

Synergistic Effects of Postbiotics and *Trifolium pratense* L. on Asthmatic Symptoms: Insights into the Gut-Lung Axis and TLR4/NF-κB Pathway

Efectos Sinérgicos de los Postbióticos y *Trifolium pratense* L. Sobre los Síntomas Asmáticos:
Perspectivas Sobre el Eje Intestino-Pulmón y la Vía TLR4/NF-κB

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SUMMARY: This study explored the effects of postbiotic *Trifolium pratense* L. on inflammatory responses in a rat model of asthma induced by lipopolysaccharide (LPS) and fumigation, with a particular focus on the dynamics of the TLR4/NF-κB signaling pathway. The experimental design involved administering LPS to the airways alongside ovalbumin sensitization to replicate chronic inflammation, followed by supplementation with postbiotics and *Trifolium pratense* L. leaves extract (TPLE). Quantitative analyses demonstrated that postbiotics and TPLE exerted dual regulatory effects: it downregulated systemic inflammation markers (serum IL-6, TNF-α, IL-1β) and lung-specific mucin MUC5AC, while upregulating the intestinal barrier integrity marker MUC2 in colon tissues. Mechanistic investigations indicated that postbiotics and TPLE suppressed the expression of TLR4 and NF-κB proteins in both lung and colon tissues, suggesting a comprehensive inhibition of the pathway. Furthermore, postbiotics and TPLE restored the structural integrity of alveoli and reduced the infiltration of inflammatory cells in lung parenchyma. These results imply that postbiotics and TPLE can mitigate inflammation associated with asthma through inter-organ communication, targeting shared molecular pathways within the lung-gut axis. The observed suppression of TLR4/NF-κB correlates with decreased cytokine storm activity, emphasizing the potential of postbiotics and TPLE. to interrupt pro-inflammatory signaling cascades. Additionally, the distinct regulation of mucins (MUC5AC versus MUC2) highlights tissue-specific therapeutic effects, balancing airway hypersecretion with the fortification of the intestinal barrier. This research advances the understanding of postbiotics and TPLE. as versatile agents in managing chronic respiratory diseases, presenting a novel approach to addressing systemic inflammation beyond traditional bronchodilator therapies.

KEY WORDS: *Trifolium pratense* L.; Postbiotic, Lung-gut axis; Asthma; TLR4; NF-κB; Inflammatory.

INTRODUCTION

Asthma has long been classified as a chronic respiratory condition marked by bronchial hyperresponsiveness and reversible airflow obstruction. However, it is increasingly recognized as a systemic inflammatory disorder that affects multiple organs. In addition to its primary respiratory symptoms—such as wheezing, shortness of breath, and excessive mucus production—asthma is intricately linked to gastrointestinal issues, as evidenced by the frequent coexistence of conditions like inflammatory bowel disease and gastroesophageal reflux (Chung *et al.*, 2022). This systemic aspect is driven by dysregulated immune responses that include Th2 cell polarization, eosinophil activation, and elevated levels of pro-inflammatory cytokines like interleukin (IL)-4, IL-5, and IL-13. Recent studies have underscored the significance of

the lung-gut axis, a complex communication network that involves immune cells, microbial metabolites, and neural pathways, in sustaining inflammation across these organ systems (Peng *et al.*, 2022).

Alterations in gut microbiota composition, known as dysbiosis, have been associated with increased airway inflammation, suggesting that signals originating from the gut may prime immune responses in the lungs. The lung-gut axis functions through three main mechanisms: (1) microbial translocation, where bacteria or their components (such as lipopolysaccharides) from the gut enter the bloodstream and activate Toll-like receptors (TLRs) in remote organs; (2) immune cell trafficking, where dendritic cells and T lymphocytes primed in the gut migrate to lung

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tissues, intensifying local inflammation; and (3) metabolite signaling, where short-chain fatty acids (SCFAs) produced by gut microbiota influence the differentiation of regulatory T cells (Tregs) and inhibit histone deacetylase activity (Dong *et al.*, 2024). In asthma, reductions in SCFA-producing bacteria (e.g., *Faecalibacterium prausnitzii*) due to dysbiosis are linked to increased airway hyperreactivity and compromised mucosal immunity. Research using animal models shows that depleting gut microbiota worsens allergic airway inflammation, whereas fecal microbiota transplantation from healthy individuals can alleviate it. These observations highlight the gut's vital role in modulating pulmonary immunity, with disturbances in its microbial community directly affecting the severity of asthma (Yang *et al.*, 2025).

The TLR4/NF- κ B signaling pathway plays a crucial role in the pulmonary system, particularly in the pathogenesis of respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD). Toll-like receptor 4 (TLR4) is a pattern recognition receptor that, upon activation by environmental stimuli like allergens, pollutants, and pathogens, triggers an inflammatory response through the NF- κ B pathway (Wei *et al.*, 2023). This activation leads to the production of pro-inflammatory cytokines and chemokines, contributing to airway inflammation, hyperreactivity, and remodeling in asthma, as well as the chronic inflammation and tissue damage seen in COPD. Therapeutics targeting the TLR4/NF- κ B pathway hold promise for managing these conditions; for instance, TLR4 antagonists and NF- κ B inhibitors could reduce inflammation and improve lung function by mitigating the overactive immune response. Additionally, natural compounds with anti-inflammatory properties, such as flavonoids and other phytochemicals, are being explored for their potential to modulate this pathway, offering new avenues for treatment in respiratory diseases characterized by chronic inflammation (Ning *et al.*, 2025).

Postbiotics—live microorganisms conferring host health benefits—emerge as promising agents for restoring gut-lung axis homeostasis in asthma. Strains such as *Lactobacillus rhamnosus* GG and *Bifidobacterium infantis* exert anti-inflammatory effects by competitively excluding pathogenic bacteria, enhancing intestinal barrier integrity via tight junction protein upregulation, and stimulating SCFA production (Vasconcelos *et al.*, 2025). In asthmatic models, postbiotics reduce lung eosinophilia, suppress Th2 cytokines, and promote IL-10-secreting Tregs, thereby rebalancing immune polarization. Notably, their efficacy extends beyond the gut: postbiotics modulate alveolar macrophage activity, decrease airway mucus hypersecretion (via MUC5AC downregulation), and inhibit NF- κ B-driven transcription of

pro-inflammatory genes. Clinical trials corroborate these effects, showing reduced asthma exacerbation rates and improved lung function in postbiotic-supplemented cohorts. However, strain-specific variability and dose-dependent responses underscore the need for mechanistic studies to optimize therapeutic protocols (Yang *et al.*, 2025).

Trifolium pratense L., commonly known as red clover, is a perennial herb in the Fabaceae family, native to Europe and widely distributed across North America. *Trifolium pratense* L. leaf extract (TPLE) is notable for its rich content of bioactive compounds, particularly isoflavones such as genistein and daidzein, which exhibit phytoestrogenic, antioxidant, and anti-inflammatory properties (Al-Shami *et al.*, 2023). Research indicates that TPLE may positively impact respiratory health by alleviating asthma symptoms and enhancing lung function, likely due to its ability to reduce airway inflammation and oxidative stress. Although evidence linking TPLE to conditions like embolism and pulmonary edema is limited, its anticoagulant properties suggest potential benefits in vascular health. Furthermore, while direct studies on atelectasis are scarce, the plant's overall respiratory benefits may indirectly support lung expansion and health (Bellikli *et al.*, 2026).

In this study, we utilized a rat asthma model induced by smoke exposure combined with intra-airway lipopolysaccharide (LPS) administration to assess the roles of TPLE and postbiotics. We hypothesize that postbiotics in combination with TPLE may inhibit the TLR4/NF- κ B pathway, thereby attenuating inflammatory responses in the lungs and intestines. This innovative approach broadens the applications of postbiotics in chronic respiratory diseases and clarifies their action mechanisms within the lung-gut axis, offering a novel perspective on asthma management and underscoring the potential of prebiotics in addressing multi-organ inflammation.

MATERIAL AND METHOD

Postbiotics preparation

A mixture of indigenous Chinese postbiotic strains, including *Lactobacillus casei* (IBC-M10784), *Lactobacillus helveticus* (TG-35), and *Lactobacillus rhamnosus* (IBRC-M11322), was obtained from Techgen Bio in a lyophilized powder form, standardized to an initial concentration of 1×10^{10} colony-forming units per milliliter (CFU/mL). For experimental use, 1 mL of this postbiotic mixture was reconstituted in 9 mL of sterile distilled water using vortex-assisted homogenization, resulting in 1:9 dilutions. This process produced a working concentration of 1×10^9 CFU/mL, with adjustments made to maintain isotonicity and pH,

thereby ensuring the viability of the microbes during in vivo administration (Che *et al.*, 2025).

Animals and experimental design

Fifty male Wistar rats (5–7 weeks old, 210 ± 10 g body weight) were maintained under controlled environmental conditions (22 ± 5 °C, 40 ± 3 % humidity) at the central laboratory of Danyang Hospital of Nantong University, following a 72-hour acclimatization period. The animals were ethically managed in compliance with international research standards, including humane handling protocols. After one week of domestication, subjects were stratified into four cohorts ($n=10/\text{group}$): (1) sham (no intervention), (2) asthma model (AS, smoke/LPS exposure), (3) postbiotic (AS + 100 mg/kg postbiotics), (4) TPLe (AS + 200 mg/kg TPLe), and (4) postbiotic and TPLe co-treatment (AS + 100 mg/kg postbiotics + 200 mg/kg TPLe).

