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Multi-omics joint analysis: *Polygonatum sibiricum* extract ameliorates renal fibrosis in mice with chronic kidney disease via gut microbiota-dependent NLRP3 inflammasome inhibition

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ABSTRACT: *Polygonatum sibiricum* F. Delaroche (PS) is an edible traditional phytomedicine known for its various bioactivities, including anti-inflammatory effects and renal protection. However, its potential role in addressing renal fibrosis, which is irreversible and contributes to the progression from chronic kidney disease (CKD) to end-stage renal disease, remains unclear. This study aimed to elucidate the mechanisms by which the aqueous extract of PS (PSAE) ameliorates renal fibrosis in a CKD mouse model using a multi-omics approach. CKD was induced by a 0.2% adenine diet, and the therapeutic effects of PSAE on renal injury and fibrosis were systematically evaluated. Integrated metabolomic and gut microbiome analyses were employed to investigate the modulatory effects of PSAE on key metabolic pathways and gut microbiota composition. Additionally, serum pharmacochimistry combined with network pharmacology was used to identify potential targets of bioactive metabolites derived from PSAE. The core mechanism was further validated through in vitro experiments and NLRP3^{-/-} CKD mice. PSAE significantly reduced plasma creatinine and urea levels and inhibited the expression of fibrosis-related markers, including TGF- β 1, α -SMA, Fibronectin, and Coll α 1. Metabolomics revealed that PSAE modulated the plasma metabolic profile, notably impacting host-microbiota co-metabolic pathways such as phenylalanine metabolism. Gut microbiome analysis further confirmed that PSAE reshaped the gut microbial community structure. Integrated serum compound analysis and network pharmacology suggested that the gut microbiota-mediated bioavailable metabolites of PSAE may alleviate renal fibrosis by targeting the NLRP3 inflammasome pathway. This mechanism was consistently verified both in vitro and in vivo, with supporting evidence from the assessment of fibrosis-related markers in NLRP3^{-/-} CKD mice treated with PSAE. In conclusion, PSAE attenuates renal fibrosis by modulating the gut microbiota and host-microbiota co-metabolism, thereby inhibiting NLRP3 inflammasome activation. These findings highlight the crucial role of the “gut microbiota-metabolism-NLRP3 inflammasome” axis in the anti-fibrotic action of PSAE, providing foundational support for the development and application of PS as a functional food or drug aimed at alleviating renal fibrosis.

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1. Introduction

Chronic kidney disease (CKD) is characterized by a sustained abnormal condition in the kidneys, indicated by a glomerular filtration rate (GFR) of less than 60 mL/min/1.73 m² or albuminuria of more than 30 mg/24 h. It represents a clinical syndrome of gradually progressive renal dysfunction caused by various underlying kidney diseases [1]. Presently, the global prevalence of CKD is estimated to be 9.5%, and this figure is rising each year [2]. CKD is often asymptomatic during its early stages. As the condition advances, a process known as renal fibrosis is activated, leading to irreversible loss of kidney function and, ultimately, end-stage renal disease (ESRD), which is the leading cause of death among patients with CKD [3]. The clinical course of CKD is further complicated by systemic manifestations, including cardiovascular diseases, anemia, mineral and bone disorders, and uremic syndrome, which collectively contribute to high morbidity and mortality [4]. In clinical practice, various medications—such as renin-angiotensin system blockers, mineralocorticoid receptor antagonists, and sodium/glucose cotransporter-2 inhibitors—are commonly used to manage the pathological indicators of CKD. These drugs primarily work by influencing blood pressure, tubular epithelial metabolism, blood glucose levels, or inflammation. While these agents demonstrate efficacy in slowing disease progression to some extent, they are often limited by several drawbacks. These include the inability to directly halt or reverse established renal fibrosis, the emergence of adverse effects such as hyperkalemia and hypotension, and a primarily single-target mechanism that may not adequately address the multifaceted pathogenesis of CKD [4–5]. Consequently, there remains a critical, unmet need for approved therapies that specifically target renal fibrosis.

Renal fibrosis is a pathological extension of the wound healing process characterized by the excessive accumulation of extracellular matrix (ECM) proteins, such as collagen I and fibronectin [6]. In cases of mild kidney damage, the inflammatory response is activated along with fibroblasts, leading to increased ECM synthesis to promote tissue repair. Normally, this ECM is later resorbed. However, during chronic injury in CKD, persistent damage triggers ongoing ECM accumulation, resulting in progressive sclerosis of the renal parenchyma and the formation of scar tissue [7]. Additionally, the kidney undergoes several post-injury changes, including renal tubule atrophy, reduced capillary density, and tissue hypoxia, all of which accelerate the progressive loss of renal function and ultimately lead to renal failure [8]. The inflammatory response and related injuries are significant contributors to renal fibrosis. Furthermore, substantial evidence indicates that dysbiosis of the gut microbiota can induce or worsen the process of renal fibrosis through various mechanisms. In CKD patients and experimental animals, there is a notable reduction in the diversity of gut microbiota, with an increase in Firmicutes and a decrease in Bacteroidota [9]. This structural alteration of gut microbiota leads to elevated concentrations of secondary metabolites, such as indoxyl sulfate, trimethylamine oxide, p-cresol sulfate, and other uremic toxins, which accumulate in the bloodstream. These toxins exacerbate nephrotoxicity and the progression of renal fibrosis [10–11]. Moreover, gut microbiota dysbiosis can compromise the intestinal

mucosal barrier, allowing harmful substances (e.g., lipopolysaccharides, uremic toxins, and bacterial DNA) to translocate from the intestine into the bloodstream [12–13]. This translocation activates inflammatory signaling pathways, such as the NOD-like receptor family, pyrin domain-containing-3 (NLRP3) inflammasome, and Toll-like receptors (TLRs). This activation promotes the secretion of inflammatory factors (including IL-1 β , IL-6, and TNF) and pro-fibrotic factors (such as TGF- β) in the kidneys. Consequently, this process induces inflammatory cell infiltration, tubular injury, and ECM deposition, thereby accelerating renal fibrosis [13–15]. As a result, the gut microbiota has emerged as a potential therapeutic target for managing renal fibrosis.

In contrast to the single-target paradigm of many clinical drugs, natural products represent a holistic and multi-targeted therapeutic strategy with a potentially superior safety profile. Bioactive compounds derived from these natural sources can concurrently modulate inflammation, oxidative stress, metabolic pathways, and gut microbiota—core drivers of CKD progression and renal fibrosis [16–17]. *Polygonatum sibiricum* F. Delaroche (PS) is a member of the *Polygonatum* genus within the Liliaceae family, commonly found in East Asia, including regions of China, Mongolia, and Korea [18]. The rhizome of PS is rich in various components, such as carbohydrates, proteins, saponins, alkaloids, flavonoids, and more [19–20]. According to *Shennong's* Classic of Materia Medica, PS is believed to tonify the kidneys, support the spleen, and nourish the lungs. Notably, PS is a classic “medicine and food homology” substance, traditionally used both as a nutritional food and for therapeutic purposes [21]. For over 2,000 years, PS has been used in China as a nutritional supplement and functional food to address kidney disease, diabetes, and respiratory infections [22–24]. Research indicates that the polysaccharides, oligosaccharides, and saponins present in PS can positively influence the gut microbiota in aging, diabetic and colitis mice [25–28]. For instance, *Polygonatum sibiricum* polysaccharides have been shown to alleviate oxidative stress and exhibit potent anti-aging activity by modulating intestinal microbes and related metabolites, specifically by increasing *Bacteroidota* and decreasing *Firmicutes*, *Patescibacteria*, *Actinobacteriota*, and *Campylobacterota* [28]. Besides, PS polysaccharides exhibit nephroprotective properties by promoting mitochondrial biogenesis and suppressing renal interstitial fibrosis in mice with hyperuricemia-induced nephropathy [29]. However, the specific mechanisms through which PS exerts its protective effects against kidney damage remain unclear, highlighting the need for further exploration into the pharmacodynamic mechanisms of PS against CKD.

