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Protective effect and mechanism of baicalin on lung inflammatory injury in BALB/cJ mice induced by PM2.5

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ABSTRACT

The public health harms caused by fine particulate matter (PM2.5) have become a global focus, with PM2.5 exposure recognized as a critical risk factor for global morbidity and mortality. Chronic inflammation is the common pathophysiological feature of respiratory diseases induced by PM2.5 and is the most critical cause of all these diseases. However, presently there is a lack of effective preventive and therapeutic approaches for inflammatory lung injuries caused by PM2.5 exposure. Baicalin is a herb-derived effective flavonoid compound with multiple health benefits. This study established a murine lung inflammatory injury model via inhalation of PM2.5 aerosols. The data showed that after baicalin intervention, lung injury pathological score of baicalin (4.16 ± 0.54, 3.33 ± 0.76, 4.00 ± 0.45) and claricid (3.00 ± 0.78) treatments were markedly lower than PM2.5-treated mice (6.17 ± 0.31), and pathological damage was alleviated. Compared to the PM2.5 group, the spleen and lung indexes in the baicalin and claricid groups were significantly reduced. The inflammatory cytokines of TNF-α, IL-18, and IL-1β in serum, alveolar lavage fluid, and lung tissue were significantly decreased in the baicalin and claricid groups. The expressions of inflammatory pathway-related genes and proteins HMGB1, NLRP3, ASC, and caspase-1 were up-regulated in the PM2.5 group. The expressions of these genes and proteins were significantly decreased following baicalin treatment. The lung function indicators showed that the MV (65.94 ± 8.19 mL), sRaw (1.79 ± 0.08 cm H2O·s), and FRC (0.52 ± 0.01 mL) in the PM2.5 group were higher than in the control and baicalin groups, and respiratory function was improved by baicalin. PM2.5 exposure markedly altered the bacterial composition at the genus level. The dominant flora relative abundances of uncultured_bacterium_f_Muribaculaceae, Streptococcus, and Lactobacillus, were decreased from the control group (9.20%, 8.53%, 6.21%) to PM2.5 group (6.26%, 5.49%, 4.77%), respectively. Following baicalin intervention, the relative abundances were 9.72%, 6.65%, and 3.57%, respectively. Therefore, baicalin could potentially prevent and improve mice lung inflammatory injury induced by PM2.5 exposure. Baicalin might provide a protective role by balancing oropharyngeal microbiota and affecting the expression of the HMGB1/Caspase1 pathway.

1. Introduction

Research indicates that 87% of the global population lives in an environment where the average annual PM2.5 concentration exceeds the concentration specified in the WHO Air Quality Guidelines (Brauer et al., 2016). According to epidemiological studies, various lung diseases and respiratory symptoms are attributed to exposure to the environmental PM2.5 (Chen et al., 2019; Cohen et al., 2017; Lin et al., 2018; Sacramento et al., 2020). Inflammation is the most critical event in all of these diseases (Racanelli et al., 2018; Shamsollahi et al., 2021). However, long-term anti-inflammatory drug use is likely to cause many side effects, including drug resistance and bacterial community disorder in

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the body (Qiao et al., 2018). Therefore, identifying novel preventive and therapeutic approaches for inflammatory injury induced by PM2.5 exposure is critical and is increasingly a focus of researchers in the field. Baicalin is an effective herb-derived flavonoid compound extracted from the root of Scutellariabaicalens (Li et al., 2021). Due to its good stability and bioactivity, baicalin has the potential to activate and modulate diverse relevant mechanisms and signaling pathways (Chen et al., 2014; Gong et al., 2017), which are widely applied in traditional Chinese medicine (TCM). Liu and Liu (2017) found that baicalin could improve depressive behavior induced by chronic unpredictable mild stress though inhibiting NLRP3 activation. Zhu et al. (2022) reported that baicalin could alleviate LPS-induced acute lung injury. However, despite its great promise, few studies have examined the anti-inflammatory effects of baicalin against PM2.5-induced inflammatory injury, and the specific protective mechanism remains to be investigated. The current study developed a PM2.5 exposure model in mice, which was used to explore the role of baicalin in lung inflammatory injury. HE was used to confirm pathological changes in lung tissues. The changes of inflammatory cytokines in mice were evaluated by ELISA, and the alteration of microbiota in upper respiratory tract was estimated by 16sRNA. Immunohistochemistry (IHC), qRT-PCR, and westernblots were subsequently applied to monitor the effects of PM2.5 and/or baicalin on the expression of inflammation-related genes and cytokines. This study was designed to investigate whether baicalin played a specific protective role against PM2.5 toxicity and to define the potential mechanisms underlying the effects of baicalin on PM2.5-induced lung injury in mice. It is anticipated that the present study will provide information for clinical uses of baicalin and demonstrate whether it is a possible candidate for preventing PM2.5-induced respiratory diseases.

2. Materials and methods

2.1. Collection and preparation of PM2.5

A high-volume PM2.5 particulate collector Tisch Environ-Mental Inc. (America), and nitrocotton filter membrane (General Electric Company GE, America), were used to collect PM2.5 samples (Zhong et al., 2019). The sampling site was at the gate of Shenyang Medical College, North Huanghe Street, Shenyang City, Liaoning Province, and sampling was performed at a height of 1.5 m. The sampling flow was 1000 L/min, and sampling was performed continuously for 24 h every day during China's winter season. After sampling, the filter membrane was cut to1 cm^2 and filtrate was collected with six layers of sterile gauze after filtration. Then the filtrate was placed into a vacuum condition for freeze-drying. The samples were then frozen and stored at − 80 ℃ in a refrigerator.

2.2. Animals and PM2.5 exposure

All animal protocols utilized in this research complied with the guidelines of the Animal Protection Law of the People's Republic of China, 2009. The research was approved by the Laboratory Animal Ethics Committee of Shenyang Medical College (NO. SYYXY 2021061502). Seventy-two adult male BALB/cJ mice (age: 7–8 weeks; weight: 20 ± 2 g) were provided by Beijing Charles River Laboratory Animal Technology Co., LTD (Beijing, China). The mice were maintained at 22–24 °C and 50–60% humidity under a 12-h light-dark cycle in specific pathogen-free (SPF) facilities. After one week of routine feeding, the mice were divided into six groups (12 mice/group): control group (saline solution), PM2.5 exposure group (saline solution), PM2.5 +baicalin (25 mg/kg), PM2.5 +baicalin (50 mg/kg), PM2.5 +baicalin (100 mg/kg), and PM2.5 +claricid (65 mg/kg). Mice in the experimental groups were treated with PM2.5 at a concentration of 750 μg/m^3 (Yang et al., 2018a) for four hour/d over six weeks within a single-concentration oral-nasal exposure system (Beijing Huironghe Science and Technology Co., LTD.). The control group was not treated with PM2.5 dust but was maintained under the same feeding and housing conditions. The intervention groups were given baicalin or claricid by intragastric administration every day, 1 h before PM2.5 exposure. In contrast, the control and model groups were treated with an equal amount of normal saline. The schematic of the experimental design is shown in Figure1A.