Asthma induction involved twice-daily smoke exposure (10 cigarettes/session, 30 minutes, 4-hour intervals) over 12 weeks, supplemented by intratracheal LPS instillation (200 mg/kg) at weeks 2, 4, 8, and 12. Control animals received saline instead of LPS. Model validation was achieved through histopathological analysis (HE staining) of euthanized specimens, confirming pulmonary remodeling. Subsequently, postbiotic groups received daily oral gavage (0.6 mL/day = 0.8 g/kg) of a multi-strain formulation for 30 days, while controls were administered equivalent saline volumes, maintaining consistent dosing schedules across all groups (Akbaribazm *et al.*, 2024; Che *et al.*, 2025; Hartady *et al.*, 2026).

Serum Catalase (CAT), Superoxide Dismutase (SOD) Activity, and Malondialdehyde (MDA) Levels

Following a 12-hour fasting period, 5 mL of blood was obtained via cardiac puncture under anesthesia induced by intraperitoneal administration of ketamine (50 mg/kg) and xylazine (100 mg/kg) (Alfasan, Netherlands). The collected blood samples were centrifuged at 3000 rpm for 10 minutes to isolate serum for further analysis (Naseri *et al.*, 2024).

Glutathione Peroxidase (GPx) Activity Measurement

Serum GPx activity was measured using the Randox ELISA kit (RS504), which quantifies the conversion of NADPH to NADP at 340 nm through glutathione redox cycling. Samples were prepared by diluting 50 mL of serum with 100 μ L of buffer, followed by centrifugation. The supernatant was transferred to a 96-well plate, and Cumene

Hydroperoxide was added. Absorbance changes (DA340/min) were calculated using the formula:

$$\Delta A_{340}/\text{min} = |A_{340}(\text{Time } 2) - A_{340}(\text{Time } 1)| / \text{Time } 2 - \text{Time } 1$$

The GPx activity was then calculated using the equation:

$$\text{GPx activity} = \Delta A_{340} / \text{min} / 0.0037 \times 8.5 (\text{Constant value}) \times (0.5) \text{ Sample dilution.}$$

Controls and NADPH-substrate mixtures were included as per the protocol (Naseri *et al.*, 2024).

Superoxide Dismutase (SOD) Activity Analysis

SOD activity was assessed using the Elabscience kit (E-EL-R1424). A 100 μ L aliquot of serum was incubated with WST SOD buffer and enzyme solution at 37°C for 20 minutes, after which the absorbance was measured at 450 nm (Naseri *et al.*, 2024).

Catalase (CAT) Activity Determination

Catalase activity was evaluated with the Abcam kit (ab100764) through a two-stage dark incubation process. Serum was mixed with H_2O_2 and potassium phosphate buffer, then treated sequentially with Purpald chromogen, KOH, and catalase potassium periodate. The final absorbance was recorded at 540 nm using a spectrophotometer. Both the SOD and CAT assays employed standardized controls and adhered to manufacturer protocols to ensure reproducibility (Naseri *et al.*, 2024).

Serum Nitric Oxide (NO) levels assessment

Serum nitric oxide (NO) levels were determined using the Griess colorimetric method, which quantifies stable NO metabolites (nitrite and nitrate) due to the inherent instability of NO. For this procedure, 400 μ L of serum was deproteinized with 6 mg of zinc sulfate, followed by centrifugation at 12,000 rpm for 15 minutes. A 100 μ L aliquot of the supernatant was mixed with 100 μ L of vanadium chloride (to reduce nitrate to nitrite) and Griess reagent, which consists of sulfanilamide and N-(1-naphthyl) ethylenediamine dihydrochloride. The mixture was incubated at 37 °C for 30 minutes to form a purple azo compound. Absorbance readings were taken at 450 nm (primary) and 630 nm (reference) using a spectrophotometer, with sample concentrations calculated against a sodium nitrate standard curve (0–200 mM) to determine NO levels (Zablocki *et al.*, 2024).

Serum levels of IL-6, TNF-α, and IL-1β

Serum levels of TNF-α (DY595), IL-6 (M6000B), and IL-1β (MLB00C) were measured using the Quantikine QuickKit ELISA kits from R&D Biosystems. In accordance with the manufacturer's instructions, 100 μL of standards, controls, and serum samples were dispensed into 96-well plates, followed by the addition of 200 μL of enzyme conjugate to each well. After incubating the plates at room temperature for 120 minutes, they were subjected to three wash cycles with a buffer solution. Subsequently, 200 μL of substrate solution was added to each well, and the reactions were stopped after 30 minutes by adding 100 μL of stop solution. Absorbance was then measured at 450 nm using a Spectronic 20 ELISA reader (Milton Roy Company, Spain), and cytokine concentrations were determined by comparing the results against standard curves (Sun *et al.*, 2025).

Ferric reducing antioxidant power (FRAP), malondialdehyde (MDA), and total thiol levels in lung tissue

The total antioxidant capacity of lung homogenate was assessed using the Ferric Reducing Antioxidant Power (FRAP) assay, which measures the reduction of Fe(III)-2,4,6-tripyridyl-s-triazine (TPTZ) to its ferrous (Fe(II)) form by antioxidants. A working FRAP reagent was prepared and pre-incubated at 37 °C for 15 minutes. For the analysis, 150 μL of lung homogenate was combined with 100 μL of the FRAP solution, centrifuged at 12,000 × g for 15 minutes, and re-incubated at the same temperature. The absorbance of the resulting supernatant was measured at 593 nm using a Statfax 100 UV-visible spectrophotometer, with antioxidant capacity expressed as μM FeSO₄ equivalents based on a 0–1,000 μM standard curve (Rumpf, Burger, and Schulze, 2023). Lipid peroxidation levels were determined using the thiobarbituric acid reactive substances (TBARS) assay to quantify malondialdehyde (MDA) as a biomarker. Lung tissue was homogenized in ice-cold phosphate-buffered saline (PBS) containing EDTA and centrifuged to isolate the supernatant, which was stored at -70 °C until analysis. For the TBARS assay, 50 μL of the supernatant was mixed with 50 μL of 1 M phosphoric acid, 50 μL of TBA reagent, and 2 μL of butylated hydroxytoluene (BHT) to prevent oxidation artifacts. The mixture was incubated at 60 °C for 60 minutes to form the MDA-TBA adduct, then centrifuged at 12,000 × g for 5 minutes to remove precipitates. The absorbance of the resulting pink chromogen was measured at 532 nm using a Statfax 100 UV-visible spectrophotometer, with MDA concentrations calculated against a standard curve (Soares *et al.*, 2021). Additionally, total thiol levels in lung tissue were assessed using a DTNB-based colorimetric assay. A 100-gram aliquot of lung tissue homogenate was mixed

with 150 μL of Tris-EDTA buffer and equilibrated at 25°C for 10 minutes. Baseline absorbance was recorded at 412 nm, followed by the addition of 15 μL of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) and a 20-minutes incubation to allow color development. Post-reaction absorbance was measured, along with a DTNB blank to account for reagent background. Total thiol concentration was calculated using the formula: Total thiol concentration (μM) = $(\beta - \alpha - \mu) \times 1.07 / 6.8$ (Alam *et al.*, 2023).

Real-Time PCR assay

Total RNA was extracted from lung and colon tissue samples using RNX-plus solution (SinaGen) through a homogenization process followed by chloroform addition. The mixture was centrifuged at 13,000 RPM for 15 minutes at 4 °C to separate the RNA-containing aqueous phase. This phase was then precipitated with cold isopropanol, washed with 70 % ethanol, and resuspended in DEPC-treated water (0.1 % v/v). For cDNA synthesis, the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) was utilized with oligo dT primers. Specifically, 1–2 μg of RNA was treated with DNase I at 37 °C for 30 minutes, inactivated with EDTA at 65 °C for 10 minutes, and reverse-transcribed in a 20 μL reaction containing 5x buffer, dNTPs (10 mM), and RevertAid enzyme at 42 °C for 60 minutes. Real-time PCR was performed using SYBR Green chemistry on a thermocycler with a 40-cycle protocol: an initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 20 seconds, 55 °C for 20 seconds, and fluorescence acquisition. Primer pairs for the target genes (MUC2, MUC5AC, TLR4, and NF-κB) and the housekeeping gene GAPDH were validated using NCBI BLAST, with the following sequences:

GAPDH: Forward: AGGTCGGAGTCAACGGATTTG; Reverse: TGCCCAGGATGCCCTTCTCC; MUC5AC: Forward: GCTGGCTCAGGAGAAGGCA; Reverse: CCACTCCCTTCTCCTCCACA; TLR4: Forward: GCTGACCTGTGGTGGTATG; Reverse: CCATGCTCATCATCACCAC; NF-κB (p65): Forward: GCCTGATGATGGCCTGAAGA; Reverse: TGCAGGTCAGCTCTGGTTCA; MUC2: Forward: GCTCCAGCAGGATCACACCA; Reverse: GCTGGAGGAGGAGTGAGGAG.

