DNA extraction kit (E.Z.N.A® Soli DNA Kit, Omega Bio-Tek, USA) was used to extract total DNA from the caeca of mice with ALI. A NanoDrop2000 Ultra-micro Spectrophotometre (Thermo Fisher Scientific, USA) was used to determine the DNA concentration, and 1% agarose gel electrophoresis was used to determine the DNA quality. Sanshu Biotech Co. (Shanghai, China) provided the Nova Seq PE250 platform (Illumina, San Diego, CA, USA) for the amplification and sequencing of region V3–V4 of the 16S rRNA gene. Primers used were: 338F (5'-ACTCCTACGGGAGGACAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3').

2.11 Metabolomic analysis

Lung tissue samples (100 mg) were prepared and analyzed for metabolomics using an ultra performance liquid chromatography (UPLC) system (Vanquis, UPLC, Thermo, USA) and mass spectrometry with high resolution (Q Exactive HF-X, Thermo, USA). Detailed condition information is given in the Supporting Information 3.

2.12 Statistical analysis

The data were statistically analyzed via SPSS 26.0 software; all the quantitative data are presented as the mean ± SDs. Each dataset is subjected to a normality test. One-way ANOVA was used to compare

homogeneous groups, and the least significant difference (LSD) test was used for multiple comparisons. GraphPad Prism software (version 8.0) was used for graphing.

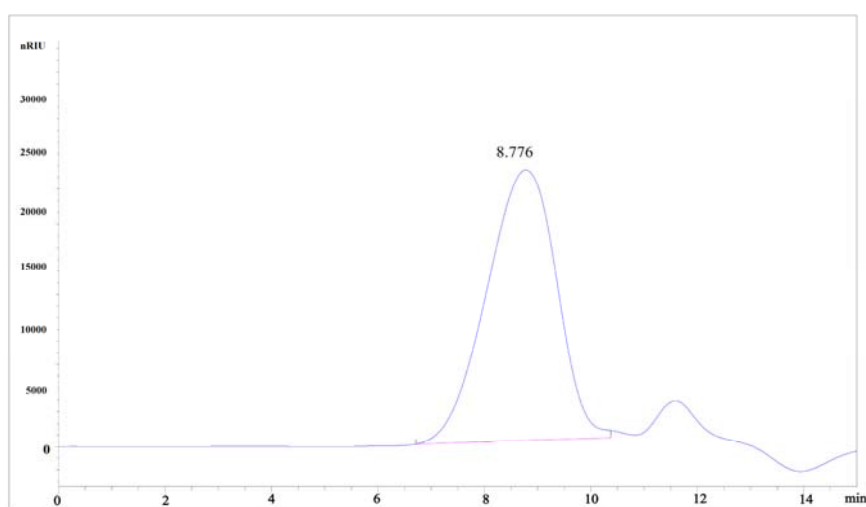
3. Results

3.1 PIRP purity and preliminary characterization

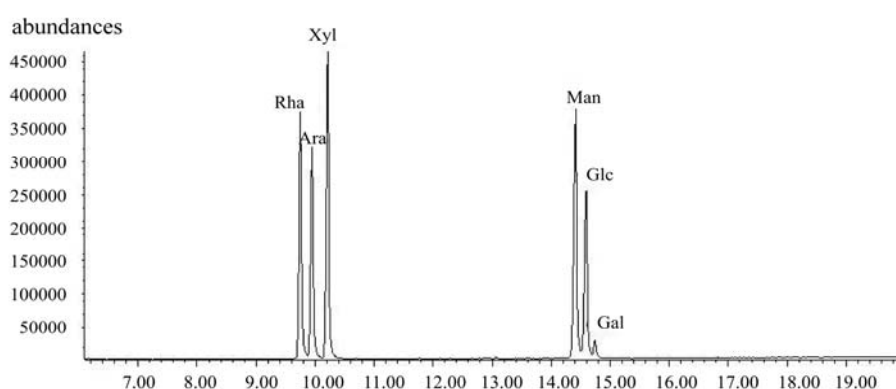
The total sugar content of PIRP was determined to be $93.40 \pm 0.03\%$. PIRP had no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of proteins and nucleic acids. Moreover, PIRP was analyzed by high performance gel permeation chromatography (Fig. 2A), and the major chromatographic peak showed a single symmetrical peak shape, and the weight-average molecular weight (M_w) and number-average molecular weight (M_n) of PIRP were 56.68 and 26.26 KDa, respectively, and the molecular weight distribution coefficient (M_w/M_n) was 2.158.

Trifluoroacetic acid (TFA) hydrolysis, reduction, and acetylation produced alditol acetates, which were used to determine the composition of PIRP. The GC–MS total ion current (TIC) chromatograms were shown in Fig. 2B–C (GC–MS performance report are provided in Fig.S1–S2). In contrast to the standard monosaccharide's retention period, PIRP consisted of arabinose, mannose, glucose and galactose at a molar ratio of 1.00:1.11:2.88:1.92.

A



B



C

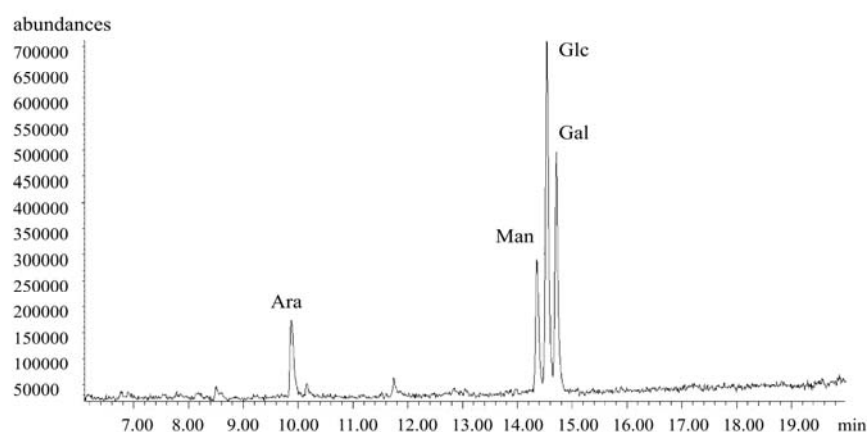


Fig. 2 Purity and monosaccharide composition of PIRP. High performance gel permeation chromatography showing the elution profiles of PIRP monitored by refractive index detector, the major chromatographic peak showed a single symmetrical peak shape (A). The GC–MS total ion current (TIC) chromatogram of monosaccharide standard mixture (B) and hydrolysis product of PIRP (C), PIRP consisted of Ara, Man, Glc and Gal.

3.2 Effect of PIRP on organ index and W/D value in mice with ALI

The spleen and thymus are often used as indicators of immunocompetence in animal experiments [34]. As shown in Fig. 3A–C, the thymus and spleen indices of the LPS group decreased by 30.43% and 25.00%, respectively, and those of the Dex group were the most affected, with values that were 68.75% and 30.30% lower than those of the LPS group, respectively. These findings suggest that adverse reactions to Dex may include damage to the thymus and spleen tissue. Compared with those of the LPS group, the thymus indices of the PIRP group tended to increase. Among these groups, PIRP-M showed the most significant effect, with a increase of 37.50%. The spleen indices of the PIRP-L, PIRP-M, and PIRP-H groups increased by 19.51%, 19.51%, and 21.21%, respectively. Compared with that of the control group, the W/D ratio of the LPS group significantly increased by 26.94%, whereas that of the Dex group was decreased by 12.24%. The W/D ratios for PIRP-L, PIRP-M, and PIRP-H decreased by 7.84%, 10.33%, and 14.91%, respectively. (Detailed data are provided in Table S1)

3.3 Effect of PIRP on total protein concentration and cell number in BALF of mice with ALI

An important feature of successful ALI modelling is increased pulmonary vascular permeability [35]. The increase in protein concentration and total cell number in BALF is closely related to increased pulmonary vascular permeability and lung injury. [36]. Compared with that of the control group, the total protein concentration in BALF of the LPS group increased 2-fold, while those of the PIRP-H and Dex groups were 25.56% and 28.89% lower than that of the LPS group, respectively. Trypan blue staining showed that the total cell count in BALF from the LPS group increased by 7.38-fold compared to the Control group, while counts in the PIRP-L, PIRP-M, and PIRP-H groups decreased by 43.45%, 50.10%, and 66.50%, respectively, compared to the LPS group (Detailed data are provided in Table S2.) (Fig. 3D–E).