In this study, we hypothesize that the aqueous extract of *Polygonatum sibiricum* (PSAE) may improve CKD by inhibiting renal fibrosis through a gut microbiota-dependent anti-inflammatory mechanism. We established a mouse model of adenine-induced CKD to evaluate the effects of PSAE on renal fibrosis, and performed integrated microbiome and metabolomics analyses to delineate the regulatory network involving significantly altered core microbiota, key metabolic pathways, and host phenotypes under PSAE intervention. Additionally, we examined the role of gut microbiota in the anti-fibrotic effects of PSAE using germ-depleted CKD mice. Through serum analysis and network pharmacology, we aimed to elucidate the potential signaling pathways associated with the effects of PSAE against CKD. This study will provide new insights into the

mechanism by which PSAE ameliorates CKD and offer a scientific basis for developing PSAE as a potential gut microbiota-targeted therapeutic strategy.

2. Materials and methods

2.1. Materials and reagents

The rhizome of *Polygonatum sibiricum* was obtained from Sichuan Xishujiujin Modern Chinese Medicine Co., Ltd. (Sichuan, China) and was authenticated by Dr. Xiongjie Sun from Hubei University of Chinese Medicine (Wuhan, China). Adenine hydrochloride was purchased from Aladdin (Shanghai, China), while phorbol-12-myristate-13-acetate (PMA) and lipopolysaccharide (LPS) were sourced from Sigma-Aldrich (St. Louis, MO, USA). ATP was supplied by Amresco (Solon, USA). Cell culture materials, including Roswell Park Memorial Institute (RPMI) 1640 medium, Opti-MEM, and fetal bovine serum (FBS), were obtained from Gibco (Grand Island, NY, USA). For molecular analysis, a first-strand cDNA synthesis kit and a 2× FastHS SYBR QPCR mixture were acquired from Allmeek Co., Ltd. (Beijing, China). The BCA protein assay kit was obtained from Beyotime (Shanghai, China), and both the Creatinine Colorimetric Assay Kit and Urea Colorimetric Assay Kit were supplied by Elabscience Biotechnology Co., Ltd. (Wuhan, China). Primary antibodies were sourced as follows: antibodies against E-cadherin and epithelial Na⁺ channel (ENaC)- γ were purchased from Abcam (Cambridge, UK); ENaC- α and aquaporin (AQP) 3 antibodies were obtained from GeneTex (CA, USA); antibodies for fibronectin, α -smooth muscle actin (α -SMA), collagen type I alpha 1 (Col1 α 1), transforming growth factor- β 1 (TGF- β 1), caspase-1, and interleukin (IL)-1 β were supplied by ABclonal Technology Co., Ltd. (Wuhan, China); the antibody for apoptosis-associated speck-like protein containing CARD (ASC) was purchased from Elk Biotechnology (Wuhan, China); and NLRP3, cyclooxygenase 2 (COX-2), and gasdermin D (GSDMD) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin antibodies were sourced from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 Preparation and composition analysis of PSAE

PSAE was extracted using distilled water at a temperature of 100 °C for 60, 40, and 30 minutes, respectively. The extraction solutions from these three different time periods were combined and concentrated under reduced pressure to obtain the PSAE at a concentration of 1 g/mL of the crude material. The chemical composition of the PSAE was analyzed using a colorimetric method as well as ultra-performance liquid chromatography tandem quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS), as detailed in the Supplementary Method.

2.3. Animal experiment

Male C57BL/6 mice (6 weeks old, weighing (22 $\pm$ 2) g) and male SD rats (6 weeks old, weighing ((200 $\pm$ 20) g) were obtained from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China). Additionally, male Nlrp3 gene deletion mice on a C57BL/6 background were sourced from Cyagen Biosciences (Suzhou,

China). All animals were housed under standard conditions, including a 12-hour light/dark cycle, $(55 \pm 5)\%$ humidity, and a temperature of $(23 \pm 2)^\circ\text{C}$, with access to food and water available ad libitum. All animal experiments were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethical Experimentation Committee of Hubei University of Chinese Medicine.

For the pharmacodynamic study on the ability of PSAE to ameliorate renal fibrosis, male C57BL/6 mice were randomly divided into five groups ($n = 8$ mice per group) after a one-week acclimation period. The Ctrl group was fed a normal diet. The Adenine group received a diet containing 0.2% adenine for three weeks, along with daily intragastric gavage of distilled water. The Adenine + PSAEL, Adenine + PSAEM, and Adenine + PSAEH groups were also fed a diet containing 0.2% adenine for three weeks, but with different doses of PSAE administered daily by intragastric gavage (2.5, 5, and 10 g crude PS/kg, respectively).

To investigate the necessity of gut microbiota for the anti-renal fibrosis effects of PSAE, a germ-depletion experiment was designed. Following a previously described protocol [30], male C57BL/6 mice ($n = 8$ mice per group) were pretreated with a combination of antibiotics (1.0 mg/mL ampicillin, 1.0 mg/mL neomycin, 0.5 mg/mL vancomycin, and 0.5 mg/mL metronidazole added to their drinking water) for eight weeks to deplete the gut microbiota. After the antibiotic treatment, these mice were then fed a diet containing 0.2% adenine and received daily intragastric gavage of PSAE (10 g crude PS/kg) for three weeks.

Further, to explore the target of PSAE, both wild-type and NLRP3-deficient ($\text{NLRP3}^{-/-}$) mice ($n = 8$ mice per group) were fed a diet containing 0.2% adenine for three weeks. During this period, $\text{NLRP3}^{-/-}$ mice were treated with PSAE (10 g crude PS/kg) via intragastric gavage daily.

Throughout all experiments, the weights of the mice were recorded weekly. At the end of the experiments, all mice were euthanized via isoflurane inhalation, and blood was collected by retro-orbital plexus puncture for plasma separation. Complete blood counts were performed using an HF-3800 hematology analyzer (Hanfang Medicine Co., Ltd., Jinan, China). Major tissues were harvested. A portion of the kidney tissues was fixed in 4% paraformaldehyde, while the remaining samples were stored at -80°C for further analysis.

2.4. Creatinine and urea Analysis

Plasma creatinine and urea levels in each group of mice were measured using the Creatinine Colorimetric Assay Kit and Urea Colorimetric Assay Kit, following the manufacturer's instructions.

2.5. Histological analysis

After fixation and dehydration, kidney tissues were embedded in paraffin and sectioned into 5 μm thick slices. The sections were stained with hematoxylin and eosin (H&E) to assess renal injury. Periodic Acid-Schiff (PAS) staining was conducted to evaluate glomerular and tubular lesions. Masson's trichrome staining was employed to observe the severity of renal fibrosis. Additionally, F4/80 immunohistochemical staining

was performed to assess inflammatory cell infiltration in the renal interstitium. All images were captured using a Leica DMIL 4000B optical microscope (Wetzlar, Germany) equipped with a Leica digital camera.

2.6. RNA extraction and quantitative real-time PCR (RT-PCR)

Total RNA was extracted from kidney tissues and analyzed using RT-PCR, following the methodology described in Ding et al. [31]. Briefly, Total RNA was extracted from approximately 30 mg of snap-frozen kidney tissue using 1 mL of TRIzol reagent and reversed transcription into cDNA according to the manufacturer's instructions (Allmeek Co., Ltd, Beijing, China). After that, RT-PCR was performed using the 2× FastHS SYBR QPCR mixture (Allmeek Co., Ltd, Beijing, China) on an ABI QuantStudio 6 Flex Real-Time PCR System. The thermocycling conditions were as follows: pre-denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 30 s. The relative expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method and normalized to the expression of β -actin. The primer sequences are provided in Supplementary Table 1.

Additionally, we extracted genomic DNA from the fecal bacteria of mice involved in the germ-depletion experiment, adhering to the detailed procedures outlined in the Supplementary Method. We assessed bacterial content using RT-PCR, with the primer sequences listed in Supplementary Table 2.