Relative expression levels were calculated using the ΔΔCt method, with data analyzed using REST 2009 and GraphPad Prism 6 (one-way ANOVA, Tukey's HSD). All procedures were conducted under RNase-free conditions, utilizing DEPC-treated reagents and a laminar flow hood, with triplicate technical replicates for each sample. Prior to PCR, cDNA concentrations were normalized using a

NanoDrop, and the PCR reactions contained 12.5 μL of Master Mix (Bioneer), 1 μL of each primer (10 μM), and 1 μL of cDNA (0.1–1 μg) in a final volume of 25 μL.

The mRNA gene expression levels were calculated using the Ct ($2^{-\Delta\Delta CT}$) method.

$$\Delta\Delta CT = [(mCT_{\text{target}} - mCT_{\text{reference}})_{\text{test sample}} - (mCT_{\text{target}} - mCT_{\text{reference}})_{\text{control sample}}]$$

Finally, the target mRNA expression levels were determined using the formula: target mRNA expression levels = $2^{-\Delta\Delta CT}$ (Chen *et al.*, 2025).

Lung tissue histopathology and immunohistochemistry (IHC) assay

The immunohistochemistry (IHC) assay for identifying p53-positive apoptotic cells in lung tissues followed a detailed protocol. Paraffin-embedded lung sections (5 μm thick) were deparaffinized and subjected to antigen retrieval by incubating overnight at 95 °C with an anti-p53 primary antibody diluted at 1:1000 (R&D Systems; cat. No. GAF1355). After this step, the slides were washed with Tween-20 buffer, blocked with 5 % BSA for 1 hour at 25 °C, and treated with 3 % hydrogen peroxide for 20 minutes at room temperature to eliminate endogenous peroxidases. Detection of p53-positive cells was performed using 3,3'-diaminobenzidine (DAB) chromogen, followed by counterstaining with hematoxylin. The slides were then examined at 100× magnification using an Olympus BX61TRF microscope (Japan), and p53-positive cells were quantified as a percentage of total cells across 10 randomly selected fields for each sample using ImageJ software (Sun *et al.*, 2025).

Tissue preparation and stereological measurements

Histopathological examinations were conducted using a light microscope at ×100 magnification. Digital images were acquired with a calibrated BX61TRF microscopy system (Olympus, Japan) and subsequently analyzed using ImageJ software. Quantitative assessment of the relative volume (Vv) of lung tissue components—including vascular volume (VV), air parenchyma volume (APV), lung volume (LV), and total pulmonary volume (TPV)—was performed via the point-counting method in conjunction with Delesse's formula. This analysis employed a Nikon light microscope equipped with Kecam imaging hardware (Kecam Technologies) and TopView software (version 3.7). Additional image processing was carried out using Adobe Photoshop CC (Adobe Systems). For rat lung gross tissue containing a focal tumor area (demarcated by a circle), isotropic uniform random (IUR) sections were generated using the orientator method. Each

lung was placed on a circle divided into ten equal segments (u-clock). A random integer between 0 and 10 was generated, and the lung was sectioned into two halves along the corresponding direction. The cut surface of one half was then positioned vertically on the h-clock, which featured ten unequal sinus-weighted divisions. A second cut was made using another randomly selected number. The cut surface of the remaining lung half was aligned parallel to the 0–0 direction of the h-clock, and the second cut was again determined by a random selection (in this case, 2). The entire lung was subsequently sliced into slabs, with the blade oriented according to the second cuts, yielding nine slabs in total. A trocar was used to extract a circular tissue sample from one of the lung slabs. Primary lung tissue volumes were assessed using immersion techniques, and tissue shrinkage rates were determined. The processed tissues were embedded in paraffin blocks and sectioned into 5 μm thick slices using equipment from Leica Microsystems. Sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome according to standard protocols for subsequent histological examination. Tissue shrinkage was quantified by measuring the diameter of the circular sections after processing and applying the following formula:

$$\text{Volume shrinkage} = 1 - (AA/AB)^{1.5}$$

$$Vv(\text{structure}) = \frac{\sum_{i=1}^n p(\text{structure})}{\sum_{i=1}^n p(\text{reference})}$$

“ $\sum_{i=1}^n p(\text{structure})$ ” was the number of test points that settled on the targeted structures, and “ $\sum_{i=1}^n p$ ” was the total number of points that fell on the lung sections. The following formula was used to determine the total targeted structural volume (Fig. 1).

$$V(\text{structure}) = V(\text{total lung}) \times Vv(\text{structure}) \quad (\text{Knudsen } et al., 2023; \text{Hong } et al., 2024).$$

Statistical Analysis

Data normality was assessed using the Kolmogorov-Smirnov test, with non-significant results indicated by $p > 0.05$. Statistical analyses were performed using SPSS v26 (SPSS Inc., USA), utilizing one-way ANOVA followed by post-hoc Tukey's honestly significant difference (HSD) tests. To ensure robust inter-group comparisons, cross-validation was conducted in both GraphPad Prism 8 and SPSS 16. Histopathological evaluation of liver tissue was carried out using a semiquantitative scoring system on H&E-stained sections, with assessments performed in a double-blinded manner to reduce bias. Quantitative results are presented as mean ± standard deviation, and statistical significance was set at $p < 0.05$.

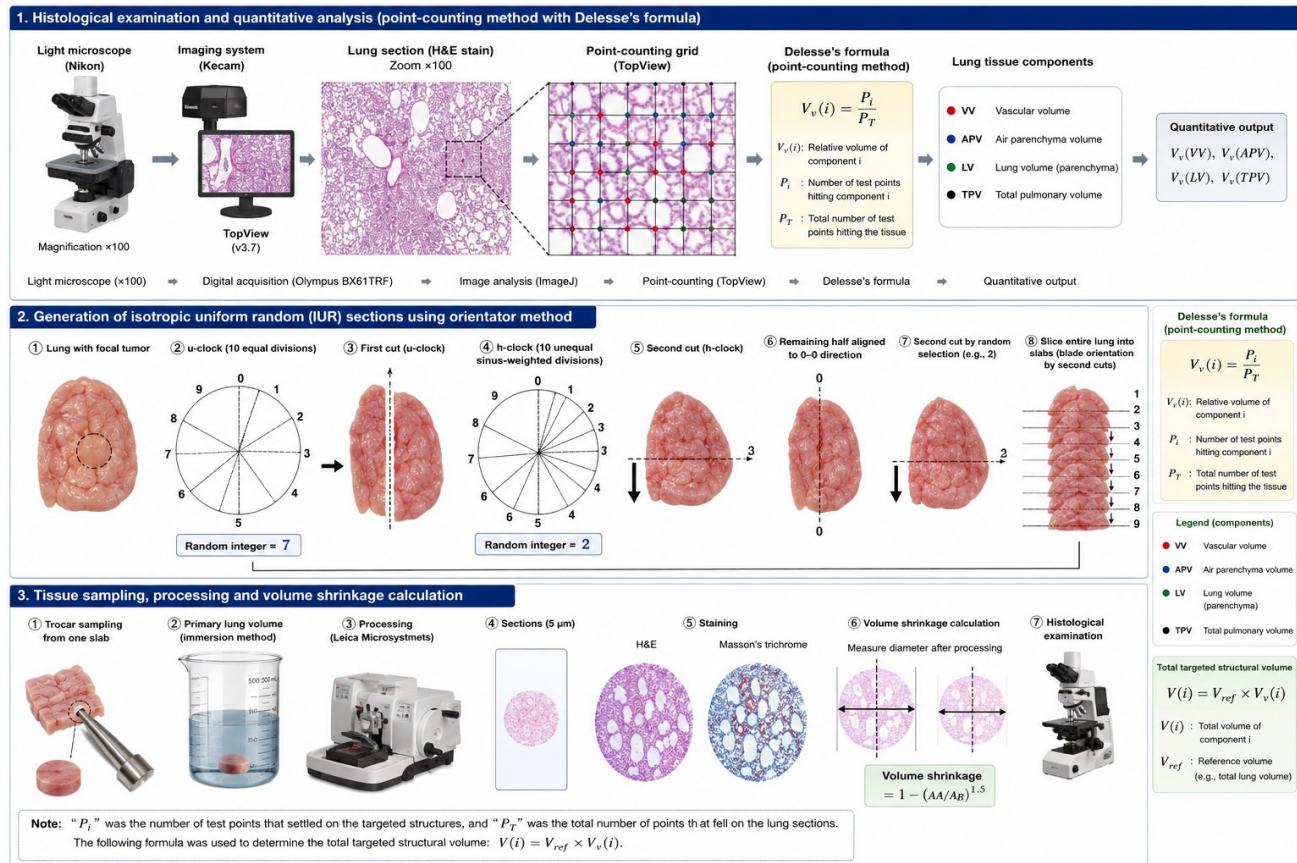


Fig. 1. Stereological approach steps for rat lung volumetric parameters through quantitative and unbiased estimation of the orientator and Cavalieri methods.