3.4 Effects of PIRP on antioxidant enzymes/peroxidases and inflammatory factors in mice with ALI

The degree of neutrophil infiltration in the lung tissue can be indicated by MPO enzyme activity, and neutrophil activation and migration are hallmarks of ALI progression [37]. Compared with that in the control group, MPO activity increased 2-fold in the LPS group. Compared with that in the LPS group, MPO activity reduced by 45.00%, 47.50%, and 52.50% in the PIRP-L, PIRP-M, and PIRP-H groups, respectively, and by 25.00% in the Dex group (Fig. 3F).

SOD is an antioxidant metalloenzyme crucial for maintaining oxidative-antioxidant balance in organism [38]. GSH-Px is an important peroxidation-degrading enzyme and indicator of bodily antiperoxidation capacity [39]. In this study, SOD and GSH-Px levels in serum decreased by 43.13% and 30.30%, respectively, in the LPS group versus the Control group. Compared to LPS, PIRP and Dex reversed the decreasing trend of SOD and GSH-Px; SOD increased by 27.47%, 37.36%, and 42.86% in the PIRP-L, PIRP-M, and PIRP-H groups, respectively, and GSH-PX increased by 17.39%, 24.78%, and 33.91%, respectively, showing a dose-effect relationship. In the Dex group, SOD and GSH-PX increased by 48.35% and 21.74%, respectively (Fig. 3G-H).

Elevated lung inflammation levels cause inflammatory mediators to enter alveoli and trachea, inducing high expression of pro-inflammatory cytokines. Compared to the Control group, IL-1 β and NO were significantly increased in the BALF of mice treated with LPS, which was up-regulated by 1.82 times and 2.76 times, as shown in Fig. 3I-J. Compared with the LPS group, PIRP and Dex interventions can downregulate the secretion levels of IL-1 β and NO in BALF. The IL-1 β secretion was reduced by 36.48%, 14.05%, 24.13%, and 28.73% in the Dex, PIRP-L, PIRP-M, and PIRP-H groups, respectively; and NO secretion was reduced by 57.97%, 15.93%, 57.69%, and 54.12%, respectively. (Detailed data are provided in Table S3.)

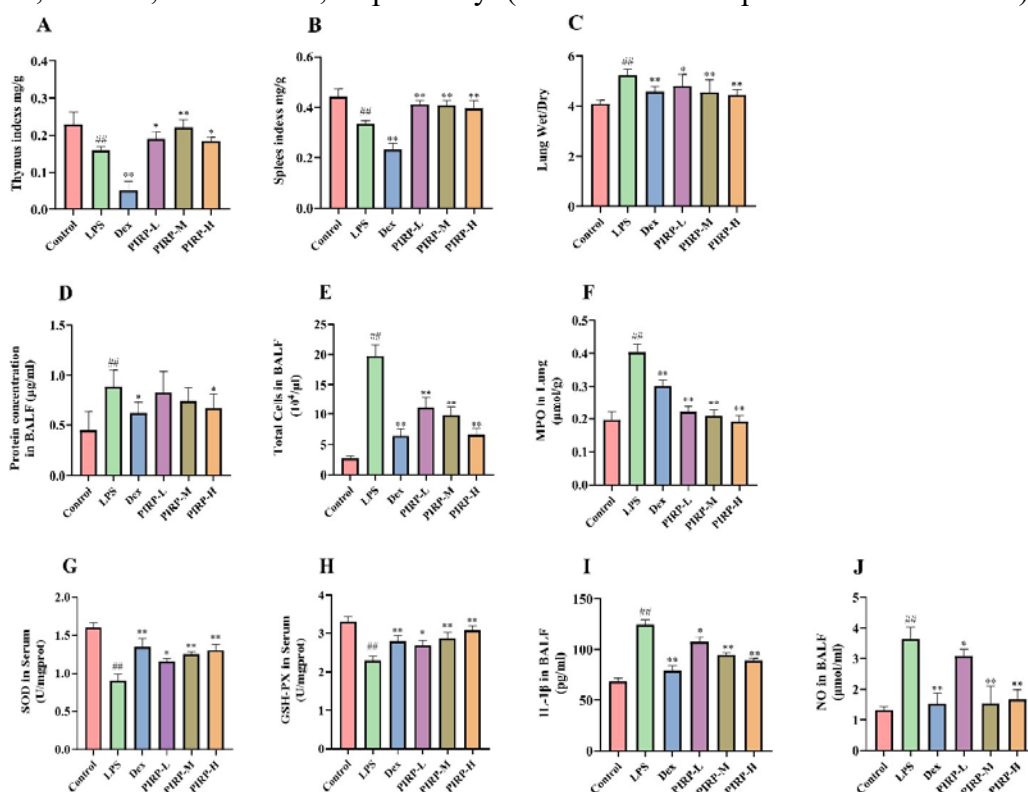


Fig. 3 PIRP protects against LPS-induced ALI. (A) Thymus index. (B) Spleen index. (C) Lung wet/dry weight ratio. (D) Protein concentration in BALF. (E) Total cells in BALF. (F) MPO activity in lung tissue. (G) SOD in serum. (H) GSH-PX in serum. (I) IL-1 β in BALF. (J) NO in BALF. n = 6. Data are expressed as mean $\pm$ SD. ## P < 0.01, # P < 0.05 vs. Control group; ** P < 0.01, * P < 0.05 vs. LPS group.

3.5 Effect of PIRP on histopathologic changes in the lungs of mice with ALI

As shown in Fig. 4A, the control group exhibited normal lung morphology. After LPS induction, the LPS group had a large number of neutrophils and macrophages infiltrated the lung interstitium in the LPS group. Moreover, the lung tissues showed obvious alveolar wall thickening and alveolar capillary congestion, suggesting histopathological damage to the lungs. Compared with those in the LPS group, the lung injury scores were significantly lower in the PIRP-L, PIRP-M, PIRP-H, and Dex groups ($P < 0.05$ and $P < 0.01$; Fig. 4B). These results indicate that PIRP effectively maintained lung tissue morphology and alleviated damage.

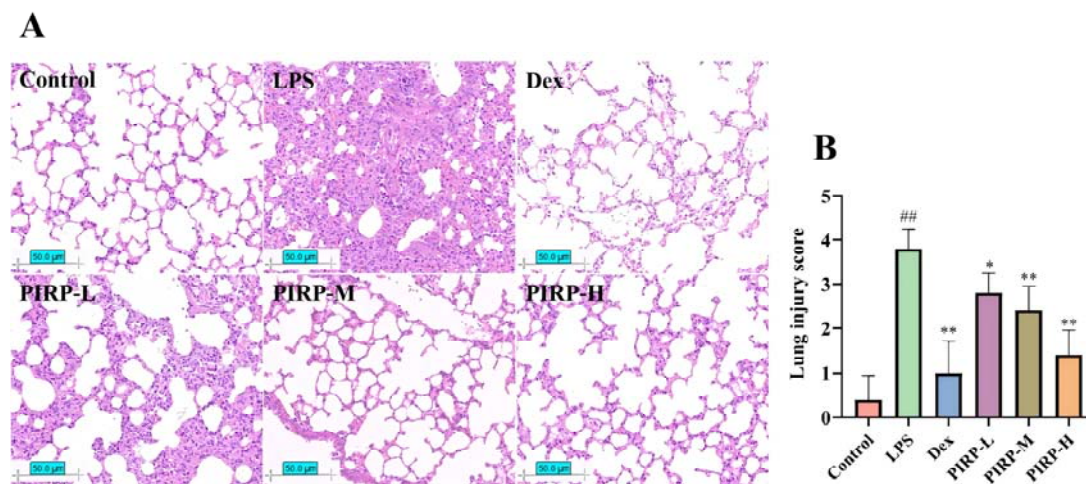


Fig. 4 PIRP exerts protective effects on lung tissue pathological change against LPS-induced ALI *in vivo*. (A) Different groups of the histopathological of lung tissues after LPS treatment were evaluated by H&E staining, scale bar = 50 μm. (B) Morphological damage score for the lung tissues, $n = 3$. Data are expressed as mean $\pm$ SD. ## $P < 0.01$, * $P < 0.05$ vs. Control group; ** $P < 0.01$, * $P < 0.05$ vs. LPS group.