2.3. Body, spleen, and lung weights

Mice body weights were analyzed on days 0, 5, 10, 15, 20, 25, 30, 35, 40, and 43. After the above treatments for six weeks, all mice were sacrificed, and serum, bronchoalveolar lavage fluid (BALF), and spleen and lung tissue were collected for further experiments. The organ index was calculated using the formula: organ index = organ weight (g)/ body weight (g)× 100%.

2.4. Non-invasive measurement of lung function

After six weeks of PM2.5 dust exposure and intervention, non-invasive double chamber plethysmography (Buxco systems, USA) was performed to analyze the airway function of the mice, as previously described (Alghetaa et al., 2018; Sultan et al., 2021). The respiratory flow was measured using a pneumotachograph and transmitted to Buxco Fine Pointe software to calculate the respiratory frequency (f), minute volume (MV), functional residual capacity (FRC), and specific airway resistance (sRaw) of the lungs. Each mouse was placed in a two-compartment scanner, and a moderate size rubber neck pad was fixed on its neck and waist. After the mouse was stable and quiet, data were collected continuously and dynamically for 5 min.

2.5. Hematoxylin-eosin and immunohistochemistry staining of lung tissues

The morphological changes in the lung samples were detected using Hematoxylin-Eosin (HE) and immunohistochemistry (IHC) staining. Each mouse's upper lobe of the right lung was removed from the chest and fixed in 4% neutral buffered formaldehyde. Then, the tissues were pruned, dehydrated, embedded, sectioned, stained, and sealed strictly according to the pathological test's standard operating procedure (SOP). Finally, the tissue sections were observed in detail under multiple different scopes by two pathologists blinded to each animal's conditions. Mc Guigan's pathological score (Kim et al., 2020; Zhou et al., 2019) was used to evaluate the degree of lung injury. The observation indicators were as follows: 1, alveolar hyperemia; 2, bleeding; 3, pulmonary macrophages, neutrophils, and other inflammatory cells infiltrated or aggregated in the vascular wall or alveolar cavity; and 4, thickening of alveolar wall or formation of a transparent membrane. The lesion degree was scored as follows: 0, no lesions or slight lesions; 1, mild; 2, moderate; 3, severe. Immunohistochemistry was detected with the SP (streptavidin peroxidase) method. Briefly, lung tissues were deparaffinized, antigen repaired, serum closed, and stained with primary antibodies against NLRP3 (1:200, ABclonal), Caspase-1 (1:100, ABclonal), and HMGB1 (1:100, ABclonal) overnight at 4 ℃ and then stained with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Images of the immunohistochemical sections were collected, and the staining status was analyzed using an image analysis system. The stain grades (I) were classified as follows: 0, no staining (negative); 1, light yellow (weak positive); 2, brownish yellow (medium positive); 3, tan (strongly positive). Next, the staining areas were measured and recorded for statistical analysis. The histochemistry score (H-score) was analyzed. The tissue staining was semi-quantified: H-score (H-score $=\sum$ (PI ×I)) = (percentage of weak intensity area ×1) + (percentage of moderate intensity area ×2) + (percentage of strong intensity area×3), where PI is the ratio of the positive pixel area and I is the grade of stain.(Craig et al., 2021; Ram et al., 2021).

2.6. Enzyme-linked immunosorbent assays (ELISA)

BALF, serum, and lung tissues were collected from the mice in each group. The BALF, serum was centrifuged at 3000 g for 10 min at 4 ℃, and the supernatant collected and stored at − 80 °C. The weighed lung tissues were homogenized with PBS at a ratio of 1:9, and then centrifuged at 3000 g for 20 min at 4 ℃. The supernatant was collected and kept at − 20 ℃ in a refrigerator until analysis. IL-1β, IL-18, and TNF-α levels in the serum, BALF, and lung samples were assessed using specific ELISA kits (mlbio, China) according to the manufacturer's instructions. Standard wells, blank wells, and sample wells were set on the HRP-coated plate. 50 μl of different concentrations of standards were added to the standard wells, and no sample or HRP-conjugated reagent was added to the blank wells. 40ul of sample diluent and 10ul of each sample were added to the sample wells. After adding the enzyme, incubating, washing, and adding the stop solution, the absorbance was analyzed at 450 nm. All samples were detected in triplicate simultaneously.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA of lung tissues was extracted using Trizol (Invitrogen), and its concentration and purity were detected by a nucleic acid concentration detector (Nanodrop2000; Thermo Fisher Scientific, USA). A PrimeScript™ RT Reagent Kit (Perfect Real Time) was used for reverse transcription. The primers used in this research were synthesized by Sango Biotech Company (Shanghai, China) and Ding Guo Chang Sheng Biotechnology Co. LTD. (Beijing, China). qPCR was carried out using an SYBR Premix Ex Taq II (TaKaRa) reaction system on an ABI 7500 Real-time PCR System. The relative mRNA levels were measured using the $2^{-\Delta\Delta CT}$ method with GAPDH as the internal reference.

2.8. Westernblotting

Lung tissue samples (mass 10 mg) were weighed and placed in a centrifuge tube. Pre-cooled RIPA buffer and 1% phosphatase inhibitor were added, and two steel balls were placed in the tube. The centrifuge tube was homogenized in a tissue homogenizer. The sample lysate was centrifuged at 12 000 g for 15 min at 4 °C, and the protein was collected. A BCA kit (Beyotime, Shanghai, China) was used for protein quantification. Proteins were submitted to sodiumdodecyldsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). 50 μg of proteins from each group were separated through SDS-PAGE and transferred to a 0.22 μm Nitrocellulose membrane (NC membrane). After being blocked with 5% nonfat milk in Tris-Buffered Saline and Tween 20 (TBST), the proteins were incubated with NLRP3 (1:1000, ABclonal, China), Caspase-1 (1:1000, ABclonal, China), ASC (1:1000, ABclonal, China), HGMB1 (1:1000, ABclonal, China), TNF-α (1:1000, Proteintech, China), IL-18 (1:1000, ABclonal, China), IL-1β (1:1000, ABclonal, China), or β-actin (1:20000, ABclonal, China) antibodies overnight at 4 °C, and then with the secondary antibody at room temperature for one hour. The proteins were detected using a Tanon High-sig ECL kit following the product instructions. Gray values were analyzed by Image-J, and the fold expression was taken as the relative protein level, using β-actin as the internal reference.

2.9. 16SrRNA

After six weeks of PM2.5 dust exposure and intervention, sterile cotton swabs were used to collect posterior oropharyngeal wall samples from the experimental mice. Five repeated samples were collected in each group. After labeling and sampling, they were immediately placed into ice boxes and stored in a refrigerator at − 80 ℃. The samples were then analyzed to determine the composition of the upper respiratory tract. High-through put sequencing was performed to evaluate changes in the microbiota of the upper respiratory tract by targeting the v3-v4 region of the 16SrRNA gene.