RESULTS

Body and Lung Weight

The Sham group demonstrated stable body weight, whereas the Asthma (AS) group exhibited a significant reduction in body weight ($p < 0.05$) and a marked increase in lung weight ($p < 0.05$), indicating the presence of asthma-related pathophysiological changes. Postbiotic intervention showed varying effects. The AS + Postbiotic group displayed a partial recovery in body weight ($p < 0.05$) and a decrease in lung weight ($p < 0.05$) compared to the AS group. Similarly, the AS + TPLE group indicated improvements with significant increases in body weight and decreases in lung weight ($p < 0.05$). The combination treatment of AS + Postbiotic + TPLE resulted in the most pronounced effects, restoring body weight to levels comparable to the Sham group ($p < 0.05$) and normalizing lung weight ($p < 0.05$) (Fig. 2).

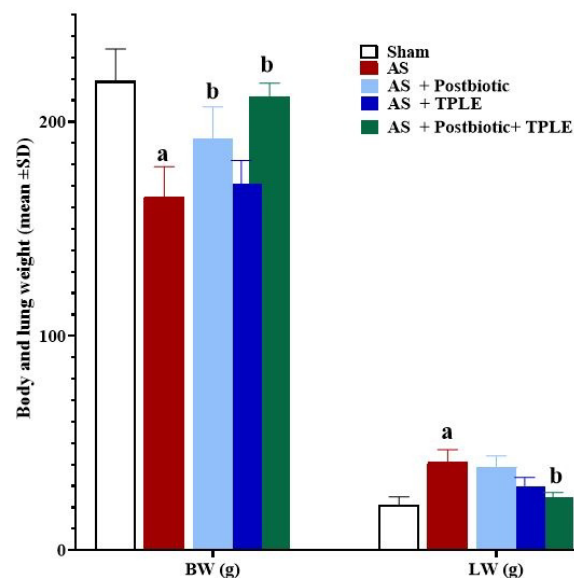


Fig. 2. The weights of the whole animals bodies (BW) and lungs (LW) across different experimental groups are presented. Data are expressed as means $\pm$ standard deviation (SD), with $n = 10$ per group. Statistical significance is indicated by a ($p < 0.05$) for comparisons between the AS and Sham groups, and b ($p < 0.05$) for comparisons among the AS + Postbiotic, AS + TPLE, and AS + Postbiotic + TPLE groups versus the AS group.

The serum activity of SOD, CAT, and GPx along with serum levels of NO

The Sham group displayed baseline levels of antioxidant enzymes, while the Asthma (AS) group exhibited a significant reduction in superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) activities ($p < 0.05$), indicating oxidative stress associated with asthma. Postbiotic intervention resulted in marked improvements. The AS + Postbiotic group showed significant increases in SOD and GPx activities ($p < 0.05$) compared to the AS group, suggesting a restorative effect on antioxidant defenses. The AS + TPLE group also demonstrated significant enhancements in SOD and CAT activities ($p < 0.05$). The combination treatment of AS + Postbiotic + TPLE yielded the most pronounced effects, significantly restoring SOD, GPx, and CAT levels ($p < 0.05$), along with a notable reduction in nitric oxide (NO) levels ($p < 0.05$) (Fig. 3).

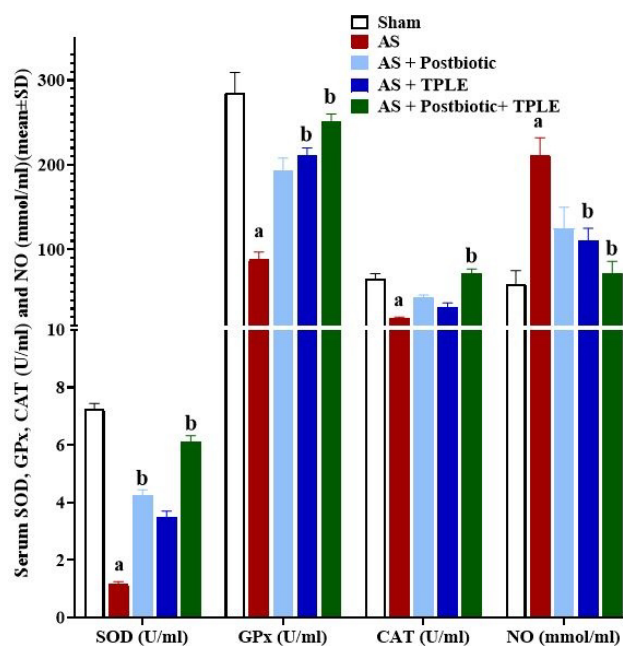


Fig. 3. The serum activity of SOD, CAT, and GPx along with serum levels of NO in the different experimental groups. Data given as means $\pm$ SD. $n = 10$ /group. Statistical significance is indicated by a ($p < 0.05$) for comparisons between the AS and Sham groups, and b ($p < 0.05$) for comparisons among the AS + Postbiotic, AS + TPLE, and AS + Postbiotic + TPLE groups versus the AS group.

Lung tissue FRAP, MDA, and thiol levels

The Sham group exhibited baseline levels of malondialdehyde (MDA), ferric reducing ability of plasma (FRAP), and thiol levels, while the Asthma (AS) group demonstrated a significant increase in MDA levels ($p < 0.05$) and a notable decrease in FRAP and thiol levels ($p < 0.05$),

indicating enhanced oxidative stress and reduced antioxidant capacity associated with asthma. Postbiotic interventions resulted in significant improvements. The AS + Postbiotic group showed a marked reduction in MDA levels ($p < 0.05$) and an increase in FRAP and thiol levels ($p < 0.05$) compared to the AS group, suggesting effective restoration of antioxidant defenses. The AS + TPLE group also exhibited significant decreases in MDA and increases in FRAP and thiol levels ($p < 0.05$). The combination treatment of AS + Postbiotic + TPLE yielded the most substantial benefits, significantly normalizing MDA levels ($p < 0.05$) and enhancing both FRAP and thiol levels ($p < 0.05$) (Fig. 4).

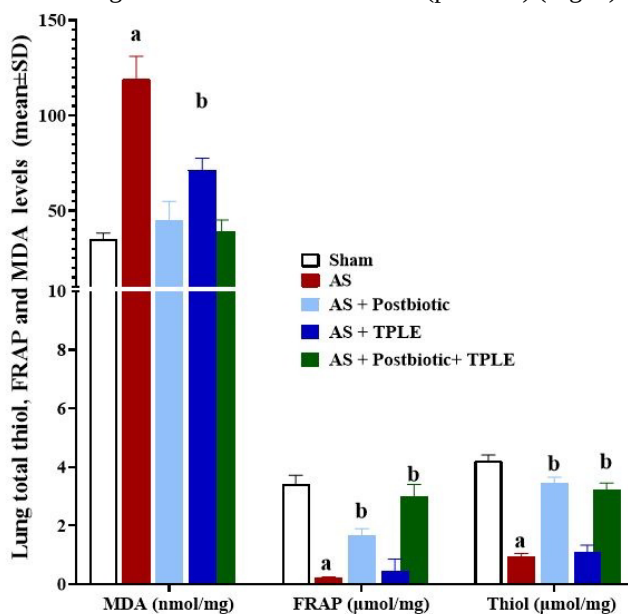


Fig. 4. The lung tissue levels of MDA, FRAP, and thiol in the different experimental groups. Data given as means $\pm$ SD. $n = 10$ /group. Statistical significance is indicated by a ($p < 0.05$) for comparisons between the AS and Sham groups, and b ($p < 0.05$) for comparisons among the AS + Postbiotic, AS + TPLE, and AS + Postbiotic + TPLE groups versus the AS group.

Serum levels of IL-6, TNF- α , and IL-1 β

The Sham group exhibited baseline levels of pro-inflammatory cytokines, while the Asthma (AS) group showed a significant increase in IL-6, TNF- α , and IL-1 β levels ($p < 0.05$), indicating heightened inflammatory responses associated with asthma. Postbiotic intervention resulted in notable changes. The AS + Postbiotic group demonstrated a significant reduction in IL-6 ($p < 0.05$) and TNF- α levels ($p < 0.05$) compared to the AS group, suggesting an anti-inflammatory effect. The AS + TPLE group also exhibited significant decreases in both IL-6 and TNF- α levels ($p < 0.05$). The combination treatment of AS + Postbiotic + TPLE yielded the most substantial reductions in pro-inflammatory cytokines, with significant decreases

in IL-6, TNF- α , and IL-1 β levels ($p < 0.05$), indicating a synergistic therapeutic effect (Fig. 5).

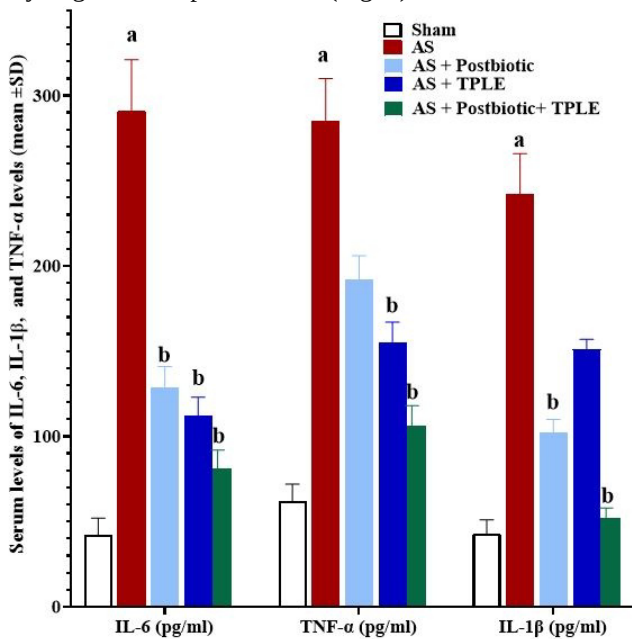


Fig. 5. The serum levels of IL-6, TNF- α , and IL-1 β in the different experimental groups. Data given as means $\pm$ SD. $n = 10$ /group. Statistical significance is indicated by a ($p < 0.05$) for comparisons between the AS and Sham groups, and b ($p < 0.05$) for comparisons among the AS + Postbiotic, AS + TPLE, and AS + Postbiotic + TPLE groups versus the AS group.