3.6 Effect of PIRP on the expression of occludin and F4/80 proteins in lung tissue of mice with ALI

In this study, changes in capillary permeability and inflammatory cell infiltration in mouse lungs were assessed by observing occludin and F4/80 proteins expression. Fig. 5A-C showed that after LPS stimulation, occludin fluorescence intensity significantly decreased compared with that in the control group, whereas the F4/80 fluorescence intensity increased significantly ($P < 0.01$). After PIRP intervention, occludin fluorescence intensity significantly increased in the PIRP-M and PIRP-H groups ($P < 0.01$), whereas F4/80 fluorescence intensity significantly decreased in all three PIRP dose groups ($P < 0.05$ or $P < 0.01$). In the LPS group, occludin expression was significantly reduced, whereas F4/80 expression was significantly higher than that in the Control group ($P < 0.01$). Compared to the LPS group, the expression of occludin protein was significantly upregulated in the three PIRP dose groups, whereas the expression of F4/80 protein was markedly downregulated in the PIRP-M and PIRP-H groups, with the PIRP-H group showing the best effect (Fig. 5D-F). (The original western blot images of Occludin and F4/80 are shown in Fig. S3-S4.)

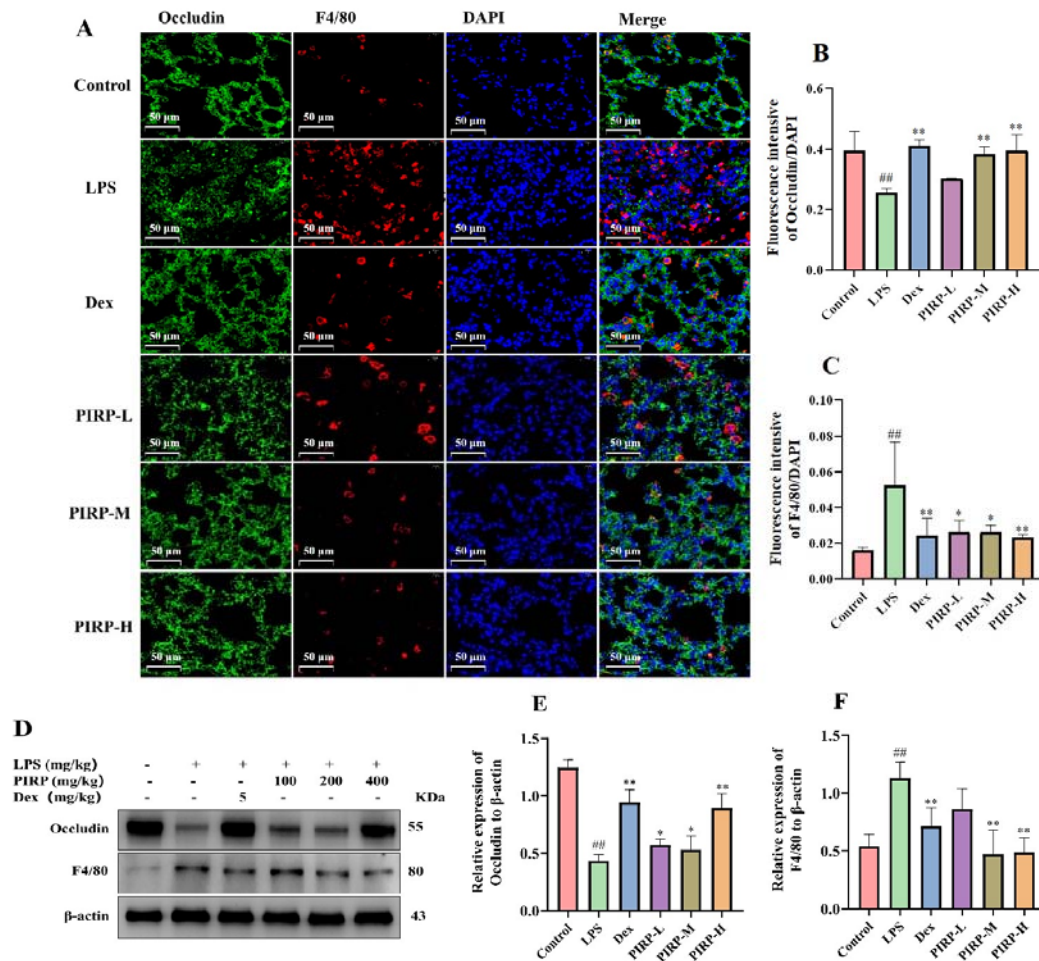


Fig. 5 PIRP increases Occludin and inhibits F4/80 protein expression in lung tissue *in vivo*. (A) Immunofluorescence images of Occludin and F4/80 in lung tissue of mice. Scale bar, 20 μ m. The quantification of Occludin (B) and F4/80 (C) fluorescence intensity in lung tissue of mice, $n = 3$. (D) Representative western blots for Occludin and F4/80 protein expression in lung tissue extracts, semi-quantitative analysis of Occludin (E) and F4/80 (F) protein expression, $n = 3$. Data are expressed as the mean $\pm$ SDs. ^{##} $P < 0.01$, ^{*} $P < 0.05$ vs. Control group; ^{**} $P < 0.01$, ^{*} $P < 0.05$ vs. the LPS group.

3.7 Effect of PIRP on mitochondrial structure in mice with ALI

Mitochondrial dysfunction is associated with progression of lung injury. Reactive oxygen species are predominantly produced in mitochondria. Therefore, we observed the mitochondrial structure in lung tissues of ALI mice using TEM. As shown in Fig. 6, the lung tissue mitochondria in the LPS group showed high oedema, mitochondrial membrane rupture, disappearance or vacuolization of most cristae, unclear boundaries between organelles, reduced intercellular matrix (Shown by the red arrow in Fig. 6). Compared with the LPS group, the PIRP-L group showed significant interstitial changes, reduced mitochondrial swelling, unclear observation of some structures, and no clear boundaries. The number of mitochondria in the PIRP-M group increased, internal structure was normal, cytoplasm was clear, boundaries between organelles were distinct. In the PIRP-H group, the number of mitochondria increased, structures remained normal, organelle boundaries were clear, and nuclei were distinctly visible (shown by the green arrow in Fig. 6). These results indicate that PIRP ameliorates mitochondrial damage in lung tissue of ALI mice.

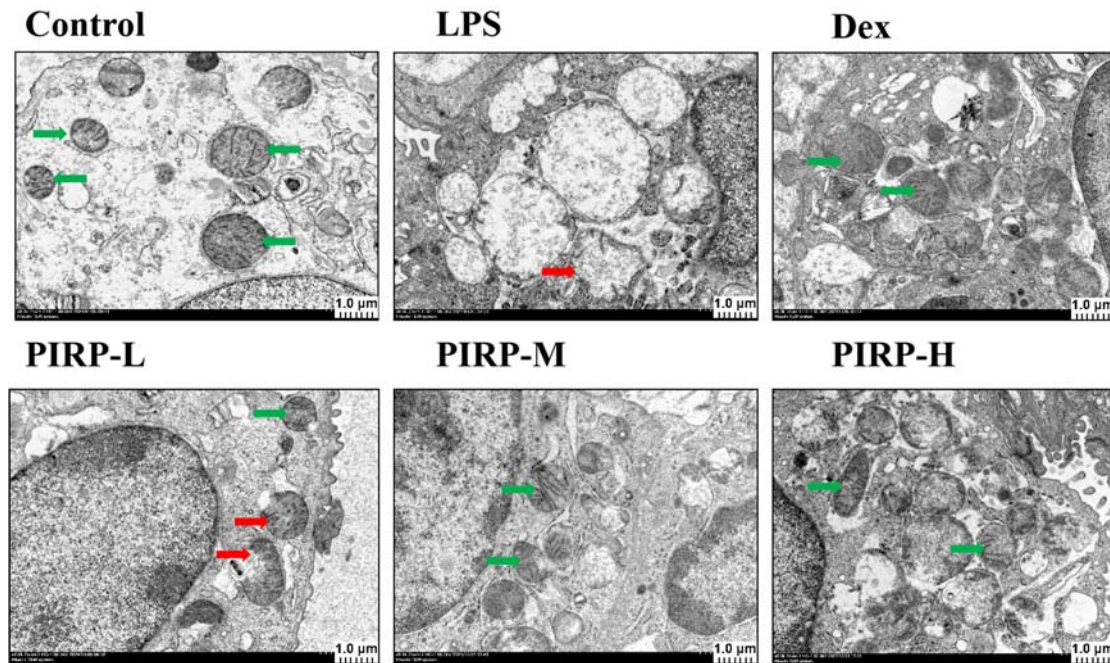


Fig. 6 Observation of PIRP improving LPS induced mitochondrial structural damage in ALI mice lung tissue by TEM.