2.7. Western blotting (WB)

Total protein was extracted from approximately 40 mg of kidney tissue by homogenization in 800 μ L of RIPA lysis buffer supplemented with 1% protease and phosphatase inhibitor cocktail (Beyotime Biotechnology, Shanghai, China) on ice. The homogenate was centrifuged at $12,000 \times g$ for 15 min at 4 °C. The supernatant was collected, and the protein concentration was determined using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Next, the proteins were separated using 8-15% SDS-PAGE gels and then transferred to PVDF membranes. After blocking with 5% skim milk in TBST for 1 h at room temperature, the membranes were incubated at 4 °C for 12 hours with primary antibodies, including ENaC- α , ENaC- γ , AQP3, TGF- β 1, Fibronectin, Col1 α 1, α -SMA, E-cadherin, Vimentin, Snail 1, caspase-1, IL-1 β , ASC, NLRP3, COX-2, GSDMD, GAPDH, and β -Actin. Following this, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1.5 hours at room temperature. Finally, the protein bands were visualized using enhanced chemiluminescence and quantified with ImageJ 1.8.0 software.

2.8. Metabolomics analysis

Preprocessed serum samples were analyzed using UPLC-Q-TOF-MS/MS. Differential metabolites were screened between the Ctrl groups, Adenine groups, and PSAEH group, based on the variable importance in the projection (VIP) > 1 with $P < 0.05$. Subsequent metabolomic data analysis and visualization were conducted utilizing MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca>), and the origin of metabolites was investigated through Metorigin (<http://metorigin.met-Bioinformatics.cn/>). Detailed analysis methods are provided in the Supplementary Method.

2.9. 16S rDNA sequencing of gut microbiota

Bacterial genomic DNA was extracted from the cecum contents of mice using the E.Z.N.A. Stool DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA). The V3-V4 hypervariable region of the bacterial 16S rDNA gene was then amplified using universal primers. The PCR products were purified with the Agencourt AMPure XP Kit (Beckman Coulter, Inc., Miami, FL, USA). Following purification, libraries were created using the NEB Next Ultra II DNA Library Prep Kit (New England Biolabs, Inc., Beverly, MA, USA). Deep sequencing was conducted on an Illumina MiSeq (Illumina, Inc., San Diego, CA, USA) after performing quality control. Detailed analysis methods are provided in the Supplementary Method.

2.10. Preparation of the PSAE-containing serum

To obtain serum containing PSAE, SD rats were randomly divided into four groups. The control (Ctrl) serum group received distilled water by gavage for 13 days. The PSAE serum group was given distilled water for 6 days, followed by the administration of PSAE (7 g of crude PS per kg) by gavage for 7 days. The antibiotic (Ab) serum group was treated with a combination of antibiotics (neomycin sulphate, streptomycin, and bacitracin, 200 mg/kg each) that cannot be absorbed through the gastrointestinal tract. This group received antibiotics by gavage for 6 days to deplete the gut microbiota, followed by continued administration of the antibiotics for another 6 days and distilled water for 7 days. The Ab/PSAE serum group underwent the same initial treatment with antibiotics for 6 days to deplete the gut microbiota, then continued receiving the antibiotics for an additional 6 days, along with PSAE (7 g of crude PS per kg) for 7 days.

After the final administration, the rats were anesthetized, and serum was collected. The serum was inactivated at 56 °C for 30 minutes, then filtered through a 0.22 µm filter to remove bacteria, and stored at -80 °C for further analysis.

2.11. Analysis of components absorbed into the blood of PSAE by UPLC-Q-TOF-MS/MS

A total of 900 µL of methanol was added to 300 µL of rat serum samples from both the Ctrl and PSAE serum groups. After vortexing, the samples were sonicated in an ice water bath for 5 minutes and then incubated at -40 °C for 30 minutes. Following this, the samples were centrifuged at 12000 × g for 10 minutes at 4 °C to obtain the supernatant. The supernatant was then evaporated to dryness under a flow of nitrogen gas. The resulting residue was re-dissolved in 200 µL of acetonitrile and subjected to centrifugation at 12000 × g for 10 minutes. The supernatant was subsequently filtered through a 0.22 µm filter and injected into the UPLC-Q-TOF-MS/MS system for analysis. The chromatographic conditions are described in the Supplementary Method.

2.12. Network pharmacology analysis

The components absorbed into the bloodstream of PSAE were imported into the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and the TCMSP database (<https://old.tcmsp-e.com/index.php>) to identify potential targets. The target names were then converted to their official gene symbols using the STRING

database (<https://cn.string-db.org/>) to obtain the targets corresponding to these components. Next, the DisGeNET database (<http://www.disgenet.org/>) was utilized to search for targets related to “chronic kidney disease”. We selected genes with an EI of 1 and eliminated those with a score of less than 0.02 to refine the relevant targets for CKD.

Afterward, a Venn diagram illustrating the commonalities between the component targets and CKD targets was generated using a bioinformatics platform (<http://www.bioinformatics.com.cn/>). The identified common targets were then imported into the STRING database to construct a protein-protein interaction (PPI) network, which was visualized using Cytoscape 3.7.1 software. Additionally, the common targets were analyzed with the DAVID database (<https://david.ncifcrf.gov/>) to conduct Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The results were visualized using the bioinformatics platform. Finally, a “component-target-pathway” network was constructed using Cytoscape 3.7.1 software.

2.13. Cell culture and treatments

THP-1 cells were obtained from the China Center for Type Culture Collection in Wuhan, China. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C with 5% CO₂. To differentiate the THP-1 cells, 100 nmol/L phorbol 12-myristate 13-acetate (PMA) was added, and the cells were incubated for 24 hours.

After differentiation, the cells were treated for 24 hours with 10% control serum, PSAE serum, Ab serum, or Ab/PSAE serum. Following this treatment, the cells were stimulated with lipopolysaccharide (LPS) at a concentration of 1 mg/mL for 4 hours. The culture medium was then replaced with Opti-MEM, and 5 mmol/L ATP was added to induce the activation of the NLRP3 inflammasome, followed by a 45-minute incubation. Finally, total proteins were extracted from both the cell lysates and the culture supernatants to analyze the expression of NLRP3 inflammasome-associated factors.

2.14. Statistical analysis

Data were analyzed using GraphPad Prism 8.0 software. Results are expressed as mean ± standard deviation (SD). Statistical significance was evaluated using one-way analysis of variance (ANOVA), and a *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Component identification of PSAE

To determine the main components of PSAE, we first measured the contents of various substances: total polysaccharides ($(13.360 \pm 0.325)\%$), proteins ($(7.412 \pm 0.210)\%$), polyphenols ($0.234 \pm 0.005\%$), saponins ($(0.159 \pm 0.002)\%$), and flavonoids ($(0.058 \pm 0.003)\%$) (see Supplementary Table 3). Next, we utilized UPLC-Q-TOF-MS/MS to analyze the chemical components of PSAE. By examining the MS/MS fragmentations in both positive and negative ion modes, we identified 60 compounds by comparing them with

the Natural Standard NPs PCDL database. The base peak chromatograms of PSAE are presented in Supplementary Fig. 1, and the identified compounds can be found in Supplementary Tables 4 and 5.