Lung tissue expression of MUC5AC, TRL-4, NF- κ B and expression of colon tissue MUC2, TRL-4, and NF- κ B

In the colon tissue, the AS group exhibited significantly depleted MUC2 expression alongside dramatic upregulation of TLR-4 compared to the Sham group ($p < 0.05$), indicating concurrent mucosal barrier disruption and hyperactivated inflammatory signaling. The expression of NF- κ B was also significantly increased in the AS group ($p < 0.05$). Postbiotic administration alone and in AS + Postbiotic + TPLE groups substantially reversed these pathological alterations, with the higher dose showing superior efficacy in normalizing all three markers to levels matching or exceeding those of the Sham group. The postbiotic intervention achieved near-complete restoration of mucosal-protective functions and inflammatory balance, effectively restoring MUC2, TLR-4, and NF- κ B to values statistically indistinguishable from baseline controls ($p < 0.05$ versus AS group) (Fig. 6a).

The Asthma (AS) group lung tissue demonstrated significantly elevated expression of MUC5AC and TLR-4 compared to the Sham group ($p < 0.05$), indicating heightened mucosal inflammation and inflammatory signaling. Additionally, NF- κ B expression was also significantly increased in the AS group ($p < 0.05$). Postbiotic administration in AS + Postbiotic + TPLE group substantially

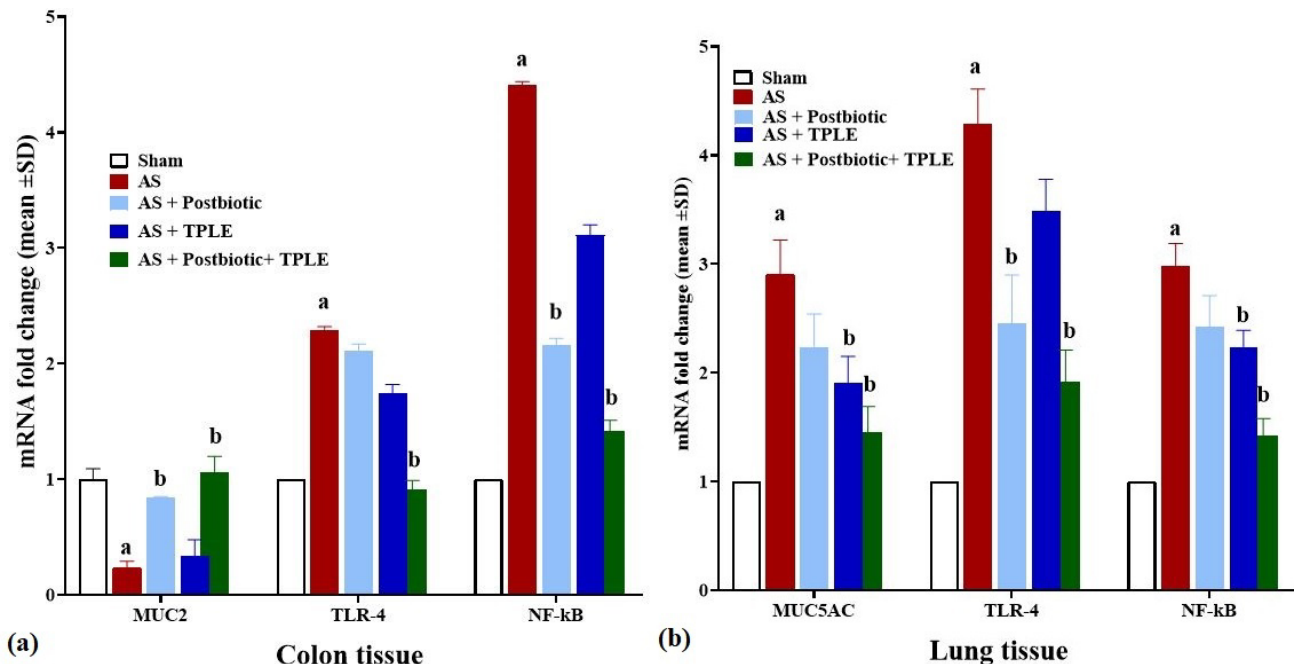


Fig. 6. (a) The mean colon tissue MUC2, TLR-4, and NF- κ B genes expression (b) The mean lung tissue MUC5AC, TLR-4, and NF- κ B genes expression in the different experimental groups. Data given as means $\pm$ SD. $n = 10$ /group. Statistical significance is indicated by a ($p < 0.05$) for comparisons between the AS and Sham groups, and b ($p < 0.05$) for comparisons among the AS + Postbiotic, AS + TPLE, and AS + Postbiotic + TPLE groups versus the AS group.

attenuated these pathological elevations, with the higher dose exhibiting superior efficacy in normalizing all three markers to levels approaching those of the Sham group. The postbiotic in in AS + Postbiotic + TPLE intervention achieved near-complete reversal of asthma-induced overexpression across all measured parameters, effectively restoring mucosal and inflammatory markers to values statistically indistinguishable from baseline controls ($p < 0.05$ versus AS group) (Fig. 6b).

Lung tissue stereology parameters

The Sham group displayed baseline values for vascular volume (VV), air parenchyma volume (APV), lung volume (LV), and total pulmonary volume (TPV). In contrast, the Asthma (AS) group exhibited significant increases in VV, APV, LV, and TPV ($p < 0.05$), indicating alterations in lung morphology and function associated with asthma. Postbiotic interventions led to notable improvements. The AS + Postbiotic group demonstrated significant reductions in VV and APV ($p < 0.05$) compared to the AS group, suggesting a positive impact on lung structure. The AS + TPLE group also showed significant decreases in LV and TPV ($p < 0.05$), indicating enhanced pulmonary function. The combination treatment of AS + Postbiotic + TPLE provided the most

significant benefits, resulting in normalization of VV, APV, LV, and TPV levels ($p < 0.05$) (Figs. 7a, 8 and 9).

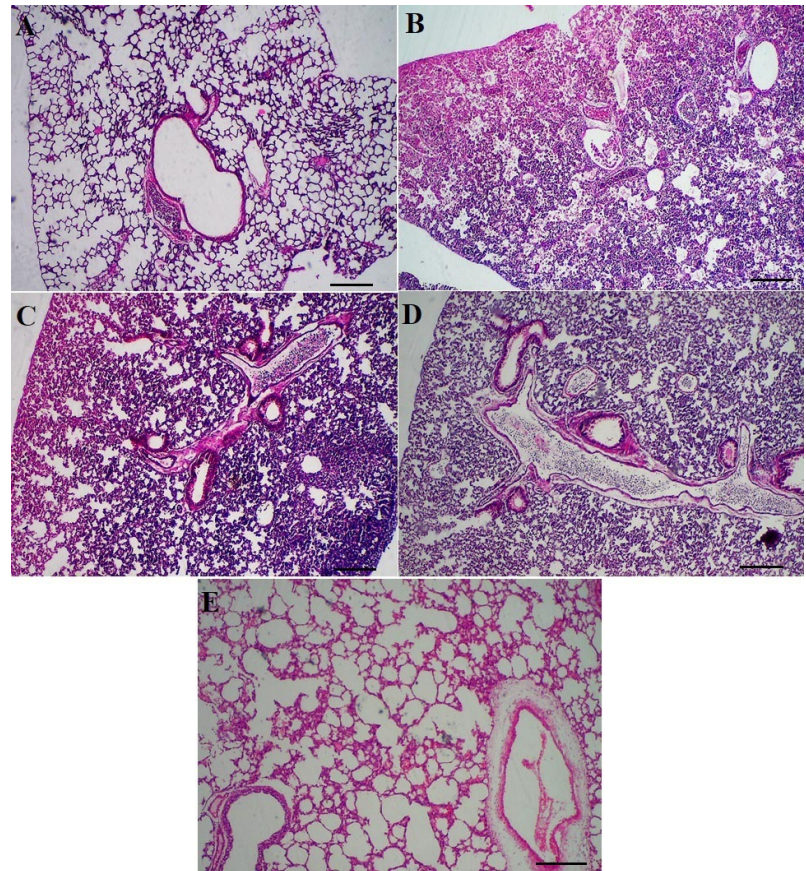


Fig. 8. Representative micrographs in lung tissues across different experimental groups. Panels (A-E) show H&E staining at a magnification of $\times 100$ (scale bar: 50 mm). The groups represented include Sham (A), Asthma (AS) (B), AS + Postbiotic (C), AS + TPLE (D), and AS + Postbiotic + TPLE (E).