2.10. Statistical analysis

Data are presented as the mean ± standard error (SE). Statistical analyses were performed using SPSS22.0 software, and graphs were constructed using GraphPad Prism8.0 software. Differences between groups were analyzed using the one-way Analysis of Variance (ANOVA), followed by the least significant difference (LSD) multiple-range test or Tamhane's test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Baicalin improved the lung inflammatory injury symptoms and reversed the lung histological alterations induced by PM2.5

The body weights of the mice were recorded every five days during the animal study. Unexpectedly, there were no significant differences in the mice's body weights in the different groups (Fig. 1B). The spleen and lung indexes of the PM2.5 group were 3.33 ± 0.42% (Fig. 1C) and 6.97 ± 0.37% (Fig. 1D), respectively, which were markedly higher than the control group. Baicalin (25, 50,100 mg/kg) or claricid (65 mg/kg) treatment led to significant reductions in the spleen and lung indexes compared with the PM2.5 group, $P < 0.05$ (Fig. 1C and D). Histological evaluations (Fig. 1F) showed that lung tissue samples from the control group had normal bronchial and alveolar structures. Tissues from the PM2.5-treated group exhibited alveolar wall thickening with prominent inflammatory cell infiltration, including infiltration of macrophages, lymphocytes, and neutrophils. Moreover, local bleeding was observed. Alveolar swelling and bronchial epithelium disorders were.

occasionally seen. The lung injury score of the PM2.5-treated mice was (6.17 ± 0.31), which was markedly higher than the control group (2.17 ± 0.65) ($P < 0.001$) (Fig. 1E). The pathological scores of baicalin groups and claricid group were significantly reduced, indicating that both could effectively alleviate pathological damage ($P < 0.05$).

3.2. Baicalin improved the lung function of mice exposed to PM2.5

The respiratory frequency of the mice after exposure to PM2.5 (301.52 ± 3.82 BPM) was markedly increased in comparison to the control group (282.72 ± 6.11 BPM), $P < 0.05$ (Fig. 2A). The minute volume in the PM2.5 group (65.94 ± 8.19 mL) was markedly lower than the control.

group (79.30 ± 10.43 mL), $P < 0.05$. The minute volumes of the baicalin groups (50 mg/kg, 100 mg/kg) and claricid group (65 mg/kg/day) were significantly increased in comparison to the PM2.5 group, $P < 0.05$ (Fig. 2B). sRaw in the PM2.5 group (1.79 ± 0.08 cmH2O·s) was markedly higher in comparison to the control group (1.21 ± 0.02 cmH2O·s) and the baicalin group (1.19 ± 0.05 cmH2O·s), $P < 0.05$ (Fig. 2C). The FRC in the PM2.5 group (0.52 ± 0.01 mL) was significantly increased in comparison to the control group (0.41 ± 0.04 mL) but was markedly decreased after treatment with baicalin (50 mg/kg) and claricid (65 mg/kg), $P < 0.05$ (Fig. 2D).

3.3. Baicalin regulated inflammatory cytokines

To confirm the effect of baicalin on inflammatory cytokine levels after continuous exposure to PM2.5, TNF-α, IL-1β, and IL-18 levels in serum (Fig. 3C-E), (BALF) (Fig. 3F-H), and lung tissue homogenate (Fig. 3I-K) were confirmed using ELISA. The results indicated that TNF-α, IL-1β, and IL-18 levels in serum, BALF, and lung tissue, in the PM2.5 group markedly increased compared to the control group ($P < 0.05$). Following treatment with 50 mg/kg or 100 mg/kg baicalin, or 65 mg/kg claricid, the TNF-α, IL-1β and IL-18 levels in serum, BALF, and lung tissue were markedly decreased in comparison to the PM2.5 group ($P < 0.05$). The levels of these three inflammatory cytokines in the 25 mg/kg group also exhibited downward trends. However, the IL-1β and IL-18 levels in serum, IL-18 levels in BALF, and TNF-α levels in

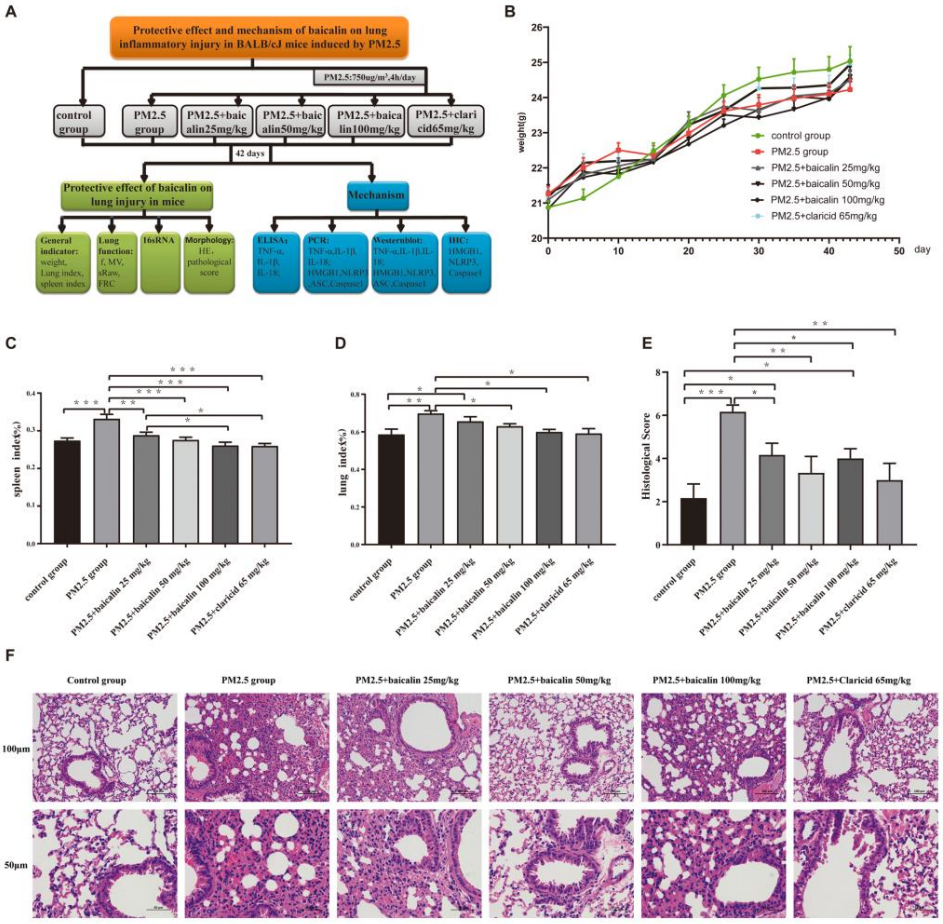

Fig. 1. (A) Schematic of the experimental design. (B) Changes in body weight (n = 12 per group). (C) Spleen index in mice (n = 12 per group). (D) Lung coefficient in mice (n = 6 per group). Spleen and lung indexes were analyzed using: organ index= organ weight (g)/body weight (g)× 100%. (E) Pathological scores among groups (n = 6 per group). (F) HE staining of lung tissues. For the upper pane, scale bar= 100 μm; for the lower pane, scale bar= 50 μm.

serum and lung tissue were not markedly decreased compared to the PM2.5 group ($P > 0.05$).