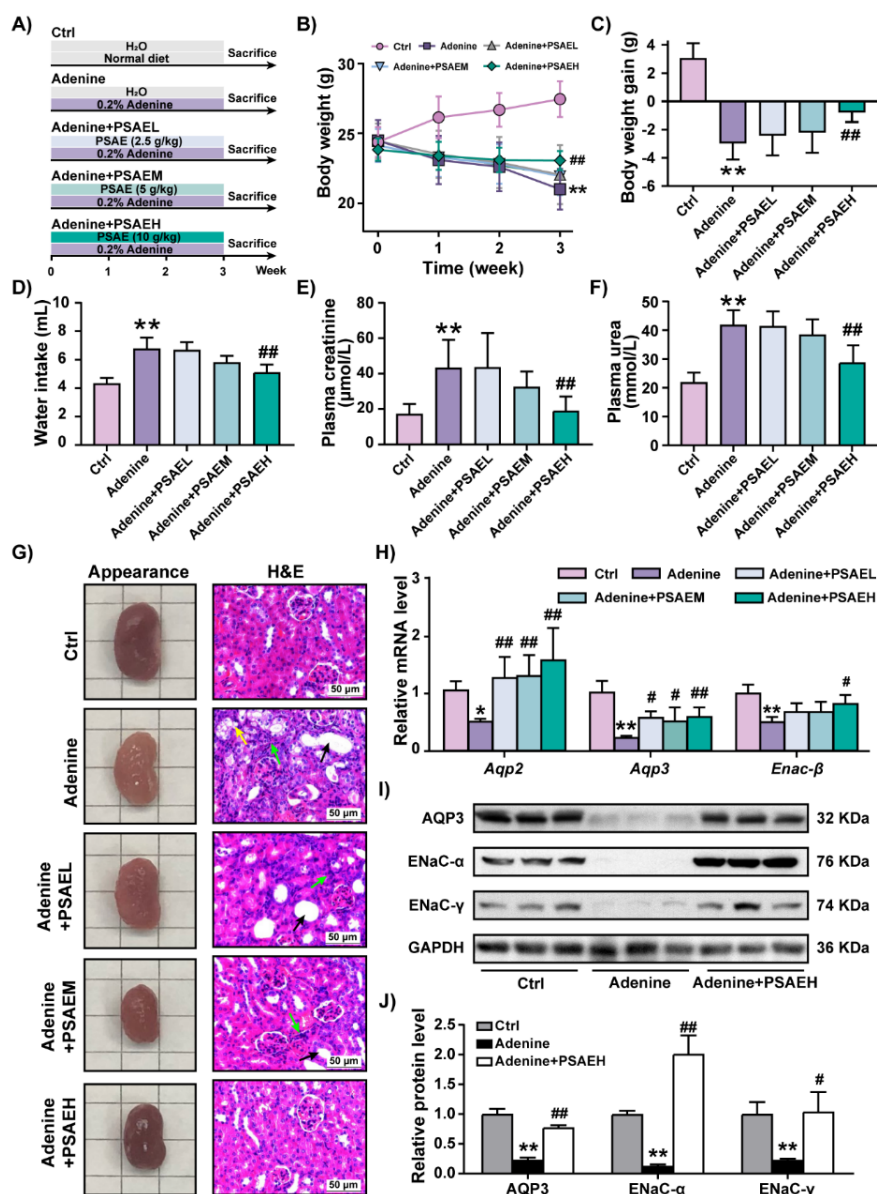


Fig. 1. PSAE reduced renal injury in mice with CKD. (A) Schedule for animal experiments. (B) Growth curve data (n = 8). (C) Body weight gain measurements (n = 8). (D) Water intake levels (n = 8). (E) Plasma creatinine levels (n = 8). (F) Plasma urea levels (n = 8). (G) Examination of renal appearance and morphological changes in the kidneys using H&E staining (magnification 400×). Specific pathological alterations are indicated by arrows: yellow arrows, vacuolar degeneration of tubular epithelium; black arrows, tubular dilation and distortion; green arrows, interstitial inflammatory cell infiltration. (H) Relative mRNA levels of Aqp2, Aqp3 and Enac-β in kidney tissues by RT-PCR (n = 8). (I) Protein expression levels of AQP3, ENaC-α, and ENaC-γ in kidney tissues by WB (n = 3). (J) Protein quantification of AQP3, ENaC-α, and ENaC-γ in renal tissues (n = 3). Data are presented as means ± SD. * $P < 0.05$, ** $P < 0.01$ vs. Ctrl group; # $P < 0.05$, ## $P < 0.01$ vs. Adenine group.

3.2 PSAE alleviated renal injury of CKD mice

The experimental procedure is illustrated in Fig. 1A, where we assessed the ameliorative effects of PSAE (2.5, 5, and 10 g/kg) on mice with adenine-induced chronic kidney disease (CKD). Compared to the Ctrl group, mice in the Adenine group exhibited a gradual loss of body weight and a significant increase in water intake. These changes were reversed in the high-dose PSAE group ($P < 0.01$, vs. Adenine group) (Fig. 1B-

1D). Additionally, the high dose of PSAE reduced levels of renal function markers in the CKD mice, including plasma creatinine and urea ($P < 0.01$, vs. Adenine group) (Fig. 1E-1F).

Histological analysis revealed that the kidneys of mice in the Ctrl group had a smooth, reddish-brown surface, while those of CKD mice appeared light red and grainy. Hematoxylin and eosin (H&E) staining revealed severe renal damage in the Adenine group, characterized by vacuolar degeneration of tubular epithelial cells (indicated by yellow arrows), dilated and distorted renal tubules (black arrows), and substantial infiltration of inflammatory cells within the renal interstitium (green arrows). In contrast, all three doses of PSAE intervention markedly alleviated these pathological alterations, as evidenced by reduced tubular injury and diminished inflammatory cell infiltration (see Fig. 1G).

Given that the regulation of water and electrolyte metabolic homeostasis is a primary physiological function of the kidneys, we examined the changes in aquaporins and epithelial sodium channel (ENaC) subunits in kidney tissues using RT-PCR and WB analysis. As illustrated in Fig. 1H-1J, the mRNA and/or protein levels of AQP2, AQP3, ENaC- α , ENaC- β , and ENaC- γ were significantly decreased in CKD mice ($P < 0.05$ or 0.01 , vs. Ctrl group). However, these levels were significantly restored by PSAE treatment ($P < 0.05$ or 0.01 , vs. Adenine group).

3.3 PSAE abrogated renal fibrosis in CKD mice

Irreversible renal fibrosis is a key mechanism in the progression of CKD to end-stage renal disease (ESRD) and is associated with a high mortality rate^[7]. In our study, we evaluated the impact of PSAE on renal fibrosis, as illustrated in Fig. 2A. Periodic Acid-Schiff (PAS) staining showed thickening of the tubular basement membrane (red arrows) and glomerular mesangial matrix expansion (blue arrows) in the Adenine group compared to the Ctrl group. Masson's trichrome staining further demonstrated extensive blue collagen fiber deposition in the interstitial areas (light blue arrows), indicating severe renal fibrosis. In contrast, PSAE intervention effectively attenuated these changes, resulting in less basement membrane thickening and a significant reduction in interstitial collagen deposition (see Fig. 2A).

We investigated the mRNA and protein levels of fibrosis-related factors in renal tissues. As shown in Fig. 2B, mice with CKD exhibited higher mRNA expression levels of Tgf- β 1, Fibronectin, and Coll α 1. These abnormalities were significantly reversed with medium or high doses of PSAE ($P < 0.05$ or 0.01 , vs. Adenine group). At the protein level, western blotting analysis confirmed a marked upregulation of TGF- β 1, Fibronectin, Coll α 1, and the myofibroblast activation marker α -smooth muscle actin (α -SMA) in CKD mice. These changes were significantly suppressed by high-dose of PSAE treatment ($P < 0.01$, vs. Adenine group) (Fig. 2C-2D).

We next examined the effect of PSAE on epithelial-mesenchymal transition (EMT), a pivotal process in renal fibrosis. At the mRNA level, CKD mice showed a significant down-regulation of the epithelial marker E-cadherin, which was restored by high-dose of PSAE treatment ($P < 0.01$, vs. Adenine group) (Fig. 2B). At the protein level, high doses of PSAE intervention effectively reversed the adenine-induced loss of E-cadherin

and concurrently suppressed the upregulation of the mesenchymal marker Vimentin and the key EMT-transcriptional repressor Snail1 ($P < 0.01$, vs. Adenine group) (Fig. 2C-2D).