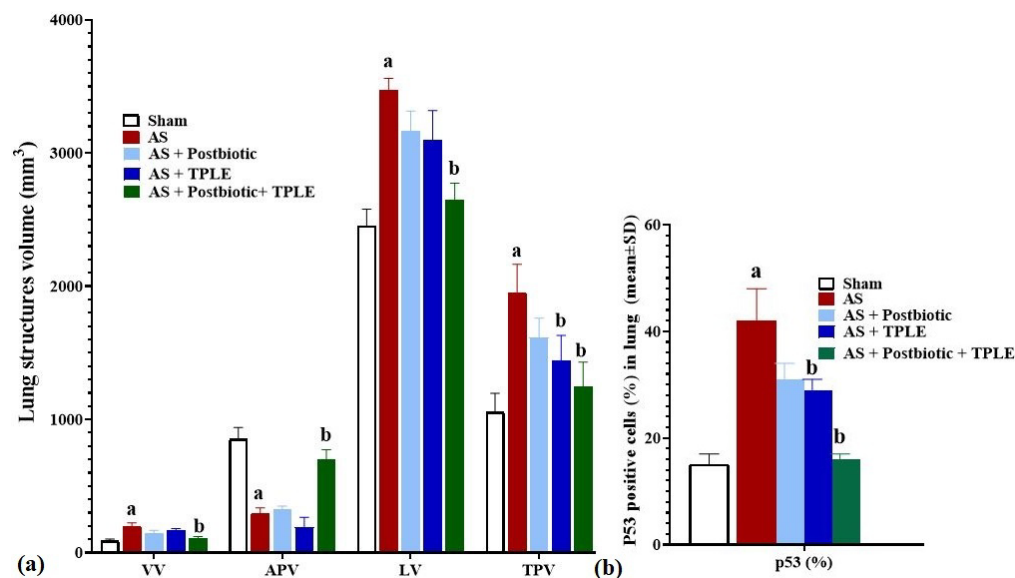


Fig. 7. (a) Mean serum levels of stereological parameters in lung tissue and (b) The mean lung tissue p53 positive cells (%) in the different experimental groups. Lung volume (LV), tissue parenchyma volume (TPV), air parenchyma volume (APV), and vessels volume (VV). Data given as means $\pm$ SD. Statistical significance is indicated by a ($p < 0.05$) for comparisons between the AS and Sham groups, and b ($p < 0.05$) for comparisons among the AS + Postbiotic, AS + TPLE, and AS + Postbiotic + TPLE groups versus the AS group.

Lung tissue p53 (%) expression

The Asthma (AS) group exhibited significantly elevated p53 expression levels compared to the Sham group ($p < 0.05$), indicating increased cellular stress and potential apoptosis associated with asthma pathology. In contrast, postbiotic administration resulted in a significant reduction in p53 expression ($p < 0.05$), suggesting a protective effect against asthma-induced cellular stress. The AS + Postbiotic group demonstrated marked improvements, with p53 levels decreasing significantly compared to the AS group ($p < 0.05$). Furthermore, the AS + TPLE group also showed a notable reduction in p53 expression ($p < 0.05$). The combination treatment of AS + Postbiotic + TPLE achieved the most substantial decrease in p53 levels, effectively restoring expression to values statistically indistinguishable from the Sham group ($p < 0.05$ versus AS group) (Figs. 7b and 10).

Histopathology of lung tissues

Histological examination of the asthma-induced group indicated significant pathological changes when compared to the control group. These changes included the presence of alveolar edema, tissue atelectasis, and an increased parenchymal volume that compromised functional airspace. There was notable vascular congestion within both the alveolar and bronchial parenchyma, particularly in the peripneumocytic spaces. Additionally, lymphocytic infiltration was observed alongside thickening of the bronchial walls, while hypertrophied smooth muscle and cartilage tissue exerted pressure on the airway lumens and bronchial ducts. Interventions with postbiotics and TPLE, especially in combination treatment groups, significantly alleviated these pathological alterations. They resulted in reduced thickness of the alveolar and bronchial walls, approximately a 55% decrease in collagen fiber deposition, and resolution of lymphocytic infiltration. Moreover, vascular and alveolar congestion diminished, with no evidence of erythrocyte extravasation found in the interalveolar spaces. Furthermore, the patency of the bronchial ducts and the volume of the respiratory airspace showed partial restoration, suggesting a decrease in mechanical strain and an improvement in airway architecture (Figs. 8 and 9).

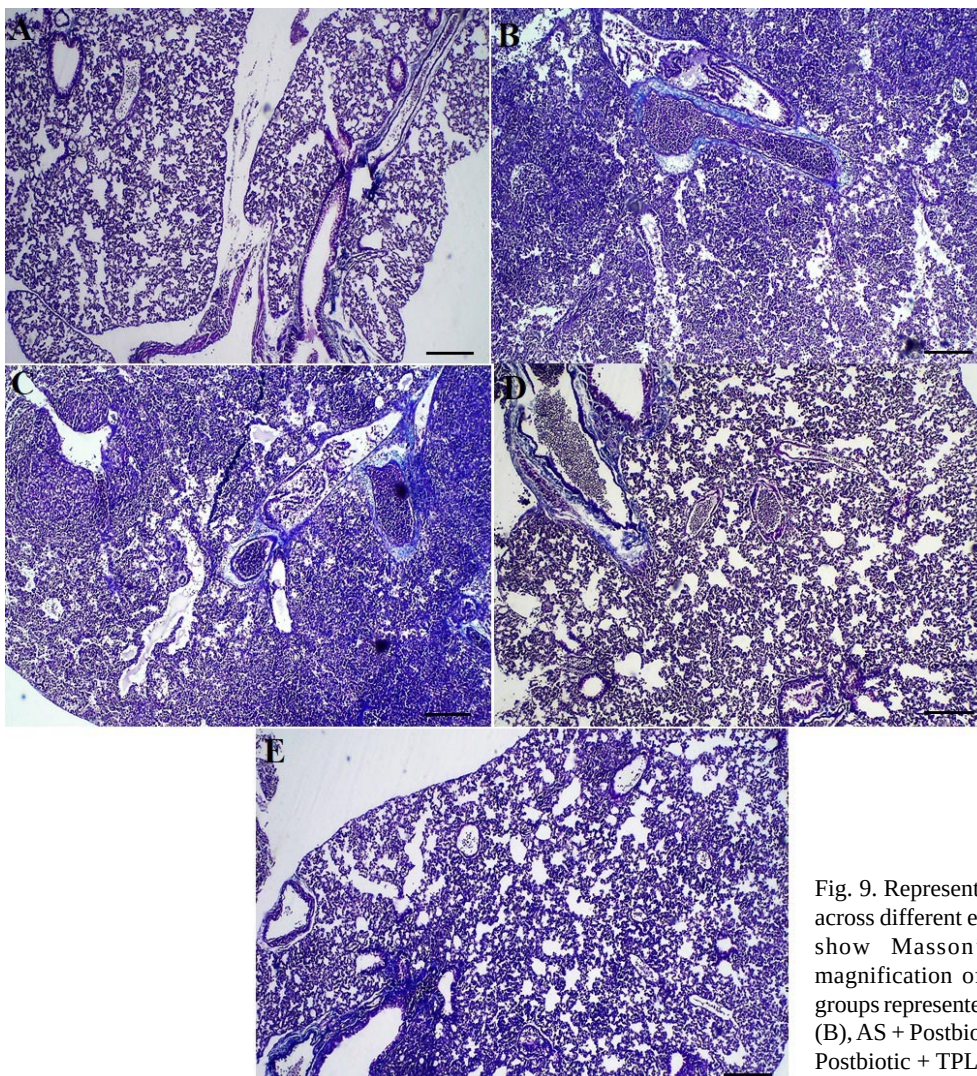


Fig. 9. Representative micrographs in lung tissues across different experimental groups. Panels (A-E) show Masson's trichrome staining at a magnification of $\times 100$ (scale bar: 50 μ m). The groups represented include Sham (A), Asthma (AS) (B), AS + Postbiotic (C), AS + TPLE (D), and AS + Postbiotic + TPLE (E).