Simultaneously, the mRNA and protein levels of the inflammatory cytokines were determined using PCR and Western blots. Compared with the PM2.5 group, the mRNA and protein levels of TNF-α, IL-1β, and IL-18 ($P < 0.05$, Fig. 4) decreased in the 50 and 100 mg/kg baicalin groups.

3.4. Baicalin reduced the mRNA and protein levels of HMGB1, NLRP3, ASC, and caspase-1

The mRNA expression levels of HMGB1, NLRP3, ASC, and Caspase-1 were determined using qRT-PCR. PM2.5 exposure significantly increased.

the mRNA expression levels of HMGB1, NLRP3, ASC, and Caspase-1, while baicalin treatment markedly decreased the expression levels of these mRNAs (Fig. 5A-D). Moreover, the Westernblot results indicated that 50 and 100 mg/kg baicalin treatments and 65 mg/kg claricid treatment markedly inhibited the protein expression levels of HMGB1, NLRP3, ASC, and Caspase-1 (Figures5E-I). The immunofluorescence assay demonstrated that the fluorescence intensities of HMGB1, NLRP3, and caspase-1 in the PM2.5 group were markedly increased. In contrast, the fluorescence intensities of HMGB1, NLRP3, and caspase-1were significantly decreased in the 50 and 100 mg/kg baicalin groups, and the 65 mg/kg claricid group (Fig. 6). These results suggested that baicalin suppressed HMGB1, decreased the expression of NLRP3, ASC, and caspase-1, and attenuated the inflammatory injury induced by PM2.5.

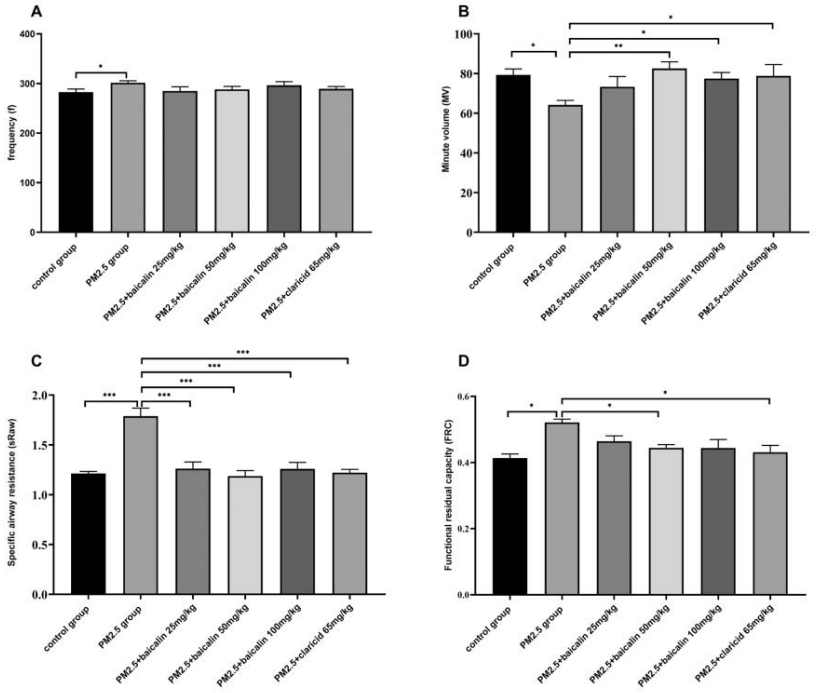

Fig. 2. The effect of baicalin on the lung function of mice treated with PM2.5. (A) Frequency (f), BPM. (B) Minute volume (MV), mL. (C) Specific airway resistance (sRaw), cmH2O•s. (D) Functional residual capacity (FRC), mL. The data are expressed as the mean±SEM (n = 12 per group). The variance in the MV, sRaw, and FRC variables is not uniform. The Welch ANOVA or Brown-Forsythe ANOVA method was used for statistical analyses, and Tamhane's test was used for multiple comparisons.

3.5. Effect of baicalin on oropharyngeal microbiota diversity modulation

The oropharyngeal microbiota of mice was analyzed according to 16SrRNA amplicon sequencing to investigate the effect of baicalin on the overall structure of the oropharyngeal microbiota. The dominant genera of the control group were uncultured_bacterium_f_Muribaculaceae (9.20%), Streptococcus (8.53%), Lactobacillus (6.21%), and uncultured_bacterium_f_Lachnospiraceae (4.04%) (Fig. 7 A). However, PM2.5 exposure markedly altered the bacterial composition at the genus level, and the relative abundances of uncultured_bacterium_f_Muribaculaceae, Streptococcus, Lactobacillus, and uncultured_bacterium_f_Lachnospiraceae were decreased to 6.26%, 5.49%, 4.77%, and 2.46%, respectively (Fig. 7 A). In contrast, the relative abundances of uncultured_bacterium_f_Muribaculaceae, Lactobacillus, and uncultured_bacterium_f_Lachnospiraceae were increased after treatment with baicalin (9.72%, 6.65%, and 3.57%, respectively) and claricid (17.14%,9.18%, and 5.66%, respectively). The relative abundances of uncultured_bacterium_f_Muribaculaceae,Lactobacillus, and uncultured_bacterium_f_Lachnospiraceae in the baicalin and claricid groups were higher compared with the PM2.5 group. Moreover, PM2.5 markedly upregulated the relative abundance of Muribacter (39.12%), while it was significantly decreased in the baicalin (20.38%) and claricid (0.02%) groups (Fig. 7 A).

A heatmap of species abundance at different taxonomic levels is shown in Fig. 7B. The values shown in the heatmap are z-scores generated by the z-normalization of the relative species abundance. The colors from blue to red indicate the species' relative abundance at each row, and the color gradient (blue to red) represents the relative richness (low to high). β-diversity was analyzed using principal component analysis (PCA) of the weighted UniFracdistances. A significant difference in the oropharyngeal microbiota was observed between the PM2.5 and control groups. Administration of baicalin and claricid eased the shift in the oropharyngeal microbiota caused by PM2.5 (Fig. 7C). The Chao1 and Shannon indexes were used to evaluate α-diversity. The Chao1 index did not differ significantly among the groups (Fig. 7D). The Shannon index was markedly decreased in the PM2.5group compared with the control group ($P < 0.05$). In contrast, the Shannon index was significantly upregulated in the baicalin and claricid groups compared to the PM2.5 group ($P < 0.05$, Fig. 7E).