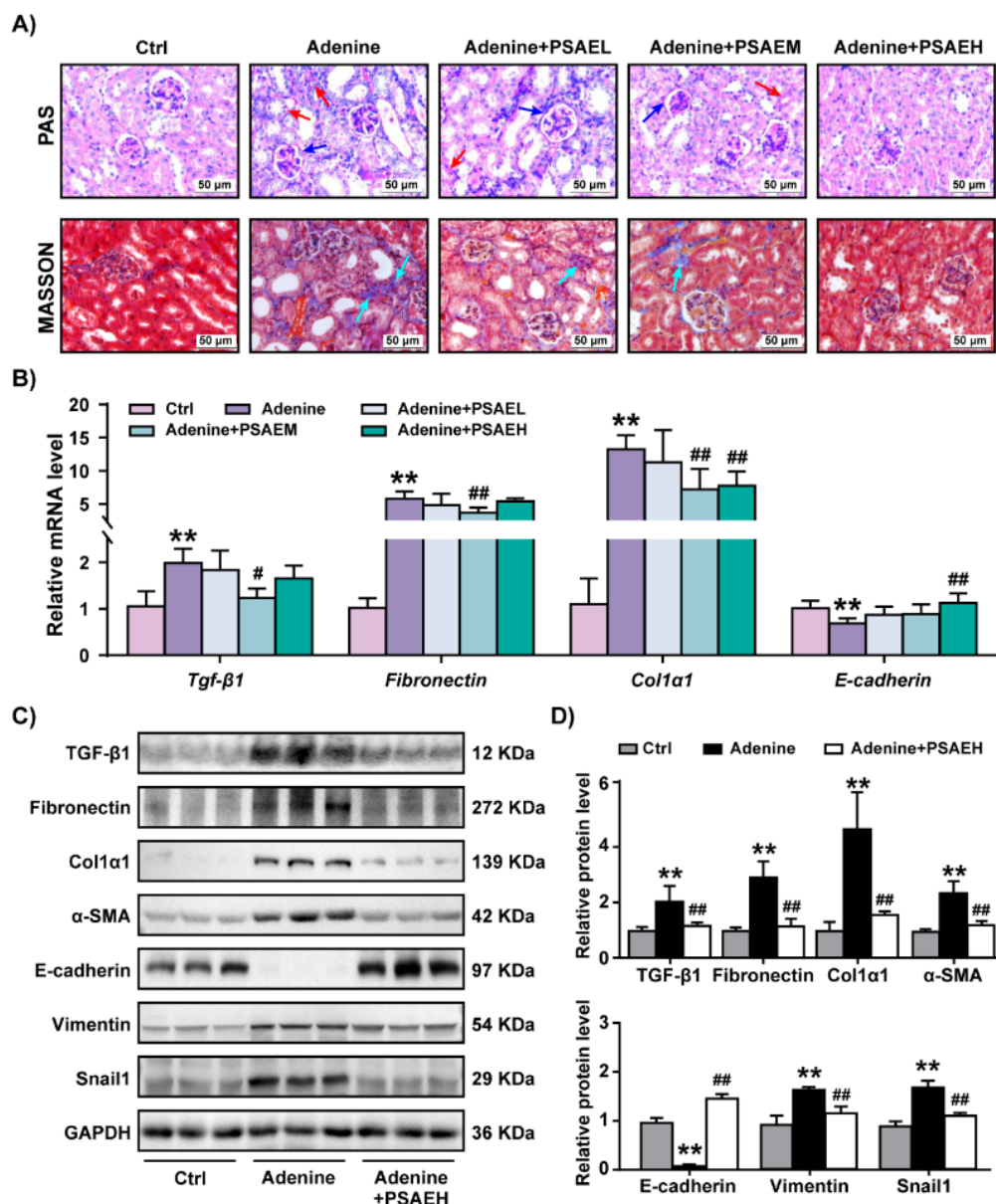


Fig. 2. PSAE delayed progression of renal fibrosis in CKD mice. (A) Morphological changes in kidney tissues were assessed using PAS and Masson's trichrome staining at a magnification of 400×. Specific pathological alterations are indicated by arrows: red arrows, thickening of the tubular basement membrane; blue arrows, expansion of the glomerular mesangial matrix; light blue arrows, interstitial collagen deposition. (B) Relative mRNA levels of renal fibrosis-related factors in kidney tissues, including Tgf-β1, Fibronectin, Col1α1, and E-cadherin by RT-PCR ($n = 8$). (C) Protein expression levels of renal fibrosis-related factors in kidney tissues, including TGF-β1, Fibronectin, Col1α1, α-SMA, E-cadherin, Vimentin, and Snail1 by WB ($n = 3$). (D) Quantification of relative protein levels of these target proteins ($n = 3$). Data are presented as means $\pm$ SD. ** $P < 0.01$ vs. Ctrl group; # $P < 0.05$, ## $P < 0.01$ vs. Adenine group.

3.4 PSAE modulated the plasma metabolomic profile in CKD mice

This study employed untargeted metabolomics to analyze the effects of PSAE on endogenous plasma metabolites in mice with CKD. Principal component analysis (PCA) revealed distinct separation of metabolic profiles among the experimental groups, with tight intra-group clustering of samples (Fig. 3A). Orthogonal partial least squares-discriminant analysis (OPLS-DA) further confirmed significant discrimination of

metabolite profiles among the Ctrl, Adenine, and Adenine + PSAEH groups (Fig. 3B-3C). A 200-permutation test ruled out model overfitting (Supplementary Fig. 2A-2B). Based on the screening criteria ($VIP > 1$, $P < 0.05$, fold change > 1.5), we systematically identified differential metabolites across groups. Volcano plot analysis demonstrated that, compared to the Ctrl group, the Adenine group exhibited significant alterations in 166 metabolites, with 60 down-regulated and 106 up-regulated (Fig. 3D). Notably, PSAEH intervention significantly reversed the aberrant changes in 120 metabolites in the Adenine group (29 up-regulated and 91 down-regulated) (Fig. 3E).