Scale bar, 1 μ m. The lung tissue of the LPS group was severely damaged. Compared to LPS group, the number of mitochondria in the PIRP-M and PIRP-H group increased, the structure was normal, boundaries between organelles were clear, and obvious nuclei were visible. The red arrows in the figure indicate mitochondria with structural damage, while the green arrows indicate mitochondria without significant structural damage.

3.8 Effect of PIRP on MAPK, TLR4/MyD88/NF- κ B and Nrf2/Keap1/HO-1 signalling pathways and ALOX5 protein expression in mice with ALI

Various physiological and pathological processes, including inflammatory responses, are regulated by the MAPK signalling pathway^[40]. As shown in Fig. 7A-D, the phosphorylation levels of P38, jnk, and erk1/2 in the LPS group were significantly higher than those in the control group. The increase in P38 and jnk protein phosphorylation induced by LPS stimulation were significantly reversed in PIRP-M and PIRP-H groups ($P < 0.01$), while the PIRP-H group significantly reversed increased in erk1/2 phosphorylation ($P < 0.01$). These findings illustrate that PIRP relieve ALI by inhibiting MAPK signalling pathway activation.

There is evidence that the TLR4/MyD88/NF- κ B signalling pathway is involved in ALI pathogenesis^[41]. ALOX5 is one of the gene targets associated with lung injury in the traditional chinese medicine systems pharmacology (TCMSP) database; therefore, it is necessary to explore its expression in ALI. As shown in Fig. 7E-I, protein expression of MyD88, TLR4, and ALOX5, as well as P65 phosphorylation, was markedly increased by LPS. Compared with those in the LPS group, the three dose groups of PIRP significantly downregulated the protein expression of TLR4 ($P < 0.05$ or $P < 0.01$), the PIRP-H group significantly downregulated the expression of MyD88 protein ($P < 0.01$), and the PIRP-M and PIRP-H groups significantly downregulated the protein phosphorylation level of P65 and the protein expression of ALOX5. These results indicate that inhibiting MyD88, TLR4, ALOX5, and phosphorylated NF- κ B upregulation is a therapeutic mechanism for PIRP-mediated ALI alleviation.

The Nrf2/Keap1/HO-1 pathway regulates cellular oxidative stress and redox homeostasis^[42]. As shown in Fig. 7J-M, compared with the Control group, the LPS group showed a significant decrease in Nrf2 and

HO-1 protein expression, whereas Keap1 protein expression was significantly increased ($P < 0.01$). After PIRP intervention, the expression of Nrf2 protein significantly increased in the PIRP-M and PIRP-H groups ($P < 0.01$), whereas Keap1 protein expression was significantly reduced in PIRP-H ($P < 0.01$). In the PIRP high, medium and low dose groups, HO-1 protein expression increased significantly ($P < 0.05$, $P < 0.01$). This suggests that modulating Nrf2, Keap1, and HO-1 expression is a pathway for PIRP-mediated ALI protection. (The original Western blot images of the above proteins are shown in Fig. S5-S19.)

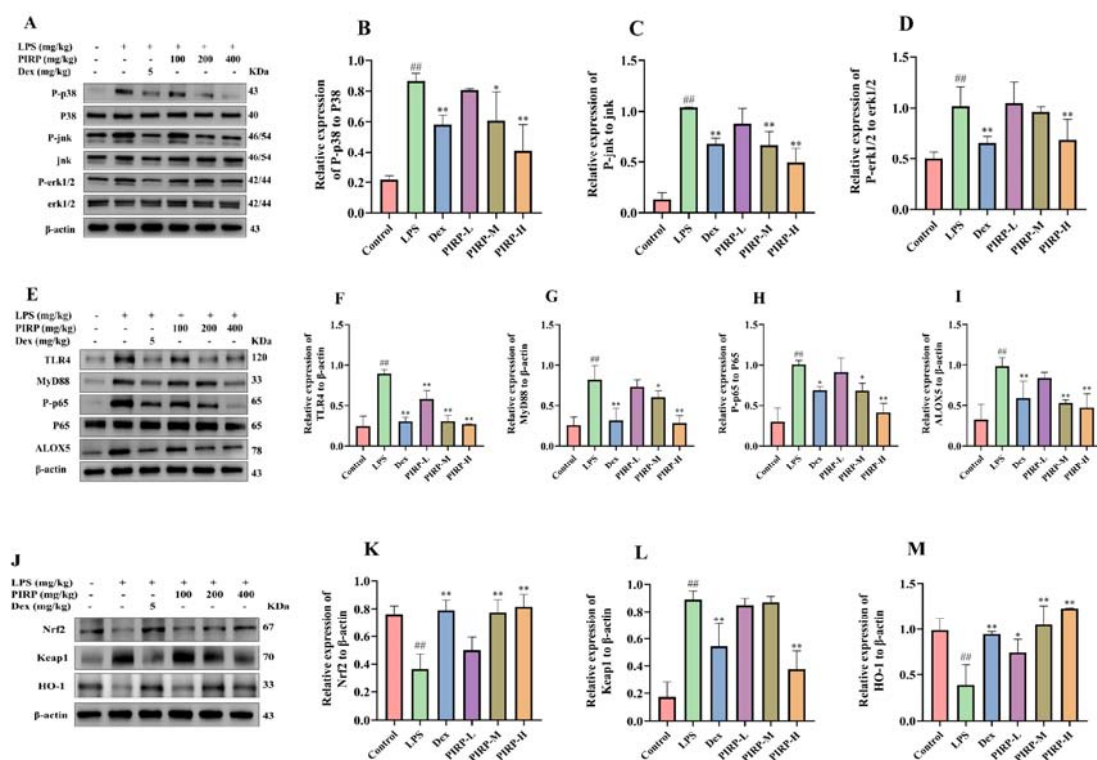


Fig. 7 Representative images of MAPK, TLR4/MyD88/NF- κ B and Nrf2/Keap1/HO-1 protein expression and statistical analysis of the relative expression levels in different group. (A) Representative bands of MAPK. Statistical chart of relative protein expression levels of phosphorylated P38 (B), jnk (C), and erk1/2 (D) in lung tissues, $n = 3$. (E) Representative bands of TLR4/MyD88/NF- κ B and ALOX5. Statistical chart of relative protein expression levels of TLR4 (F), MyD88 (G), P-p65 (H) and ALOX5 (I) in lung tissues, $n = 3$. (J) Representative bands of Nrf2/Keap1/HO1. Statistical chart of relative protein expression levels of Nrf2 (K), Keap1 (L) and HO-1 (M) in lung tissues, $n = 3$. Data are expressed as mean $\pm$ SD. ## $P < 0.01$, # $P < 0.05$ vs. Control group; ** $P < 0.01$, * $P < 0.05$ vs. LPS group.