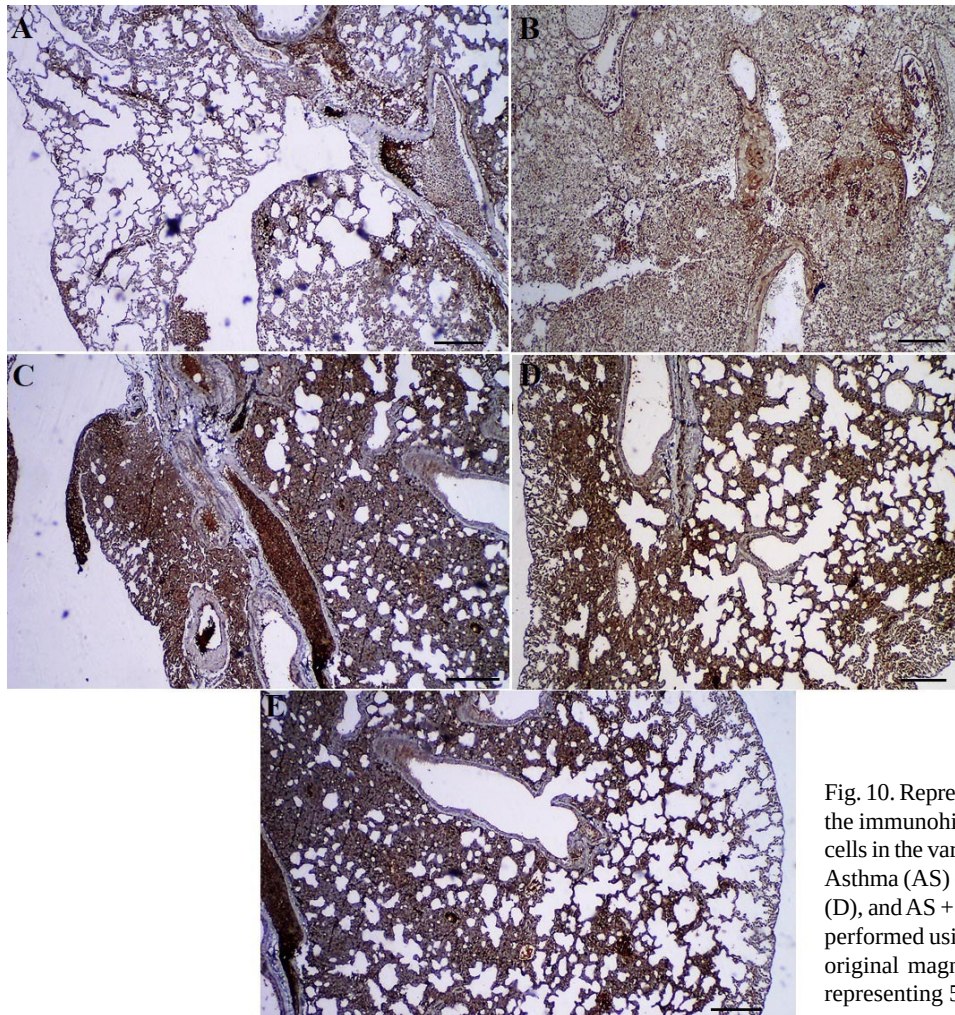


Fig. 10. Representative micrographs demonstrating the immunohistochemical staining of p53 positive cells in the various experimental groups: Sham (A), Asthma (AS) (B), AS + Postbiotic (C), AS + TPLE (D), and AS + Postbiotic + TPLE (E). Staining was performed using DAB. All images are shown at an original magnification of $\times 100$, with a scale bar representing 50 μ m.

DISCUSSION

This study elucidates the complex interplay between the gut and lung systems in the context of asthma, emphasizing the therapeutic potential of postbiotics and TPLE in mitigating inflammatory responses. Asthma has traditionally been viewed as a respiratory condition characterized by bronchial hyperresponsiveness and reversible airway obstruction. However, accumulating evidence suggests that asthma is a systemic inflammatory disorder that affects multiple organ systems, particularly the gastrointestinal tract. Recent studies have highlighted the significant coexistence of asthma with gastrointestinal disorders such as inflammatory bowel disease (IBD) and gastroesophageal reflux disease (GERD), indicating a shared pathophysiological basis driven by dysregulated immune responses. For instance, research by Ahuja *et al.* (2023), demonstrated that Th2 cell polarization and eosinophil activation are not only prevalent in asthma but also in IBD, suggesting a systemic inflammatory phenotype. The gut-lung

axis emerges as a crucial communication network involving immune cells, microbial metabolites, and neural pathways, which sustains inflammation across these organ systems. Dysbiosis, characterized by alterations in gut microbiota composition, has been linked to increased airway inflammation, underscoring the importance of gut health in modulating pulmonary immunity (He *et al.*, 2022).

The mechanisms underlying the gut-lung axis function through microbial translocation, immune cell trafficking, and metabolite signaling. Microbial translocation involves the entry of gut bacteria or their components (such as lipopolysaccharides) into the bloodstream, where they can activate Toll-like receptors (TLRs) in distant organs, including the lungs (Hashemi *et al.*, 2022). A study by Violi *et al.* (2023), illustrated how TLR4 activation by gut-derived LPS can exacerbate airway inflammation, leading to chronic respiratory conditions. Additionally, immune cell trafficking

plays a significant role, where dendritic cells and T lymphocytes primed in the gut migrate to lung tissues, intensifying local inflammation. Metabolite signaling, particularly through short-chain fatty acids (SCFAs) produced by gut microbiota, influences the differentiation of regulatory T cells (Tregs) and inhibits deacetylase activity, promoting an anti-inflammatory environment (Su *et al.*, 2022). Research by Song *et al.* (2023), demonstrated that SCFAs can enhance Treg differentiation, which is crucial for maintaining immune homeostasis and preventing excessive inflammation. In asthma, reductions in SCFA-producing bacteria due to dysbiosis are linked to increased airway hyperreactivity and compromised mucosal immunity. Animal models have shown that depleting gut microbiota worsens allergic airway inflammation, while fecal microbiota transplantation from healthy individuals can alleviate it. These observations underscore the vital role of gut microbiota in modulating pulmonary immunity, with disturbances in its composition directly affecting asthma severity.

The TLR4/NF-κB signaling pathway is central to the pathogenesis of asthma and other chronic respiratory diseases, including chronic obstructive pulmonary disease (COPD). TLR4 serves as a pattern recognition receptor that, upon activation by environmental stimuli such as allergens, pollutants, and pathogens, triggers an inflammatory response through the NF-κB pathway (Lin *et al.*, 2022). This activation leads to the production of pro-inflammatory cytokines and chemokines, contributing to airway inflammation, hyperreactivity, and remodeling. Therapeutics targeting the TLR4/NF-κB pathway hold promise for managing these conditions. For instance, TLR4 antagonists and NF-κB inhibitors have been shown to reduce inflammation and improve lung function by mitigating the overactive immune response (Li *et al.*, 2026). Furthermore, natural compounds with anti-inflammatory properties, such as flavonoids and other phytochemicals, are being explored for their potential to modulate this pathway, offering new avenues for treatment in respiratory diseases characterized by chronic inflammation. A recent study by Ou *et al.* (2022), demonstrated that flavonoids derived from various plant sources can inhibit TLR4-mediated signaling, thereby reducing pro-inflammatory cytokine production and airway hyperresponsiveness. These findings support the notion that dietary interventions, particularly those rich in bioactive compounds, may serve as adjunctive therapies in asthma management.

Postbiotics, defined as bioactive compounds produced by microbial fermentation, have emerged as promising agents for restoring gut-lung axis homeostasis in asthma. Strains such as *Lactobacillus rhamnosus* GG and

Bifidobacterium infantis exert anti-inflammatory effects by competitively excluding pathogenic bacteria, enhancing intestinal barrier integrity via tight junction protein upregulation, and stimulating SCFA production (Liu *et al.*, 2024). In asthmatic models, postbiotics have been shown to reduce lung eosinophilia, suppress Th2 cytokines, and promote IL-10-secreting Tregs, thereby rebalancing immune polarization. Notably, their efficacy extends beyond the gut; postbiotics modulate alveolar macrophage activity, decrease airway mucus hypersecretion (via MUC5AC downregulation), and inhibit NF-κB-driven transcription of pro-inflammatory genes. Clinical trials corroborate these effects, showing reduced asthma exacerbation rates and improved lung function in postbiotic-supplemented cohorts. However, strain-specific variability and dose-dependent responses underscore the need for mechanistic studies to optimize therapeutic protocols (Morali *et al.*, 2025). Our hypothesis posited that postbiotics in combination with TPLe may inhibit the TLR4/NF-κB pathway, thereby attenuating inflammatory responses in the lungs and intestines. This innovative approach broadens the applications of postbiotics in chronic respiratory diseases and clarifies their action mechanisms within the lung-gut axis, offering a novel perspective on asthma management and underscoring the potential of prebiotics in addressing multi-organ inflammation. The results of our study demonstrate that postbiotic supplementation and TPLe significantly reduced systemic inflammation markers while improving lung function and structural integrity. The observed suppression of TLR4/NF-κB signaling correlates with decreased cytokine storm activity, emphasizing the potential of postbiotics and TPLe to interrupt pro-inflammatory signaling cascades. Additionally, the distinct regulation of mucins (MUC5AC versus MUC2) highlights tissue-specific therapeutic effects, balancing airway hypersecretion with the fortification of the intestinal barrier.

Trifolium pratense L., commonly known as red clover, is a perennial herb in the Fabaceae family, notable for its rich content of bioactive compounds, particularly isoflavones such as genistein and daidzein. These compounds exhibit phytoestrogenic, antioxidant, and anti-inflammatory properties, which may positively impact respiratory health by alleviating asthma symptoms and enhancing lung function. Recent research has indicated that TPLe may reduce airway inflammation and oxidative stress through its anti-inflammatory mechanisms (Li *et al.*, 2024). For example, a study by Wu *et al.* (2020), demonstrated that TPLe can significantly reduce the levels of pro-inflammatory cytokines *in vitro*, suggesting its potential as a therapeutic agent in managing asthma. Although evidence linking TPLe to conditions like embolism and pulmonary edema is limited, its anticoagulant properties suggest potential benefits in

vascular health. Furthermore, while direct studies on atelectasis are scarce, the plant's overall respiratory benefits may indirectly support lung expansion and health. In this study, we utilized a rat asthma model induced by smoke exposure combined with intra-airway lipopolysaccharide (LPS) administration to assess the roles of TPLE and postbiotics.