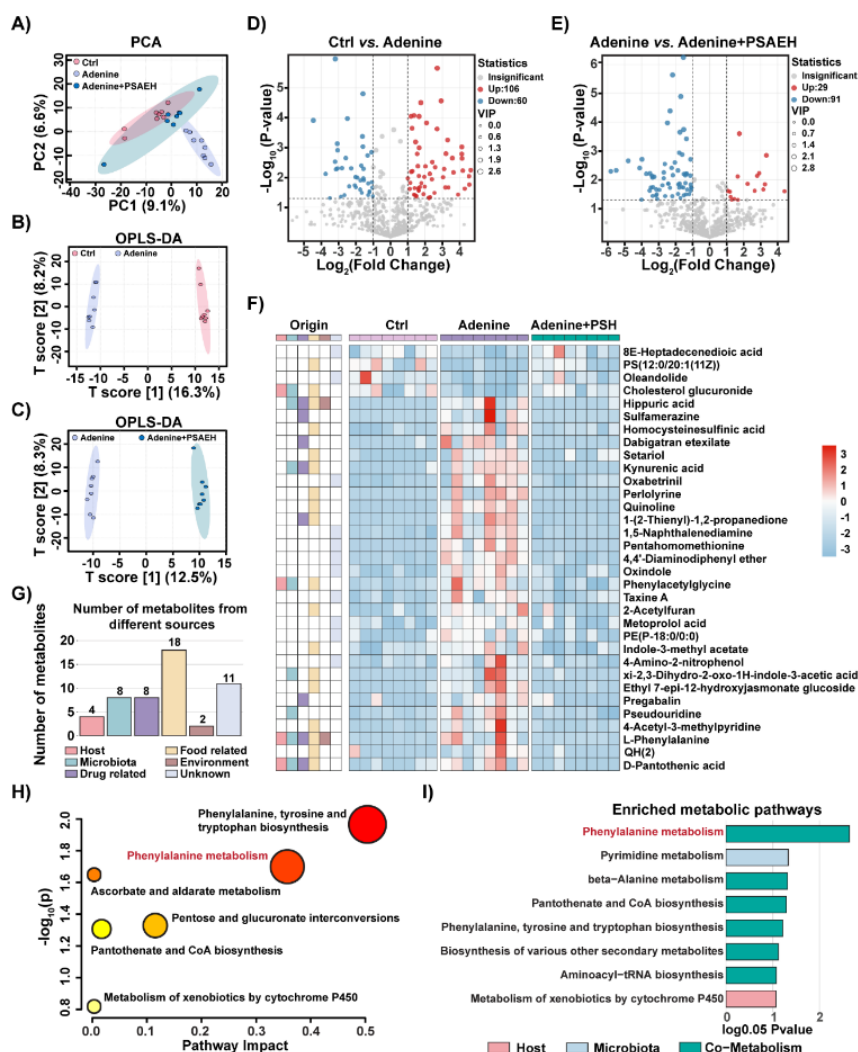


Fig. 3. PSAE modulated the plasma metabolomic profile in CKD mice. (A) Principal component analysis (PCA) of plasma metabolomes. (B) Orthogonal partial least squares-discriminant analysis (OPLS-DA) of the Ctrl group and Adenine group. (C) OPLS-DA of the Adenine group and Adenine + PSAEH group. (D) Volcano plot of differential plasma metabolite analysis between the Ctrl group and Adenine group. (E) Volcano plot of differential plasma metabolite analysis between the Adenine + PSAEH group and Adenine group. (F) Cluster heatmap of differential metabolites. (G) Bar plot of differential metabolites tracing analysis. (H) KEGG pathway enrichment analysis. (I) Bar plot of enriched differential metabolic pathways based on metabolite tracing analysis.

Integrated analysis revealed that PSAE intervention significantly reversed the alterations of 33 metabolites. Cluster heatmap analysis intuitively displayed the distinct expression patterns of these metabolites across the different groups (Fig. 3F). MetOrigin-based origin tracking analysis indicated that these metabolites derived from broad sources: 4 were attributed to host metabolism, 8 to gut microbiota metabolism,

8 were related to drug metabolism, 18 originated from food metabolism, and 2 were associated with environmental exposure (Fig. 3G).

To further elucidate the biological significance of these differential metabolites, we performed KEGG pathway analysis on the significantly altered metabolites. The results revealed the involvement of 6 major metabolic pathways (Fig. 3H), suggesting that PSAE likely influences the plasma metabolic profile in CKD mice primarily by modulating phenylalanine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, and other related pathways. Origin tracing analysis revealed significant enrichment in 1 host-related pathway, 1 microbiota-related pathway, and 6 co-metabolic pathways (Fig. 3I). Notably, among the significantly enriched host-microbiota co-metabolic pathways, the phenylalanine metabolism pathway was particularly prominent (Fig. 3I). The phenylalanine metabolic pathway has been reported to mediate renal inflammation and fibrosis through the generation of uremic toxins^[32]. Therefore, PSAE may ameliorate CKD by modulating the phenylalanine metabolism pathway and reducing the release of uremic toxins. In summary, our findings indicate that the core metabolic changes associated with the ameliorative effects of PSAE on CKD are primarily driven by host-microbiota co-metabolic pathways, underscoring the critical role of gut microbiota in the protective action of PSAE against CKD.

3.5 PSAE reversed gut microbiota dysbiosis in CKD mice

We next examined the impact of PSAE on the gut microbiota structure in mice with CKD using 16S rDNA sequencing. To evaluate α -diversity, we employed metrics such as Chao1, observed species, PD whole tree, and the Shannon index. We found that α -diversity was significantly increased in the CKD mice and that high doses of PSAE restored this diversity ($P < 0.05$ or 0.01 , vs. Adenine group) (see Fig. 4A-4B and Supplementary Fig. 3A-3B). For β -diversity assessment of the gut microbiota, we utilized principal component analysis (PCA), partial least squares discrimination analysis (PLS-DA), and non-metric multidimensional scaling (NMDS). The results displayed five distinct clusters, indicating that the gut microbiota in each group exhibited a unique structure (see Fig. 4C and Supplementary Fig. 3C-3D).

At the phylum level, PSAE treatment reversed the upregulation of Campilobacterota and Deferribacterota, as well as the downregulation of Patescibacteria and Verrucomicrobiota in CKD mice (Fig. 4D). At the family level, PSAE decreased the abundance of Lachnospiraceae while increasing the levels of Erysipelotrichaceae and Prevotellaceae (Fig. 4E). At the genus level, a heatmap was employed to highlight the bacteria that showed significant changes. The results indicate that several bacteria were enriched in the Adenine group, including Ruminococcaceae, Turicibacter, and Lachnospiraceae. These were decreased by PSAE treatment ($P < 0.05$, vs. Adenine group). Additionally, PSAE treatment statistically suppressed the reduction of Butyrivibrio, Allobaculum, and Faecalibaculum ($P < 0.05$, vs. Adenine group) (Fig. 4F).

Using linear discriminant analysis effect size (LEfSe) and linear discriminant analysis (LDA), we identified the microbial taxa characteristic of each experimental group. As shown in Fig. 4G, the unique microbiota of the Adenine group included Lachnospiraceae, and Ruminococcaceae. In contrast, the PSAE intervention group displayed significant contributions from Clostridia, Oscillospiraceae, and Actinobacteria.

Next, we performed a Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST) analysis for functional predictions. The results indicated that the PSAE intervention significantly reversed the increase in glycerolipid metabolism, as well as the decrease in lipoic acid metabolism, DNA replication, and RNA degradation in the CKD mice ($P < 0.05$ or 0.01 , vs. Adenine group) (see Supplementary Fig. 4A).

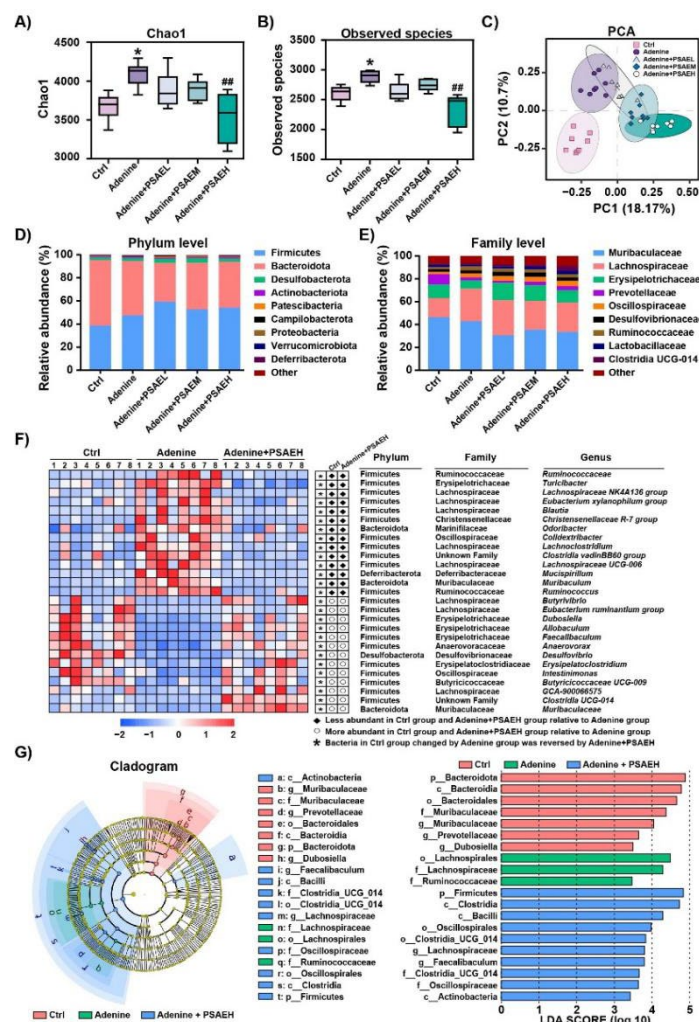


Fig. 4. Improvement of gut microbiota dysbiosis in CKD mice by PASE. (A) Chao1 index. (B) Observed species index. (C) Principal component analysis (PCA). (D) Relative abundance at the phylum level. (E) Relative abundance at the family level. (F) Heatmap showcasing characterized gut microbiota at the genus level. (G) Identification of characteristic taxa among experimental groups using linear discriminant analysis effect size (LEfSe) and Linear Discriminant Analysis (LDA). Data are presented as means $\pm$ SD ($n = 8$). * $P < 0.05$ vs. Ctrl group; # $P < 0.01$ vs. Adenine group.