3.9 Effect of PIRP on gut microorganisms in mice with ALI

The sequencing results of the 16S rRNA amplicons showed that there were 25, 36, 50, 26, and 25 unique Operational Taxonomic Units (OTUs) in the control, LPS, and PIRP-L/M/H groups, respectively (Fig. 8A). The α -diversity index was used to evaluate the homogeneity and richness of gut microbiota. As shown in Fig. 8B-D, in the LPS group, the Chao1, ACE, and Shannon indices were significantly higher than those in the Control group ($P < 0.01$). Compared with those of the LPS group, the Chao1, ACE, and Shannon indices of the PIRP-H group were significantly reduced ($P < 0.01$). β -diversity analysis was used to evaluate the species variability among samples. Principal coordinate analysis (PCoA) showed significant differences between samples in the LPS and Control groups and a certain degree of separation between samples in the PIRP-H group and the LPS and other dose groups. These findings suggest that PIRP enhanced the gut microbiota's species richness and community diversity in ALI-affected mice (Fig. 8E).

The percentages of microbial communities at the phylum and genus levels were analyzed in mice. As shown in Fig. 8F, the predominant phyla included Bacteroidetes, Firmicutes, Proteobacteria, Cyanobacteria, and other intestinal flora. Fig. 8G shows that the genus level mainly consisted of enteric flora, such as *Lachnospiraceae unclassified*, *Lachnospiraceae NK4A136 group*, and *Bacteroidetes*. At the phylum level, Firmicutes and Bacteroidetes reflect the overall status of the gut microbiota in organisms, with Firmicutes containing more harmful bacteria and Bacteroidetes containing more beneficial bacteria. Therefore, the Firmicutes/Bacteroidota (F/B) ratio is often used as a marker to evaluate gut microbiota imbalance [43]. The findings indicate that, compared with the Control group, the LPS group showed an increase F/B ratio; in contrast, the F/B ratio in the PIRP-H group was markedly reduced, suggesting that PIRP can partially restore gut microbiota balance, as shown in Fig. 8H.

To better understand the comprehensive mechanism by which PIRP improved intestinal homeostasis in mice with ALI, a correlation analysis was conducted between the microbiota and lung injury indicators at the phylum level (Fig. 8I). At the phylum level, Bacteroidetes showed a significant difference from IL-1 β and exhibited a negative correlation with IL-1 β , NO, and MPO, while displaying a positive correlation with SOD and GSH-PX. In contrast, Firmicutes was negatively correlated with SOD and GSH-PX, positively correlated with IL-1 β , NO, and MPO, and showed a significant difference from MPO.

Linear discriminant analysis of effect size (LEfse) was used to examine the differences in microbiota across groups. Differences in microbiota between groups were analyzed by LEfSe (LDA > 2, $P < 0.05$), which was used to analyze the results at all levels of classification and to identify specific phylotypes in each group, with higher Linear Discriminant Analysis (LDA) scores for colony type across genera. The main indicator bacteria in the LPS group were *Streptococcus*, *Ruminiclostridium* and *Clostridium difficile* CIEAF012 compared to the control group (Fig. 8J). The *Streptococcus*, as a very large group of microorganisms, contains a number of potent pathogens such as *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Streptococcus agalactiae*, and is indisputable to be a hazard to the organism.^[44] After PIRP intervention, there was a significant increase in indicator species such as *Saccharimonadales*, *Patescibacteria*, *GCA_900066575*, and unculturable bacteria (Fig. 8K-M). Most microorganisms such as *Saccharimonadales*, *Patescibacteria*, and uncultured bacteria exist as commensal or beneficial flora, becoming potentially harmful only under specific host conditions. This indicates that PIRP can down-regulate the proportion of harmful bacteria while up-regulating the proportion of beneficial or commensal bacteria in the gut microbiota.^[45]

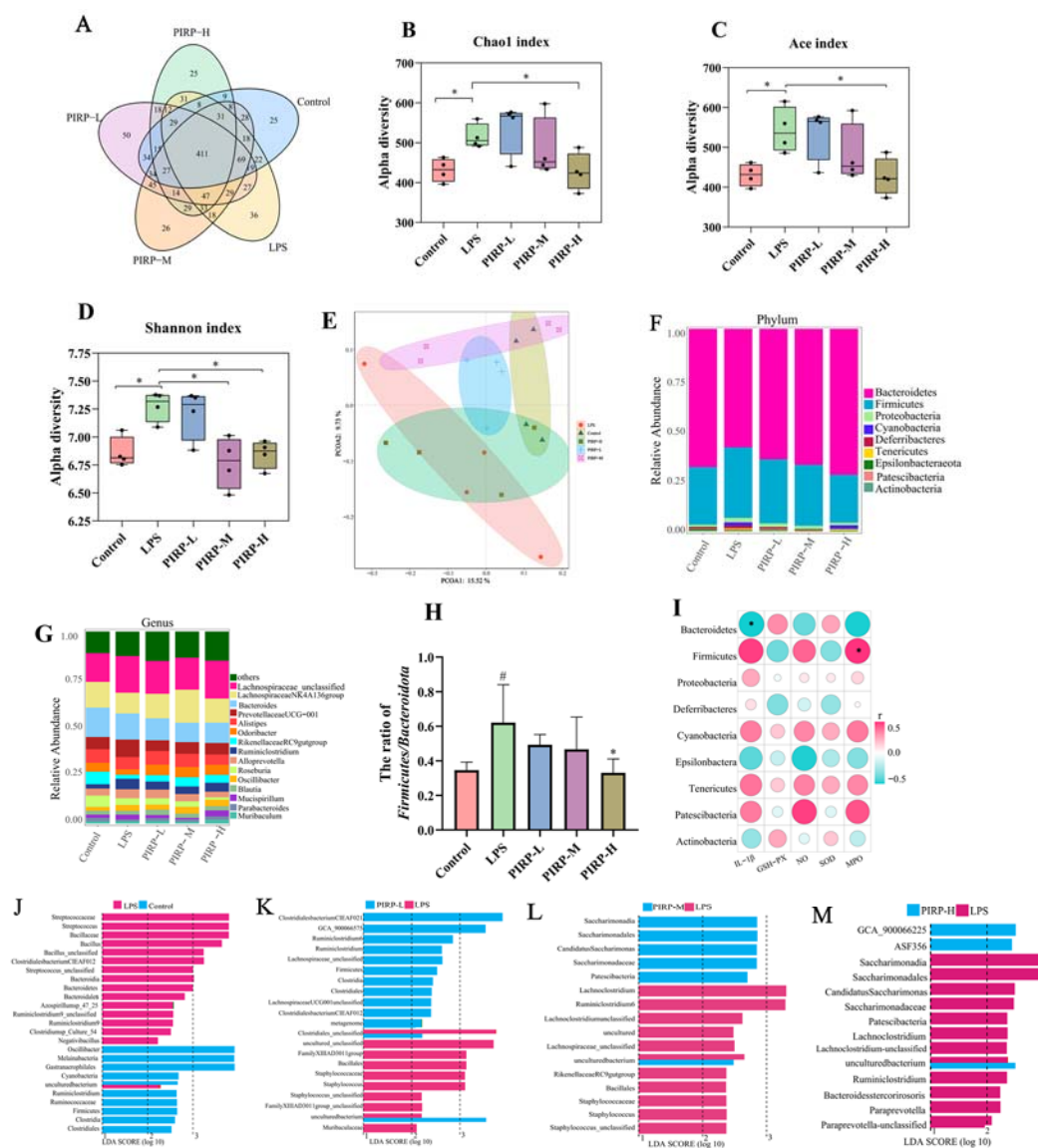


Fig. 8 PIRP regulates colonic microbiota diversity and composition in ALI mice. (A) OTU abundance. (B) Chao1 index. (C) Ace index. (D) Shannon index. (E) PCoA diagram. (F) Phylum level analysis. (G) Genus level analysis. (H) Phylum level F/B value. (I) Correlation diagram between the phylum-level microbiota and lung injury. The positive and negative correlations are shown by the colors pink and green, respectively. The correlation coefficient increases with color darkness. LefSe analysis of two-by-two comparisons of control, LPS, PIRP-L, PIRP-M and PIRP-H groups at the KEGG pathway level: Bivariate histograms of differential species in the intestinal flora of the Control and LPS group (J), LPS and PIRP-L group (K), LPS and PIRP-M group (L), LPS and PIRP-H group (M), $n = 4$. Data are expressed as mean $\pm$ SD. $^{###}P < 0.01$, $^{#}P < 0.05$ vs. Control group; $^{**}P < 0.01$, $^{*}P < 0.05$ vs. LPS group.