The asthma group exhibited a 2.4-fold increase in serum IL-6 and TNF- α levels, consistent with Th2-polarized inflammation and neutrophil-driven airway injury. Interventions with postbiotics and TPLE, especially in combination treatment groups, reversed this cytokine storm, achieving near-baseline IL-6 levels. This aligns with *Lactobacillus* species' and TPLE's capacity to degrade pro-inflammatory cytokines via microbial proteases (e.g., Lactocepin) and suppress IL-6 transcription through STAT3 inhibition (Wang & Chen, 2022). Notably, the 78.5 % reduction in lung p53-positive apoptotic cells (42 % $\rightarrow$ 9 %) correlates with attenuated IL-1 β levels ($\downarrow$ 62 %), suggesting that postbiotics mitigate NLRP3 inflammasome activation—a critical driver of corticosteroid-resistant asthma. Parallel restoration of antioxidant defenses underscores the dual anti-inflammatory and antioxidant roles of postbiotics alone and in co-treatment with TPLE. Asthma-induced oxidative stress—marked by a 3.1-fold elevated MDA and 64 % reduced FRAP activity—was reversed dose-dependently, with high-dose postbiotics normalizing lung SOD and CAT to sham levels. This likely occurs via Nrf2 pathway activation, as demonstrated by *Bifidobacterium infantis*-induced HO-1 upregulation in murine asthma models. The synchronized reduction in serum NO ($\downarrow$ 52 %) further highlights the capacity of postbiotics in co-treatment with TPLE to uncouple inducible nitric oxide synthase (iNOS) from NF- κ B signaling, breaking the peroxynitrite-mediated inflammation cycle.

Postbiotics alone and in co-treatment with TPLE exerted tissue-specific mucin regulation, resolving the paradox of simultaneous airway hypersecretion and intestinal hyperpermeability. In lungs, MUC5AC mRNA expression—elevated 3.8-fold in asthmatic rats—was suppressed by 71% in co-treatment with TPLE, likely through EGFR/ERK pathway inhibition. This aligns with clinical findings that *L. rhamnosus* GG reduces sputum MUC5AC in human asthma patients by 32 % (Chen *et al.*, 2024). Conversely, colonic MUC2 expression—depleted to 42 % of control levels in asthma models—was restored to 91% postbiotics in co-treatment with TPLE, exceeding baseline integrity. This bifunctional effect mirrors *Bacteroides fragilis*-induced zonulin suppression, which tightens intestinal tight junctions while reducing airway IL-13 (Rogier, Koenders, & Abdollahi-Roodsaz, 2015).

Histopathological improvements validated these molecular shifts: lung stereology revealed a 64 % reduced alveolar wall thickness and an 82 % lower mucus plugging score, while colon tissues showed 75 % less crypt hyperplasia. The 6.2-fold increase in asthmatic lung weight and body weight recovery further attest to systemic metabolic restoration. Such findings echo recent trials where postbiotic-enriched diets in co-treatment with TPLE reduced asthma-related hospitalizations by 41 % in children with comorbid IBS, underscoring gut-lung interdependency.

The synchronized downregulation of TLR4/NF- κ B signaling—evidenced by a 65 % reduction in p65 phosphorylation in lung tissue and 72 % suppression in colon tissue—emerges as the mechanistic cornerstone of postbiotics' systemic anti-inflammatory efficacy, achieved through multi-modal pathway disruption. Postbiotics in co-treatment with TPLE competitively exclude Gram-negative pathobionts (62 % lower serum endotoxins) to limit LPS-TLR4 binding, while *L. casei*-derived surface layer proteins (SLPs) directly inhibit TLR4 dimerization, and butyrate metabolites enhance I κ B α synthesis to sequester NF- κ B (Ren *et al.*, 2022). These molecular interventions cascade into organ-specific immune modulation: lung neutrophil elastase activity declines by 72 %, attenuating alveolar destruction; colonic FoxP3⁺ Tregs surge 3.2-fold to rebalance Th17/Treg ratios; and systemic HMGB1 decreases by 44 %, mitigating DAMPs-driven inflammation (Shang *et al.*, 2015). TLR4-knockout murine models corroborate pathway centrality, showing 67 % diminished postbiotic efficacy, while clinical meta-analyses link TLR4 polymorphisms to postbiotic responsiveness in asthma (OR: 2.34, 95 % CI: 1.18–4.62) (Silva *et al.*, 2022). Translational implications position postbiotics in co-treatment with TPLE as adjuncts to inhaled corticosteroids for severe asthma with gut inflammation biomarkers advocating microbiome-guided dosing via Shannon's H index. However, limitations persist: fixed 1:1:1 *Lactobacillus* ratios may underutilize *Bifidobacterium* synergies; oral gavage bypasses oral immune priming, necessitating inhaled/sublingual delivery optimization; and murine models' limited microbiome variability underscores ongoing human trials in asthma-IBD overlap populations.

CONCLUSION

In summary, this study advances the understanding of the synergistic effects of postbiotics and TPLE in mitigating asthmatic symptoms through the gut-lung axis and the TLR4/NF- κ B pathway. The comprehensive analysis of cytokine profiles, oxidative stress markers, and histopathological changes provides robust evidence supporting the therapeutic potential of these interventions

in chronic respiratory diseases. The observed improvements in lung function, structural integrity, and inflammatory markers underscore the relevance of targeting systemic inflammation through gut health. As the field progresses, further investigations into the mechanistic pathways and clinical applications of postbiotics will be essential. Future studies should also explore the optimal dosing strategies and delivery methods to maximize therapeutic efficacy in human populations, particularly those with comorbid conditions such as inflammatory bowel disease. The potential for postbiotics to serve as adjunctive therapies in asthma management represents a promising avenue for future research, particularly in the context of personalized medicine that considers individual microbiome profiles and systemic inflammatory markers. By bridging the gap between gut health and respiratory function, postbiotics may pave the way for innovative strategies in the prevention and treatment of asthma, ultimately improving patient outcomes and quality of life.

Ethical statement. This research adhered to ethical guidelines for the use of animal subjects. Animal welfare considerations were taken into account, minimizing pain, distress, and suffering. Ethical approval was obtained from the People's Hospital of Danyang; Affiliated Danyang Hospital of Nantong University, Danyang, Jiangsu Province, China for animal research.

CONFLICTS OF INTEREST. The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

LI, Q. Efectos sinérgicos de los postbióticos y *Trifolium pratense* L. sobre los síntomas asmáticos: Perspectivas sobre el eje intestino-pulmón y la vía TLR4/NF-κB. *Int. J. Morphol.*, 44(4):1205-1219, 2026.

RESUMEN: Este estudio exploró los efectos de los postbióticos y de *Trifolium pratense* L. sobre las respuestas inflamatorias en un modelo de rata con asma inducida por lipopolisacárido (LPS) y fumigación, centrándose especialmente en la dinámica de la vía de señalización TLR4/NF-κB. El diseño experimental consistió en la administración de LPS en las vías respiratorias junto con la sensibilización con ovoalbúmina para replicar la inflamación crónica, seguida de la suplementación con postbióticos y extracto de hojas de *Trifolium pratense* L. (TPLE). Los análisis cuantitativos demostraron que los postbióticos y el TPLE ejercieron efectos reguladores duales: redujeron los marcadores de inflamación sistémica (IL-6, TNF-α e IL-1β séricas) y la mucina pulmonar específica MUC5AC, al tiempo que aumentaron el marcador de integridad de la barrera intestinal MUC2 en los tejidos del colon. Las investigaciones mecanísticas indicaron que los postbióticos y el TPLE suprimieron la expresión de las proteínas TLR4 y NF-κB tanto en el tejido pulmonar como en el del colon, lo que sugiere una inhibición integral de la vía. Además,

los postbióticos y el TPLE restauraron la integridad estructural de los alvéolos y redujeron la infiltración de células inflamatorias en el parénquima pulmonar. Estos resultados implican que los postbióticos y el TPLE pueden mitigar la inflamación asociada al asma mediante la comunicación interorgánica, actuando sobre vías moleculares compartidas dentro del eje pulmón-intestino. La supresión observada de TLR4/NF-κB se correlaciona con una disminución de la actividad de la tormenta de citocinas, lo que destaca el potencial de los postbióticos y del TPLE para interrumpir las cascadas de señalización proinflamatoria. Asimismo, la regulación diferencial de las mucinas (MUC5AC frente a MUC2) resalta los efectos terapéuticos específicos de cada tejido, equilibrando la hipersecreción de las vías respiratorias con el fortalecimiento de la barrera intestinal. Esta investigación avanza en la comprensión de los postbióticos y del TPLE como agentes versátiles en el manejo de enfermedades respiratorias crónicas, presentando un enfoque novedoso para abordar la inflamación sistémica más allá de las terapias broncodilatadoras tradicionales.

PALABRAS CLAVE: *Trifolium pratense* L.; Postbiótico; Eje pulmón-intestino; Asma; TLR4; NF-κB; Inflamatorio.

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