The diversity of the oropharyngeal microbiota between the groups was evaluated using the histogram of Line Discriminant Analysis (LDA) value distribution, and evolutionary branch graph of the LDA effect size (LEfSe) (Segata et al., 2011). An LDA score histogram (Fig. 7F-G) was constructed to screen for significant markers and identify the dominant microorganisms in the different groups. Dominant communities of 6, 8, and 26 taxa were identified in the PM2.5, baicalin, and claricid groups, respectively. Among these, o_pasteurellales and f_pasteurellaceae dominated in the PM2.5 group, while S_uncultured_bacterium_g_Rodentibacter and g_Rodentibacter were dominant microbes in the baicalin group. Finally, p_Firmicutes and O_Clostridiales dominated the

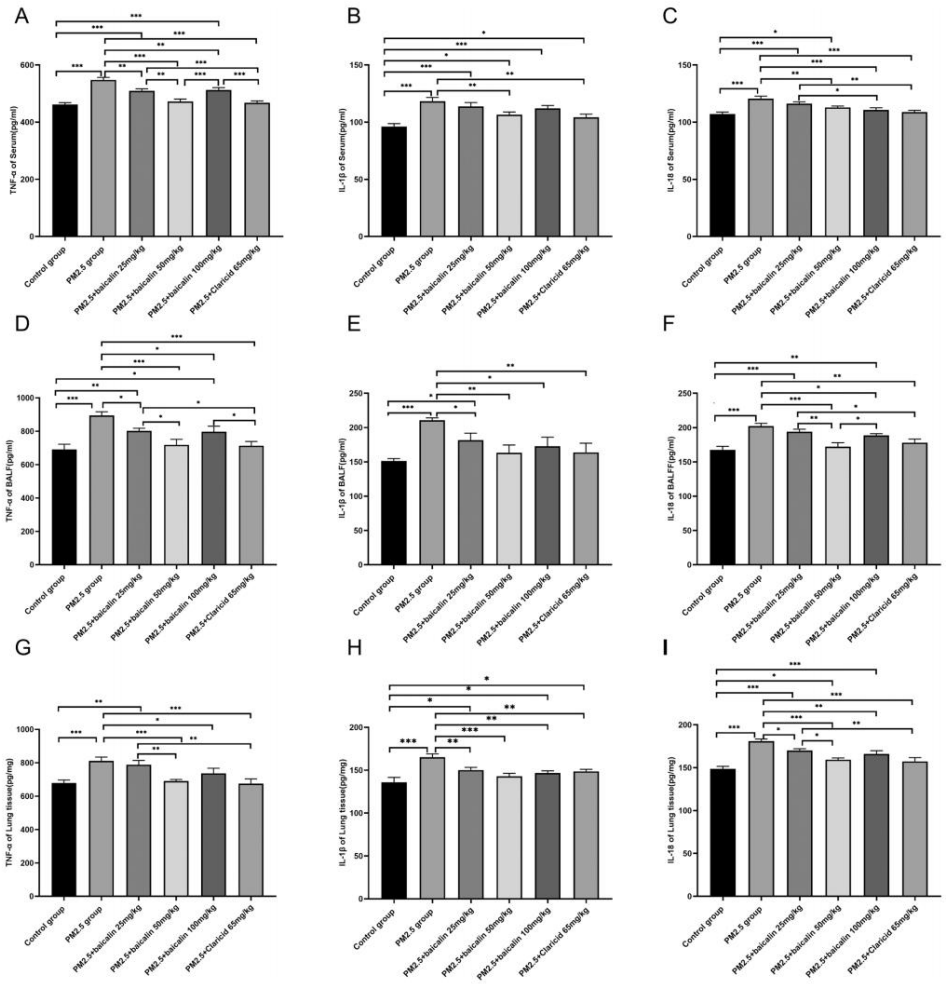

Fig. 3. The effect of baicalin on inflammatory cytokines was measured using ELISA. (A-C): TNF-α, IL-1β, and IL-18 levels in serum. (n = 12 per group). (D-F): Concentrations of TNF-α, IL-1β, and IL-18 in BALF (n = 6 per group). (H-J): TNF-α, IL-1β, and IL-18 levels in the lung tissue homogenate (n = 6 per group).

claricid group (Fig. 7F-G). Moreover, the abundance of Muribacter was increased in PM2.5 exposed mice, while the abundances of uncultured_bacterium_f_Muribaculaceae, Lactobacillus, and uncultured_bacterium_f_Lachnospiraceae were significantly decreased (Fig. 7H). The baicalin treatment upregulated abundances of uncultured_bacterium_f_Muribaculaceae, Lactobacillus, and uncultured_bacterium_f_Lachnospiraceae, and significantly reduced the abundance of Muribacterin when compared with PM2.5 group (Fig. 7H).

4. Discussion

PM2.5 is identified as a primary pollution component in air pollution, which can cause damage to multiple organ systems, especially the respiratory system (Arias-Pérez et al., 2020; Bhatnagar, 2022; Sacramento et al., 2020). The pathogenesis of respiratory diseases induced by PM2.5 exposure is strongly related to persistent inflammation (Racanelli et al., 2018). Dexamethasone, claricid, and penicillin are commonly used to treat persistent inflammation. Unfortunately, unsatisfactory outcomes and potential side effects have emerged with the above treatment strategies. Previous studies highlighted that baicalin has various pharmacological activities, including antitumor, antimicrobial, and antioxidant properties (Li et al., 2021). Whether baicalin has a protective effect on inflammatory lung injury induced by PM2.5 exposure is unclear. Our study established BALB/cJ mice lung