3.10 Effect of PIRP on metabolites in lung tissue of mice with ALI

The UPLC-Q-Exactive HF-X Orbitrap-MS analysis of metabolites in mouse lung tissue showed that a total of 555 metabolites were detected in the combined positive and negative ion modes. Based on the chemical classification information, the compounds were classified into 16 categories, as shown in Fig. 9A. Additionally, partial least squares discrimination analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) models were employed in this investigation to validate the separation of each group. As shown in the PLS-DA plot, the metabolites of the LPS and Control groups, as well as the three

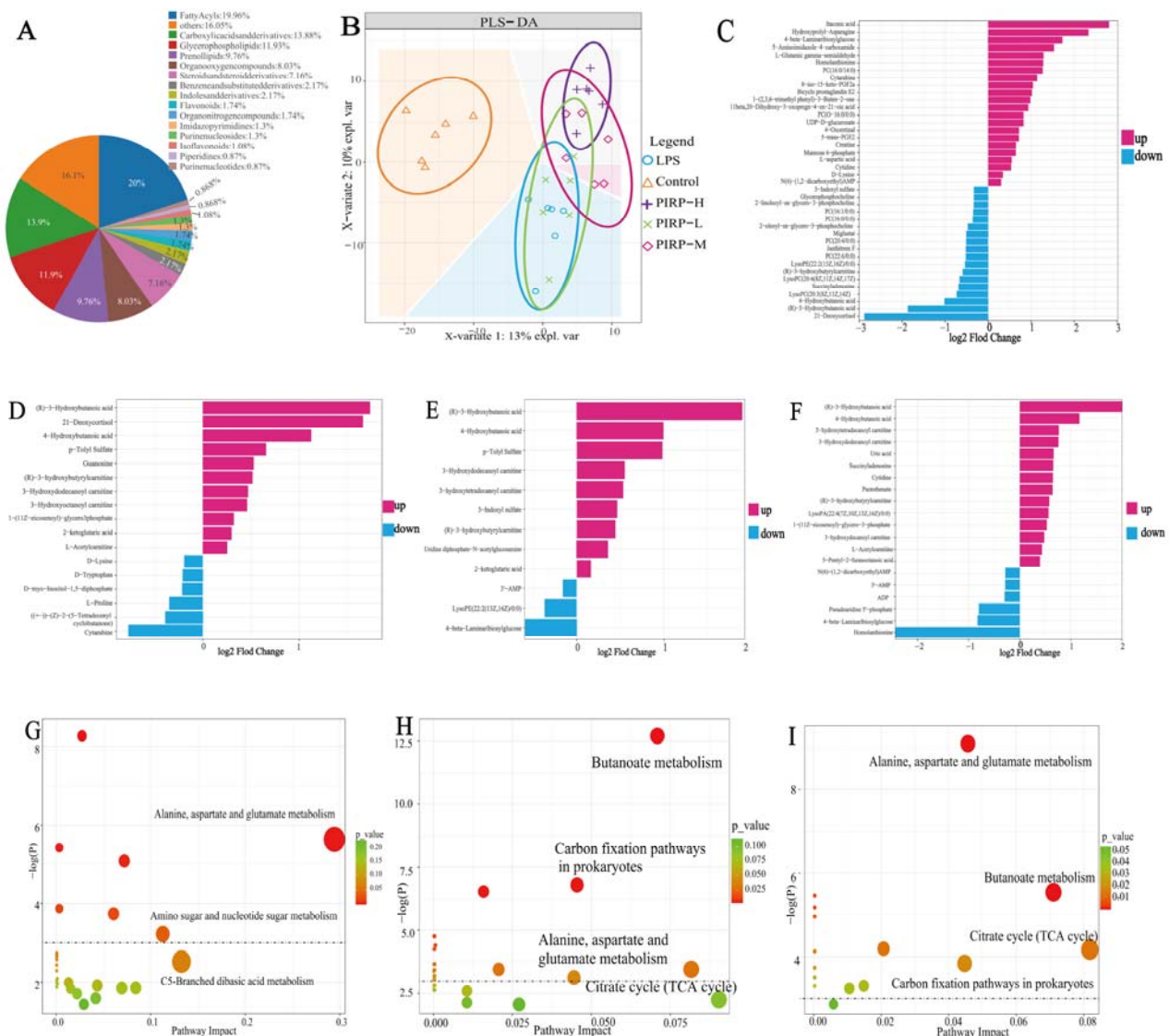
PIRP dose groups, were separated, and the PIRP group tended to approach the Control group, indicating significant differences in metabolism between the groups (Fig. 9B). The fit of the OPLS-DA model was verified for 200 permutations, and Q^2 and R^2 were found to be lower than the initial values, and the intersection of the regression line of Q^2 with the vertical axis was lower than zero ^[46]. (Fig. S20-S23.) The results indicate that the model is stable and does not suffer from overfitting.

Five hundred fifty-five metabolites with significant impact on grouping ($VIP > 1$, $P < 0.05$) were regarded as differentially expressed metabolites (DEMs). Compared to the control group, 24 DEMs were upregulated and 18 DEMs were downregulated in the LPS group. As shown in Fig. 9C-F, the PIRP-L group showed 17 DEMs compared to the LPS group (11 upregulated and 6 downregulated). The PIRP-M group exhibited 12 DEMs (9 upregulated and 3 downregulated), while the PIRP-H group demonstrated 20 DEMs (14 upregulated and 6 downregulated). These results indicate that PIRP alleviated LPS-induced lung tissue damage in mice by regulating metabolic changes.

3.11 Metabolic pathways and functional analysis of PIRP

We analyzed these metabolic pathways using MetaboAnalyst (MetPA) from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to identify potential pathways impacted by LPS or PIRP. The pathway analysis algorithm was configured to perform out-degree centrality for pathway topology analysis and hypergeometric tests for overrepresentation analysis. As shown in Fig. 9G-J, analysis of metabolites between the LPS and control groups included 28 metabolic pathways, including glycine, threonine, serine, pyruvate, amino acid sugar and nucleotide sugar, alanine, aspartic acid, glutamate, purine metabolism. Correspondingly, the LPS group had 12 DEMs that matched with metabolic pathways, further suggesting that these metabolites can serve as metabolic indicators for ALI, such as betaine, creatine, L-aspartic acid, (R) -3-hydroxybutyric acid, 4-hydroxybutyric acid, and UDP-D glucuronic acid, among others. In addition, analysis of the PIRP-L group identified 22 pathways, including glycine and threonine metabolism, serine metabolism, purine metabolism, and pyrimidine metabolism. The PIRP-M group showed 15 metabolic pathways, such as: glycine, threonine and serine metabolism, Tricarboxylic acid cycle (TCA cycle), and carbon fixation pathways in prokaryotes. The PIRP-H group involved 9 metabolic pathways, including purine metabolism, biosynthesis of pantothenic acid and coenzyme A, alanine, aspartate, and glutamate metabolism. There are a total of 15 metabolites involved in the three dose groups of PIRP, including L-aspartic acid, (R) -3-hydroxybutyric acid, 4-hydroxybutyric acid, and 2-ketoglutaric acid, N (6)-(1,2-dicarboxyethyl) AMP.