To investigate the relationship between gut microbiota dysbiosis and pathological parameters related to CKD, we calculated the correlation between significantly altered bacteria at the genus level and various CKD indices using Spearman's correlation analysis. As shown in Supplementary Fig. 4B, most of the changes in gut microbiota were found to be either positively or negatively correlated with physicochemical indices, renal function, and renal fibrosis.

3.6 Gut microbiota was vital for PSAE to treat CKD

To determine whether gut microbiota is essential for PSAE to treat CKD, we depleted the gut microbiota in mice using a combination of antibiotics prior to the PSAE intervention (see Fig. 5A). As shown in

Supplementary Fig. 5, the intestinal bacteria were significantly reduced after eight weeks of antibiotic treatment. Unlike the Adenine + Ab group, PSAE treatment did not reverse the elevated plasma creatinine levels (Fig. 5D). However, it did reduce weight loss and lower plasma urea levels in CKD mice ($P < 0.01$, vs. Adenine + Ab group) (see Fig. 5B, 5C, 5E). Furthermore, in CKD mice with depleted gut microbiota, PSAE treatment failed to improve the abnormal renal appearance or the histopathological damage observed in H&E staining. Specifically, vacuolar degeneration of the tubular epithelium (yellow arrows), along with marked tubular dilation and distortion (black arrows) and interstitial inflammatory cell infiltration (green arrows), persisted in the Adenine + Ab and Adenine + Ab + PSAE groups, with no significant amelioration by PSAE (see Fig. 5F). Additionally, PSAE failed to restore the reduced expression of aquaporin proteins at both mRNA and protein levels in the kidney tissues of CKD mice after gut microbiota depletion (Fig. 5G-5I).

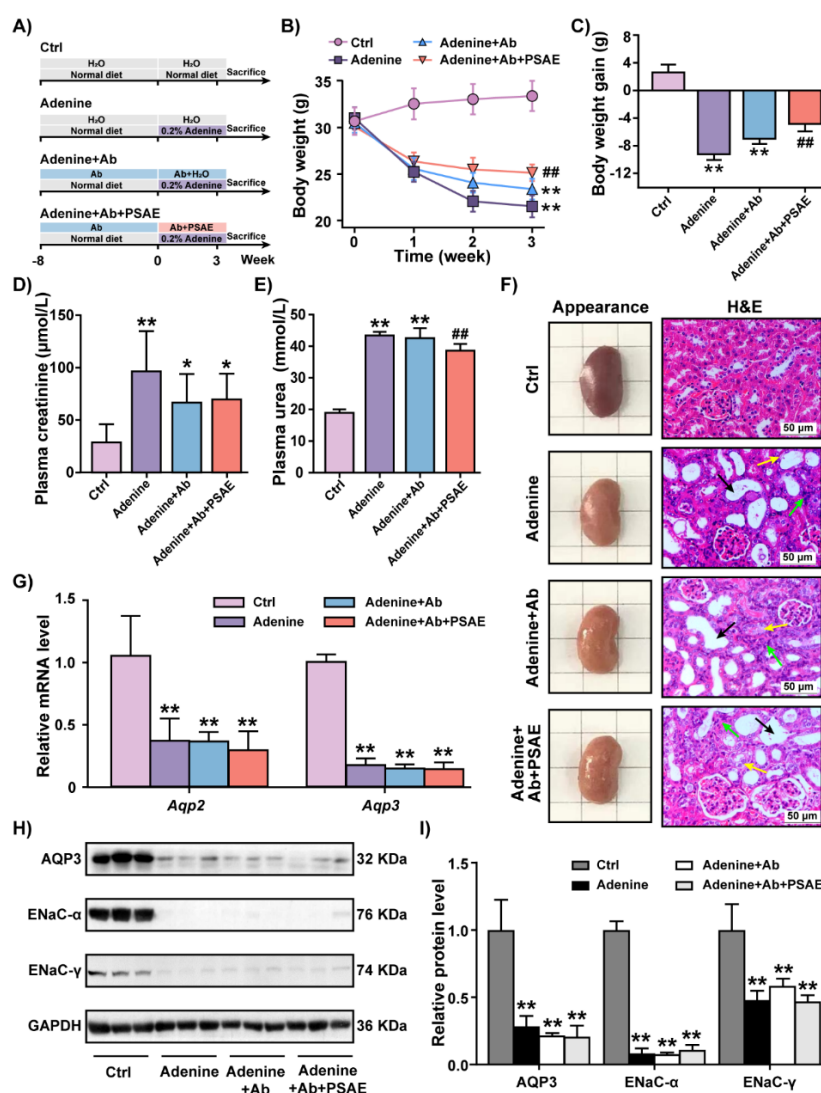


Fig. 5. Role of gut microbiota in treating kidney injury by PSAE. (A) Schedule for animal experiments. (B) Growth curve ($n = 8$). (C) Body weight gain ($n = 8$). (D) Plasma creatinine levels ($n = 8$). (E) Plasma urea levels ($n = 8$). (F) Renal appearance and morphological changes of kidneys observed via H&E staining ($400 \times$). Specific pathological alterations are indicated by arrows: yellow arrows, vacuolar degeneration of tubular epithelium; black arrows, tubular dilation and distortion; green arrows, interstitial inflammatory cell infiltration. (G) Relative mRNA levels of Aqp2 and Aqp3 in kidney tissues by RT-PCR ($n = 8$). (H) Protein expression of AQP3, ENaC- α , and ENaC- γ in kidney tissues by WB ($n = 3$). (I) Quantification of relative protein levels of AQP3, ENaC- α , and ENaC- γ in kidney tissues ($n = 3$). Data are presented as means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ vs. Ctrl group; ### $P < 0.01$ vs. Adenine+Ab group.

3.7 Anti-fibrosis effect of PSAE on CKD mice with gut microbiota depletion

We investigated whether PSAE could inhibit renal fibrosis in CKD mice with depleted gut microbiota. As shown in Fig. 6A, in gut microbiota-depleted CKD mice, PSAE treatment failed to alleviate the renal fibrosis pathology. PAS staining revealed persistent thickening of the tubular basement membrane (red arrows) and expansion of the glomerular mesangial matrix (blue arrows). Concurrently, Masson's trichrome staining showed extensive interstitial collagen deposition (light blue arrows), which was not reduced by PSAE intervention. Additionally, PSAE was ineffective in reversing the abnormal alterations of fibrosis-related factors, such as TGF- β 1, Fibronectin, Col1 α 1, E-cadherin, and α -SMA, in the kidney tissues of CKD mice at both mRNA and protein levels (Fig. 6B-6D).