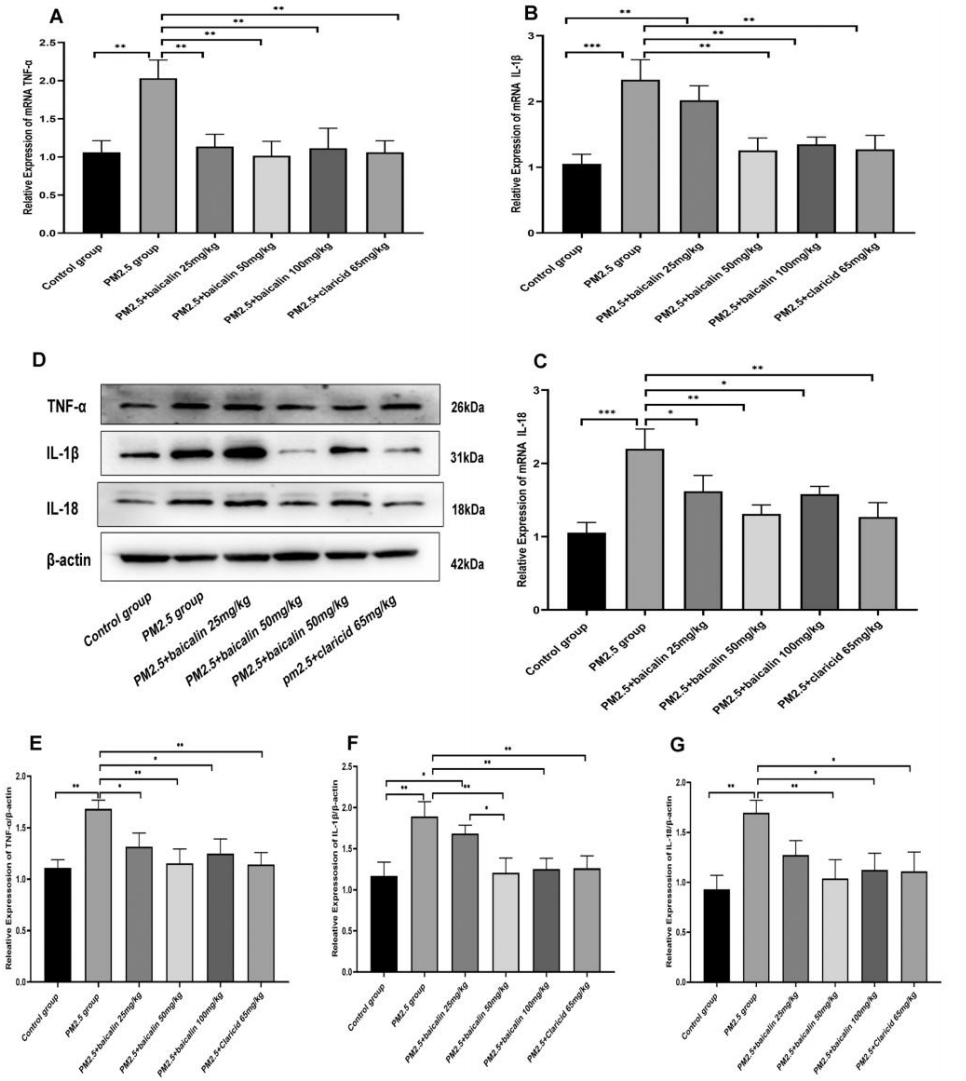

Fig. 4. The effects of baicalin on inflammatory cytokines as determined by PCR and Western blot. (A-C): The mRNA levels of TNF-α, IL-1β, and IL-18 in lung samples analyzed by qRT-PCR (n = 6 per group). (D): Protein blots of TNF-α, IL-1β, and IL-18. (E-J): The protein expression levels of TNF-α, IL-1β, and IL-18 as analyzed by Western blot (n = 5 per group).

inflammatory injury model via inhalation of PM2.5 aerosols. It reveals that baicalin can alleviate the pathological injury of lung tissue, regulate the upper respiratory flora, improve respiratory function, and reduce inflammation through inactivation of the HMGB1/Caspase1 pathway, thereby protecting mice from inflammatory lung injury.

Previous studies on the toxicity of PM2.5 exposure have shown that histological pathology changes were observed in mice. Alveolar wall thickening was accompanied by significant infiltration of inflammatory cells such as macrophages, lymphocytes and neutrophils. Additionally, alveolar swelling and bronchial epithelium disorder were occasionally observed (Xiong et al., 2021; Yang et al., 2018a). TNF-α, IL-18, and IL-1β, the inflammation markers, were increased in multiple organs of

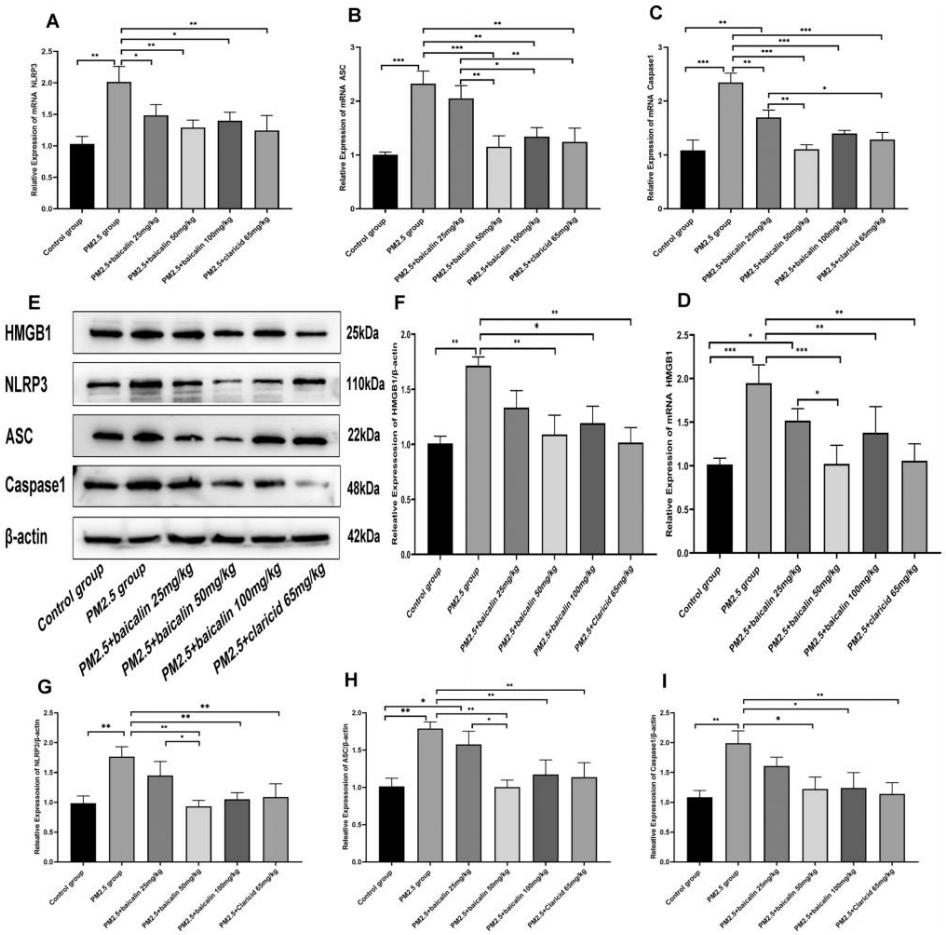

Fig. 5. The effects of baicalin on HMGBI, NLRP3, ASC, and caspase-1 expression. (A-D): The mRNA expression levels of HMGBI, NLRP3, ASC, and caspase-1 in lung samples were analyzed using qRT-PCR (n = 6 per group). (E): Protein blots of NLRP3, ASC, caspase-1, and HMGBI. (E-J): The protein levels of NLRP3, ASC, caspase-1, and HMGBI in lung samples were measured using Western blots (n = 5 per group).

PM2.5-exposed animals (Jia et al., 2021; Xiong et al., 2021). Interestingly, previous studies reported that supplementation of baicalin could reduce the secretion of TNF-α, IL-18, and IL-1β in the serum of rats, as well as moderate histological pathology in acute lung injury (ALI) in rats, induced by burns (Bai et al., 2018). Similarly, the current study showed that PM2.5 exposure led to inflammatory damage in mice lungs, characterized by the increased secretion of TNF-α , IL-18, and IL-1β, with pathological changes developing into severe lung tissue injuries. Following baicalin addition, the expression levels of these inflammatory cytokines in serum, BALF, and lung tissues were decreased. Baicalin can effectively reduce the release of pro-inflammatory inflammatory cytokines and alleviate inflammatory damage of mice model.