The impact factors of the metabolic pathways were analysed. Pathways with impact values above 0.1 are typically considered significant ^[47]. Therefore, to better understand the protective mechanism of PIRP against ALI, the metabolite list was transported to MetaboAnalyst 5.0, for pathway analysis. In this study, we used an impact value > 0.1 as the screening condition, and found that in the LPS group vs. the Control group, three metabolic pathways, including amino sugar and nucleotide sugar metabolism; alanine, aspartate, and glutamate metabolism; as well as C5-branched dibasic acid metabolism were significantly affected in lung



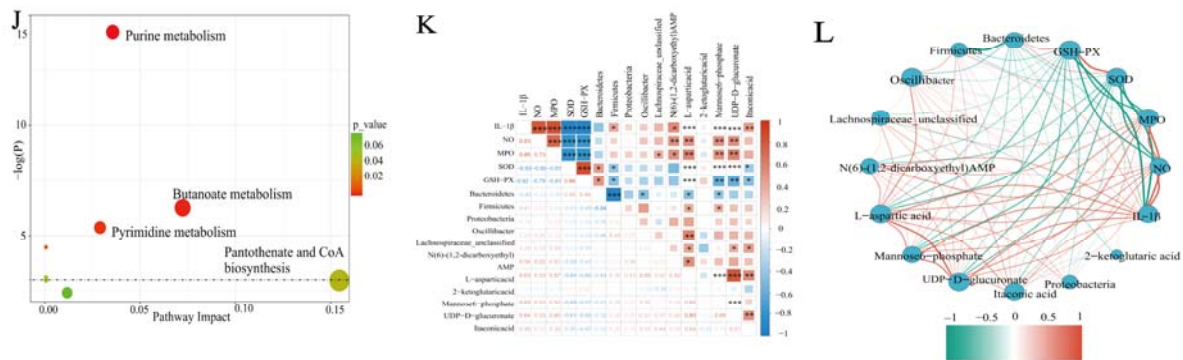


Fig. 9 Effect of PIRP on metabolites in the lung tissue of ALI mice. (A) Classification of metabolites. (B) PLS-DA diagram. Bidirectional bar chart of differentially abundant metabolites in lung tissue between the control and LPS groups (C), the LPS and PIRP-L groups (D), the LPS and PIRP-M groups (E), the LPS and PIRP-H groups (F). (C-F) Red and blue represent the upwards and downwards adjustments, respectively. Metabolic pathway diagram in lung tissue of Control vs. LPS group (G), LPS vs. PIRP-L group (H), LPS vs. PIRP-M group (I), LPS vs. PIRP-H group (J). (G-J) Each circle's size and color are determined by the P-value and route effect value; red and blue, respectively, stand for positive and negative correlations. (K) Correlation heatmap of lung injury, gut microbiota, and metabolites. The positive and negative correlations are shown by orange and blue, respectively. The degree of correlation is shown by the depth of color and the P-value. (L) Correlation network diagram of lung injury, gut microbiota, and metabolites. The orange color curve and green curve indicated the significant correlation, respectively. The correlation coefficient's size is indicated by the curve's thickness. The correlation coefficient increases with the thickness of the curve. $n = 6$. Data are expressed as the mean $\pm$ SDs. $^{##}P < 0.01$, $^{*}P < 0.05$ vs. Control group; $^{**}P < 0.01$, $^{*}P < 0.05$ vs. LPS group.

4. Discussion

ALI is marked by excessive inflammation and oxidative stress imbalance [48]. Consequently, natural functional foods with anti-inflammatory, antioxidant, antimicrobial, and immunomodulatory properties have been increasingly investigated as potential therapeutics [49]. The aim of this study was to demonstrate through *in vivo* modeling that PIRP can exert a protective effect against ALI through coordinated modulation of inflammatory signaling, redox homeostasis, mitochondrial integrity, and gut–lung axis crosstalk.

The monosaccharide composition of PIRP included Ara, Man, Glu, and Gal. Man targets macrophage mannose receptors and induces anti-inflammatory factor expression; Gal and Ara exhibit antioxidant activity; and Glu, as the main component of β -glucan, forms the core of immunomodulatory polysaccharides [50,51]. Consistent with this, PIRP markedly attenuated hallmark pathological features of ALI, including pulmonary edema (elevated wet/dry ratio), alveolar wall thickening, lobular disorganization, inflammatory cell infiltration, and increased capillary permeability—collectively preserving alveolar architecture and blood–air barrier integrity. We observed that PIRP effectively mitigated acute inflammatory responses in the lungs of the mice. During the ALI phase, there was a significant increase in inflammatory cells within the lung tissue, accompanied by a notable disruption of the lung lobule structure. In addition, alveolar wall thickening and capillary congestion were observed. The thickening of the alveolar walls, coupled with enhanced capillary permeability, leads to the leakage of plasma proteins into the alveolar space, subsequently compromising the integrity of the blood–air barrier and ultimately resulting in pulmonary edema [52]. The ensuing pulmonary edema triggered widespread infiltration of leukocytes into the lung tissue, and a surge in inflammatory cytokines could cause permanent lung damage [53]. The findings of this study demonstrate that PIRP intervention significantly reverses LPS-induced pathological changes, including an increased lung tissue W/D

weight ratio, alveolar wall thickening, disordered lung lobule structure, elevated inflammatory cell counts, and enhanced alveolar capillary permeability, indicating its potential value in ALI treatment.

The integrity of the lung-blood barrier is mainly maintained by tight junctions and gap junctions between alveolar epithelial cells. However, downregulation of occludin, claudin and ZO-1 proteins in airway epithelial cell structures has been associated with lung tissue injury in mice ^[54], while concurrently suppressing F4/80⁺macrophage activation. This dual action likely contributes to barrier stabilization and reduced leukocyte recruitment. Biochemically, PIRP reversed LPS-induced imbalances: it lowered pro-inflammatory mediators (IL-1 β , NO) in bronchoalveolar lavage fluid (BALF), enhanced systemic antioxidant defenses (SOD, GSH-Px), and suppressed myeloperoxidase (MPO) activity—indicating comprehensive mitigation of both inflammation and oxidative damage.

Numerous studies have suggested that the mitochondria are the primary source of intracellular Reactive Oxygen Species (ROS), with approximately 90% of the ROS generated within them. Mitochondria are the primary intracellular source of reactive oxygen species (ROS), and their dysfunction is a key driver of ALI pathogenesis ^[55,56]. In this study, we observed severe damage to the mitochondrial structure in LPS-induced ALI in mice. TEM revealed that the mitochondria were swollen, mitochondrial cristae structure was disrupted and disappeared. In severe cases, double membrane breakage occurred, fusing with other organelles, and the surrounding organelle structure was damaged with a reduced intercellular matrix. To further explore the antioxidant capacity of PIRP, we used TEM to closely observe the ultrastructure of lung tissue, with a particular focus on key organelles, especially the structural and quantitative changes of mitochondria. However, after intervention with PIRP, the degree of mitochondrial structural damage in mouse lung tissues was significantly reduced and normalised. These findings suggest that PIRP has a reparative impact on the levels of oxidative stress caused by LPS-induced mitochondrial damage and that it can mitigate LPS-induced mitochondrial dysfunction by shielding the mitochondrial structure from damage.

To gain deeper insight into the mechanisms by which PIRP reduces inflammation and alleviates oxidative stress in ALI, our study thoroughly investigated three important signalling pathways: MAPK, MyD88/TLR4/NF- κ B, and Nrf2/Keap1/HO-1, as well as the ALOX5 protein.

Through its activation of the NF- κ B signaling pathway, TLR4 plays a crucial role in regulating inflammation and immunological responses by promoting the production of pro-inflammatory cytokines ^[57]. Furthermore, adaptor molecules such as MyD88 are necessary mediators for the transduction of NF- κ B signal triggered by TLR4 ^[58]. In this study, the elevated expression of TLR4 and MyD88 in mouse lung tissue induced by LPS was significantly reduced by PIRP, suggesting that PIRP can prevent LPS from binding to TLR4 and MyD88 proteins and inhibit NF- κ B activation. This process further phosphorylates the P65 protein and promotes its translocation into the nucleus, thereby inducing an inflammatory response. PIRP inhibits LPS-induced P65 phosphorylation, thereby effectively inhibiting NF- κ B activation and reducing the production of inflammatory proteins and pro-inflammatory mediators.

Moreover, PIRP significantly inhibited ALOX5 expression—a key enzyme in leukotriene biosynthesis linked to neutrophil recruitment and vascular leakage in ALI^[59]. -further supporting its role in resolving inflammatory cascades.