The previous study indicated that NLRP3 assembled ASC, activated cleaved caspase-1, and induced the expression of TNF-α , IL-1β, and IL-18, thereby promoting the inflammatory response (Kesavardhana and Kanneganti, 2017; Lamkanfi and Dixit, 2012; Yang et al., 2012). HMGB1, an effective pro-inflammatory mediator, plays a crucial role in inflammation related to various diseases (Abraham et al., 2000; Andersson and Tracey, 2011; Paudel et al., 2018). A previous study indicated that the HMGB1-NLRP3 inflammatory axis played a vital role in the cognitive impairment of offspring induced by gestational exposure to PM2.5 (Zhang et al., 2021). Furthermore, baicalin has proven to be effective in ameliorating chronic unpredictable mild stress-induced depressive behavior by inhibiting NLRP3/Caspase1 inflammasome activation in rats (Liu and Liu, 2017). Besides, the treatment with baicalin significantly suppressed HMGB1, NLRP3, and caspase-1 protein

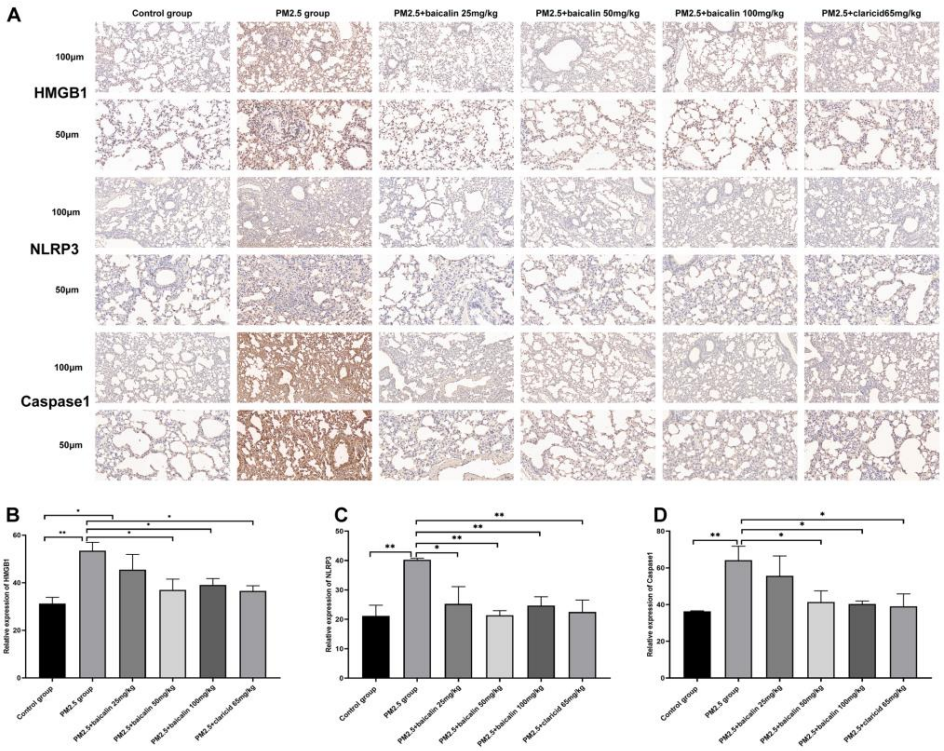

Fig. 6. IHC stains of lung tissues. (A) Representative images showing IHC staining of HMGBI, NLRP3, and caspase-1 in lung tissue. For the upper panel, scale bar= 100 μm; for the lower panel, scale bar= 50 μm. (B) Expression levels of proteins among groups (n = 3 per group).

expression in burn-induced remote ALI rats (Bai et al., 2018). The present study revealed that PM2.5 exposure increased the expression levels of HMGB1, NLRP3, ASC, and Caspase-1 in lung samples obtained from the experimental mice. Following 50 and 100 mg/kg baicalin interventions, the HMGB1, NLRP3, ASC, and Caspase-1 levels were downregulated. These data indicate that baicalin supplementation potentially ameliorated inflammatory lung injuries induced by PM2.5 in BALB/cJmice by inhibiting the HMGB1/NLRP3/Caspase-1 pathway.

Pulmonary function measurements provide a direct and objective marker of the origin and development of respiratory diseases (Glaab and Braun, 2021; Urbankowski and Przybyłowski, 2016). In the past two decades, multiple epidemiology studies have linked PM2.5 exposure and declines in respiratory functions (Yang et al., 2020). This study used a double-chambered plethysmograph system to measure lung function (Lee et al., 2011; Yang et al., 2018b). This data indicated that following PM2.5 exposure, f, sRaw, and FRC values were increased, MV was decreased, and lung function was impaired. After baicalin and claricid intervention, f, sRaw, and Frcmetrics were decreased and MV was increased, which may be because both baicalin and claricid can improve lung tissue elasticity, respiratory muscle strength, and respiratory resistance, thereby improving respiratory function injury caused by PM2.5 (Alharris et al., 2022). These findings were particularly apparent in the 50 mg/kg baicalin and claricid groups. In conclusion, these findings indicate that pulmonary function impairments induced by PM2.5 can be mitigated by baicalin or claricid treatment.

The upper respiratory tract (especially oropharynx) has a normal bacterial flora, which is an important component of respiratory tract's natural immune defense, providing a biological barrier against foreign matter or pathogenic microorganisms (Akata et al., 2016; Marsh et al., 2016). Studies demonstrated the impact of PM2.5 on airway microbiome, and reported the link between airway microbiome and respiratory functions (Wang et al., 2019; Yang et al., 2020). In addition, Pellissery et al. (2021) reported that baicalin favorably modulated gut microbiota composition without detrimentally affecting the gut microbiome diversity. Wang et al. (2021) found that applying fecal baicalin could redress the microbial dysbiosis of the gut and lungs in streptozotocin-induced diabetic mice. The current study indicated that the oropharynx microbiota of mice mainly consisted of uncultured_bacterium_f_Muribaculaceae, Streptococcus, uncultured_bacterium_f_Lachnospiraceae, and Lactobacillus at the genus level, but PM2.5 exposure caused significant differences in the relative abundances of these microbes. Muribacter and Rodentibacter were markedly upregulated in the PM2.5 group, but the microbiota imbalance was partially reversed with baicalin intervention. Additionally, the claricid treatment produced a more significant effect on the microbiota than the baicalin treatment, potentially because claricid is a macrolide drug. An increased quantity and variety of bacteria are likely attributable to the formation of communities of antibiotic-resistant bacteria (Dudek-Wicher et al.,

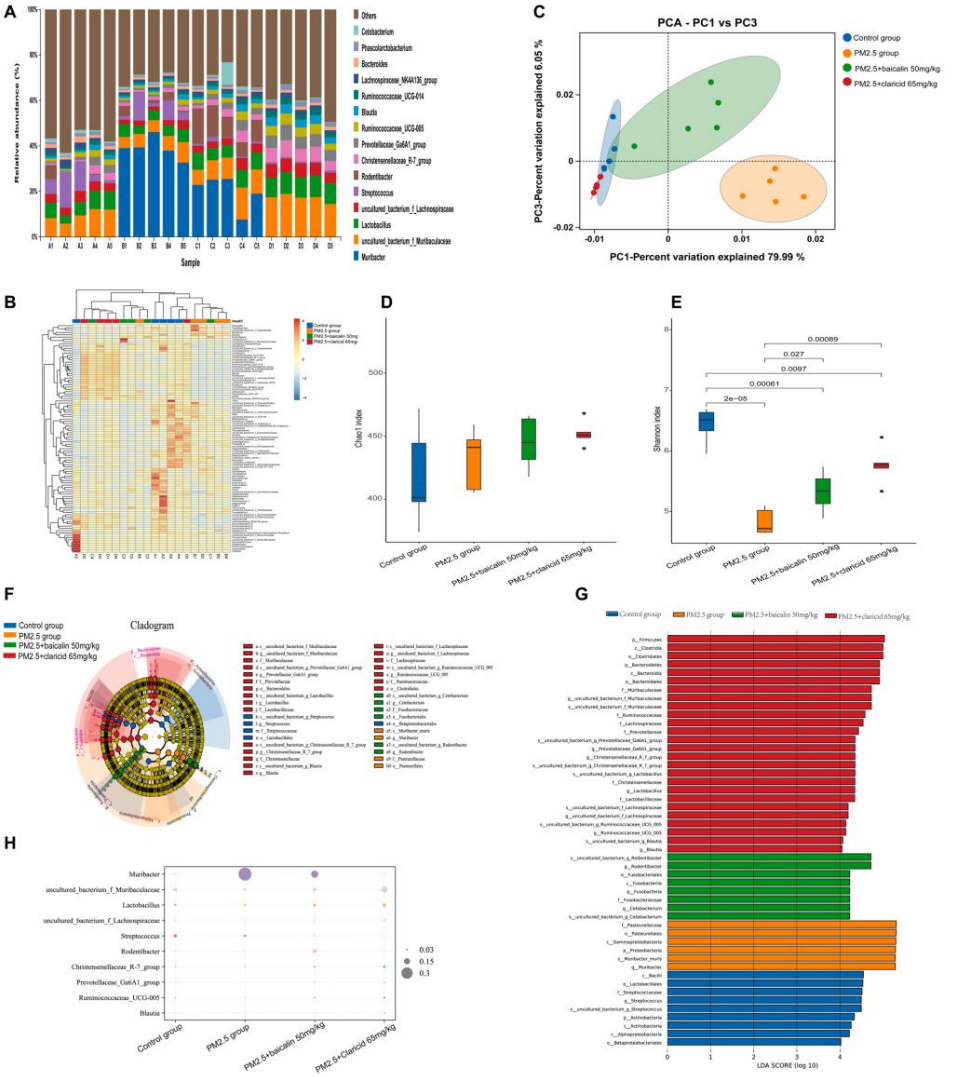

Fig. 7. Analysis of Illumina Novaseq sequencing data showed that baicalin modulated the whole structure of the oropharyngeal microbiota. (A) Microbial distribution at the genus level. (B) Heatmap of species richness clustering in genera. (C) PCA, with extended functionality for labeling groups and normal probability ellipsoids for different groups. (D) Chao1 index. (E) Shannon index. (F) Cladogram of oropharyngeal microbiota community. (G)LefSe analysis distribution histogram according to LDA (LDA>4.0). Important taxa are marked and illustrated with labels in the right panel. (H) Relative abundances of Mribacter, uncultured-bacterium-f-Muribaculaccae, Lactobacillus, uncultured-bacterium-f- Lachnospiraccae, and Streptococcus.

2018).

Previous studies have shown that PM2.5 exposure, both in humans and in mice, can increase the susceptibility of the organism to inhaled PM2.5 and affect the adhesion, colonization, and growth of oropharyngeal microorganisms, leading to changes in the microbiome of the upper respiratory tract(Chen et al., 2020; Qin et al., 2019; Yang et al., 2019). Interestingly, microbiome imbalance influenced the expression of the HMGB1 pathway (Splichalova et al., 2011).

Furthermore, over expression of HMGB1 caused activation of the inflammatory pathway, thereby promoting the release of pro-inflammatory factors (Andersson and Tracey, 2011; Zhang et al., 2021). In short, when mice were exposed to PM 2.5, the HMGB1-NLRP3 axis pathway was activated by microbiome imbalance and resulted in inflammatory lung injury (Chen et al., 2020; Wang et al., 2019; Zhang et al., 2021). Long-term inflammatory lung injury affects the lung function of the organism, bringing about restrictive and obstructive lung diseases (Shamsollahi et al., 2021; Wang et al., 2019; Wu and Segal, 2017). Our results demonstrated that baicalin intervention improved the microbiota structure in the oropharynx of mice, and reduced the expression gene and protein levels of HMGB1, NLRP3, ASC, and Caspase1, which caused a decrease in the release of inflammatory cytokines and eventually led to the improvement of pulmonary respiratory function. However, this is only a preliminary mouse experiment, and the mechanism of action of baicalin and its clinical application need further in-depth research and exploration.

5. Conclusion

This study demonstrates that baicalin has a good preventive and therapeutic effect on lung inflammatory injury caused by PM2.5 exposure in mice. It revealed that baicalin can ameliorate lung tissue pathological injury, reduce the release of inflammatory cytokines and improve respiratory function. Furthermore, our results demonstrated that baicalin inhibited the HMGB1/NLRP3/Caspase1 pathway as a potential mechanism for its ability to ameliorate lung inflammation. In addition, baicalin may interact with the airway immune system by balancing the structure of oropharyngeal flora, thereby improving lung injury. These findings provide novel insights into the potential utility of prevention and treatment in inflammatory lung injury of PM2.5 exposure, indicating that using baicalin is a potentially promising strategy for alleviating respiratory diseases induced by PM2.5. However, the detailed molecular characterization of the microbiota and lung immunity outcomes is still unknown, and further studies are required to understand the underlying mechanisms fully.

CRediT authorship contribution statement

Lili Deng: Conceptualization, Methodology, Statistical analysis, Writing – original draft. **Minyue Ma:** Conceptualization, Project administration. **Shuyin Li:** Conceptualization, Project administration. **Lin Zhou:** Conceptualization, Editing and Revision. **Sun Ye:** Conceptualization, revision. **Juan Wang:** Methodology. **Qiaoqiao Yang:** Methodology. **Chunling Xiao:** Conceptualization, Project administration, Funding acquisition, Editing and Revision. All authors have read and approved agreed to the final manuscript.

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.

Data Availability

Data will be made available on request.

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