The MAPK pathway, a classic inflammatory signalling pathway and the cornerstone of the eukaryotic inflammatory signal transmission network, is frequently considered a pivotal signalling axis for the suppression of inflammatory diseases^[60]. Research has shown that LPS within lung macrophages necessitates the engagement of MAPK to stimulate the generation of proinflammatory mediators and enzymes^[61]. Additional studies have proposed that inhibiting the activation of P38, jnk, and erk1/2 represents a potential therapeutic approach for mitigating ALI, thus offering novel insights into the treatment of this condition^[62]. The findings of this research unequivocally show that PIRP prevents P38, jnk, and erk1/2 protein phosphorylation. These results further confirm that MAPK pathways are potential therapeutic targets for PIRP in alleviating ALI, providing a strong theoretical basis for the application of PIRP in ALI treatment.

Nrf2 is a key signalling pathway that regulates the oxidative stress response in the body, primarily by promoting the expression of downstream HO-1 to exert antioxidant effects. Under physiological conditions, Keap1 and Nrf2 form a complex in which Keap1 negatively regulates Nrf2^[63]. In response to oxidative stress or other chemical stimuli, Nrf2 rapidly dissociates from Keap1 and translocates to the nucleus. It binds to particular gene promoter sequences to activate the expression of downstream genes like HO-1 and has anti-inflammatory and antioxidant effects. Previous research has revealed that oxidative stress is an upstream factor leading to lung pathology, and inflammation resulting from lung injury further exacerbates oxidative stress^[64]. Therefore, We looked into the molecular processes that underlie PIRP's antioxidant benefits in ALI-affected mice. Our results demonstrate that PIRP effectively downregulates Keap1 while upregulating Nrf2 and HO-1 protein levels. This suggests that PIRP responds to oxidative stress through the Nrf2/Keap1/HO-1 signalling pathway, thereby significantly improving LPS-induced lung peroxidation.

In many lung diseases, the close relationship between the composition of the gut microbiota and changes in lung metabolites and lung injury has received increasing attention. Gut bacteria not only play a crucial role in maintaining local immune function but also regulate systemic immune responses. Specifically, the gut microbiota is essential for maintaining immune system health, promoting lung injury recovery, and preventing bacterial translocation^[65]. These significant discoveries offer a fresh perspective on the pathogenic mechanisms underlying lung disorders by illuminating the intricate relationship between the gut and lungs ,and highlighting the critical role that gut microbiota plays in lung health and disease development. In this study, we discovered that following LPS induction, the abundance of gut microbiota in mice was altered considerably. Notably, PIRP administration significantly reduced the LPS-induced alterations in gut microbiota composition. Further analysis suggested that the dominant phyla Bacteroidetes and Firmicutes exhibited significant correlations with five lung injury indicators. Specifically, Bacteroidetes was positively correlated with SOD and GSH-PX, while negatively correlated with NO; conversely, the harmful Firmicutes were negatively correlated with SOD and GSH-PX and positively correlated with NO. Thus, PIRP modulates

the gut microbiota to mediate anti-inflammatory and antioxidant effects systemically. This aligns with established mechanisms whereby monosaccharides like galactose and arabinose—components of PIRP—promote gut microbial production of short-chain fatty acids (SCFAs, e.g.), which in turn suppress inflammatory pathways [66]. The presence of these functional monosaccharides in PIRP likely contributes to its ability to restore microbial balance and attenuate inflammation.

Predictive functional analysis of differences in the gut microbiota differences indicated that an imbalance in the gut microbiota imbalance significantly affects the amino acids, carbohydrates, cofactors, and vitamins metabolism. Metabolomic analysis further linked gut dysbiosis to perturbed amino acid metabolism in ALI, particularly in alanine/aspartate/glutamate and glycine/serine/threonine pathways—processes previously implicated in ALI recovery [67–69]. In this work, we found that LPS stimulation disrupted amino acid metabolic pathways, and PIRP treatment effectively ameliorated these metabolic disturbances. These pathways included alanine, aspartate, and glutamate metabolism and glycine, serine, and threonine metabolism. This suggests that amino acid metabolic pathways serve as important connections in gut-lung interactions. Finally, we conducted a comprehensive correlation analysis among lung injury indicators, the five dominant gut microbiota at the phylum level, and six related differential metabolites at the metabolic level. These findings further suggest that gut microbiota may influence ALI progressive in mice, as evidenced by strong correlations between these five gut bacterial phyla and lung injury scores. Furthermore, most DEMs were positively or negatively correlated with the gut microbiota, once again demonstrating the connection between lung function and gut microbiota, as well as the association between metabolic and gut microbiota disorders and ALI (Fig. 10).

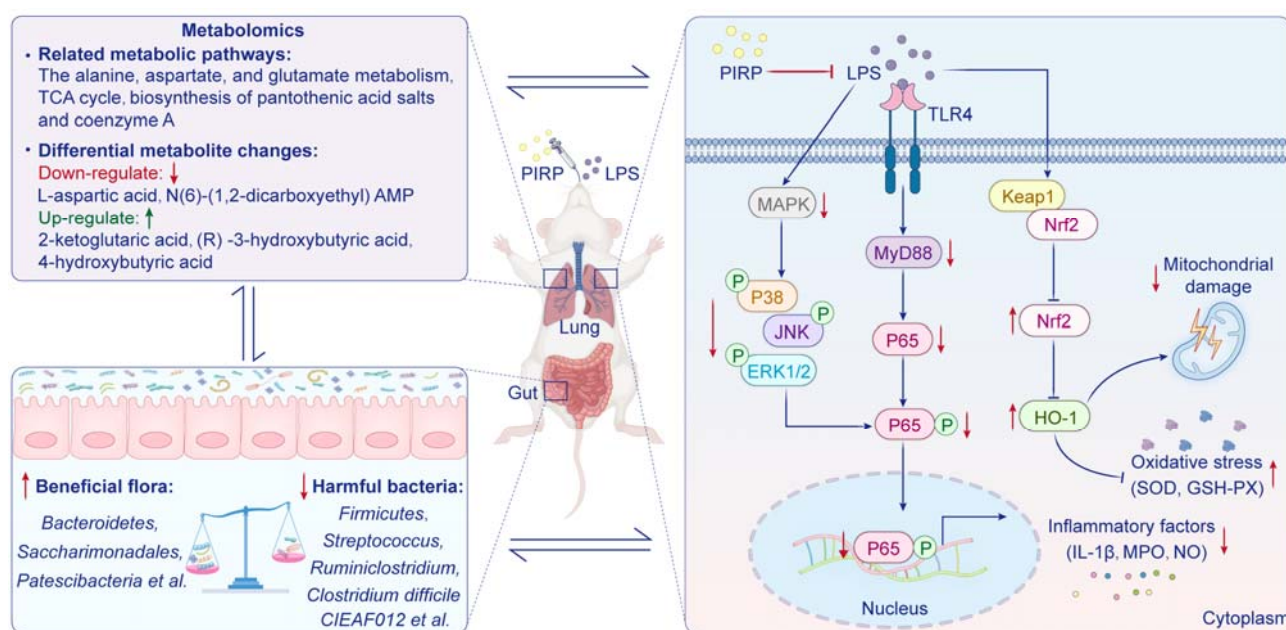


Fig. 10 Schematic representation of the molecular mechanisms by which PIRP improves lung tissue in LPS-induced ALI mice.

This study elucidates the protective effects of PIRP against ALI in mice and provides a scientific foundation for developing novel ALI therapeutics. However, while we demonstrated that PIRP's mechanism

likely involves attenuating inflammation, restoring oxidative balance, modulating microbiota composition, and altering lung tissue metabolites, key aspects remain unresolved: the precise pathways through which PIRP regulates gut microbiota were not determined, the functional contribution of microbiota remodeling to ALI protection requires validation, and the metabolomics-predicted signaling pathways need experimental confirmation. Further investigation is therefore essential to fully characterize PIRP's protective mechanisms against ALI.

5. Conclusion

In summary, our findings demonstrate the complex mechanisms of PIRP in treating ALI, including attenuation of inflammatory responses, modulation of oxidative stress, and regulation of the abundance of the gut microflora abundance and lung tissue metabolites. This study provides better ideas for development of the therapeutic approaches for ALI, which are important for disease alleviation. Furthermore, as PIRP represents a functional food ingredient, this study establishes a foundation for its future application and development.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/>.

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