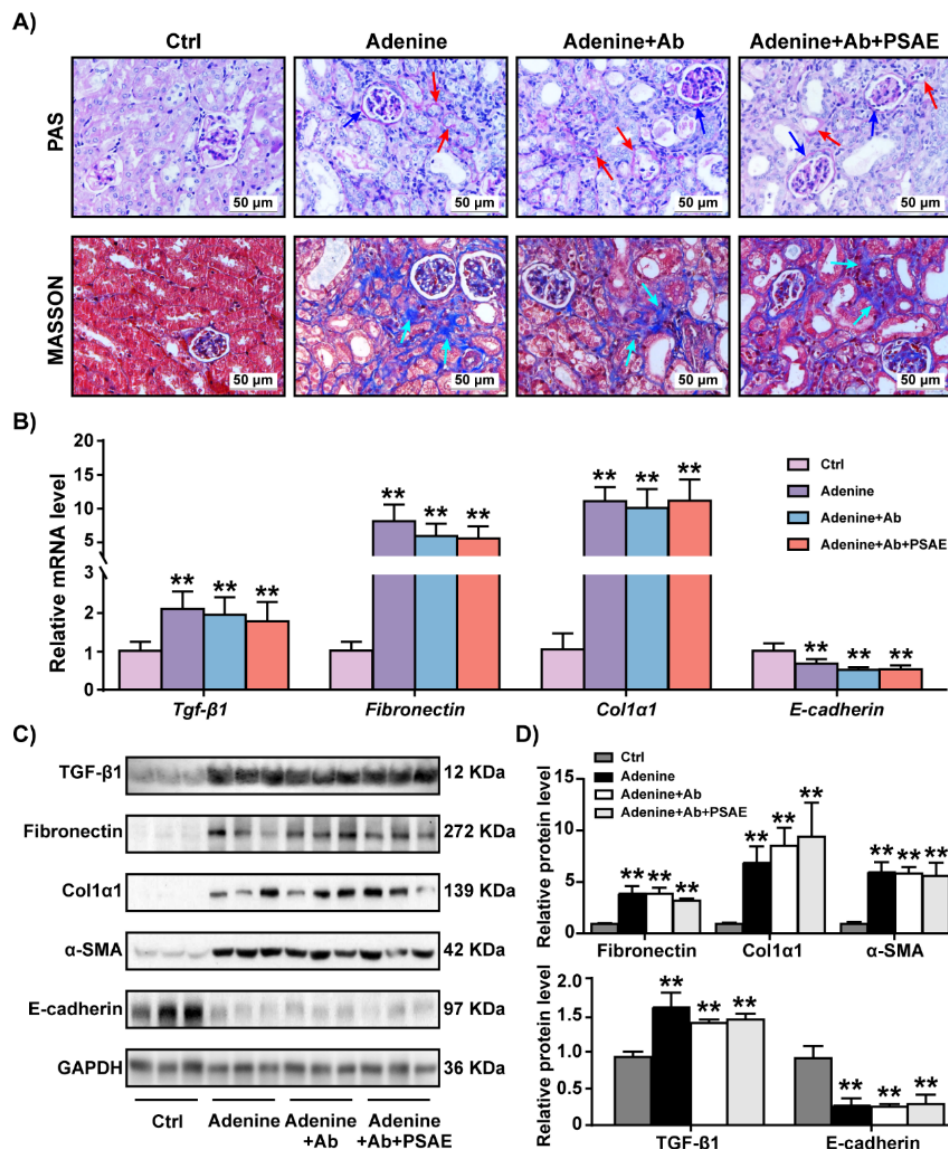


Fig. 6. Anti-fibrosis effect of PSAE was mediated by gut microbiota. (A) Morphological changes in kidney tissues were assessed using PAS staining (400 $\times$) and Masson's trichrome staining (400 $\times$). Specific pathological alterations are indicated by arrows: red arrows, thickening of the tubular basement membrane; blue arrows, expansion of the glomerular mesangial matrix; light blue arrows, interstitial collagen deposition. (B) Relative mRNA levels of renal fibrosis-related factors in kidney tissues, including Tgf- β 1, Fibronectin, Col1 α 1, and E-cadherin by RT-PCR (n = 8). (C) Protein expression of renal fibrosis-related factors in kidney tissues, including TGF- β 1, Fibronectin, Col1 α 1, α -SMA, and E-cadherin by WB (n = 3). (D) Quantification of relative protein levels of target proteins (n = 3). Data are presented as means $\pm$ SD. ***P* < 0.01 vs. Ctrl group.

3.8 Signaling pathway prediction in PSAE-mediated protection against renal fibrosis by network pharmacology

We then investigated whether PSAE was directly absorbed into the bloodstream for the treatment of CKD or was metabolized by the gut microbiota into new active compounds that subsequently entered the bloodstream to exert a therapeutic effect. PSAE-containing serum was prepared as illustrated in Fig. 7A and Supplementary Fig. 6. We analyzed the original and metabolized products of PSAE that were absorbed into the blood using UPLC-Q-TOF-MS/MS. By comparing the retention times and MS/MS fragmentations in both positive and negative ion modes to the Natural Standard NPs PCDL database, we identified 15 components in the serum from rats with normal gut microbiota (see Supplementary Table 6).

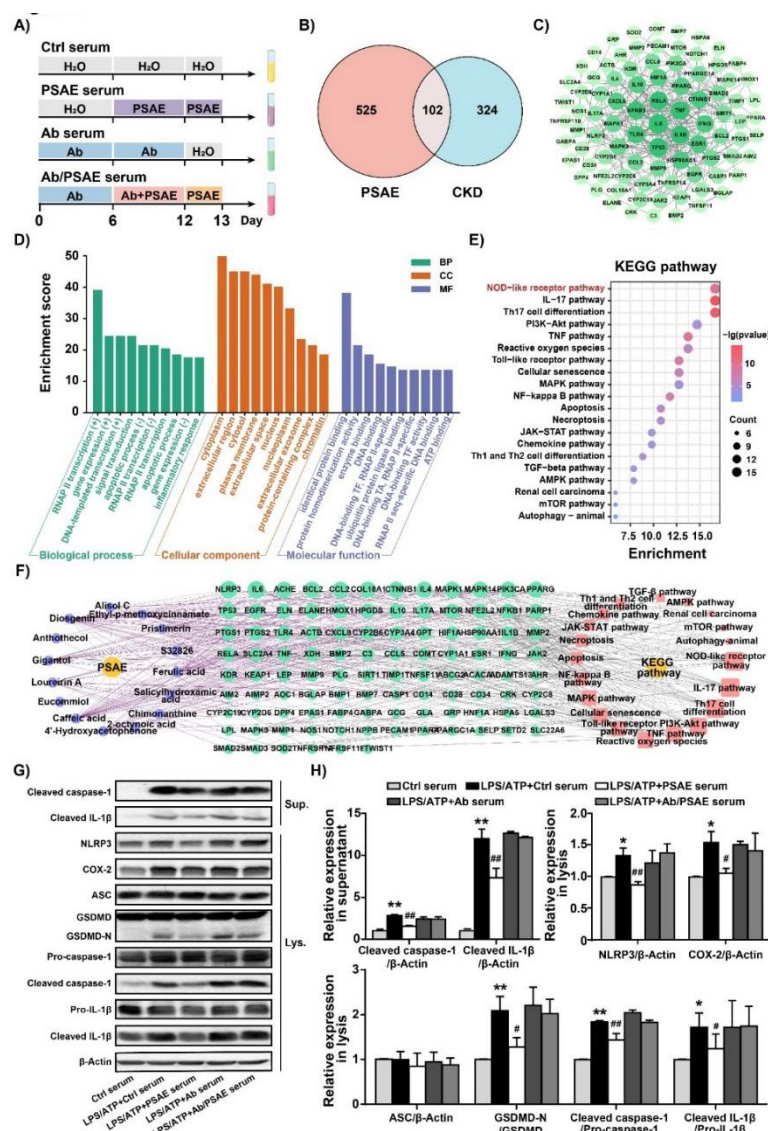


Fig. 7. Predictive analysis of gut microbiota-mediated signaling pathways in PSAE against renal fibrosis by network pharmacology. (A) Preparation of PSAE-containing serum. (B) Venn diagram illustrating shared and unique data. (C) Protein-protein interaction (PPI) network. (D) GO (Gene Ontology) enrichment analysis data, including biological processes (BP), cellular components (CC), and molecular functions (MF). (E) KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis. (F) Component-target-pathway network diagram, where purple hexagonal nodes represent components absorbed into the bloodstream from PSAE, green circular nodes represent targets, and red square nodes signify the KEGG pathways. (G) Protein expression of NLRP3, ASC, GSDMD, caspase-1, IL-1β, and COX-2 in THP-1 cell lysate and supernatant by WB. (H) Quantification of relative protein levels for target proteins (n = 3). Data are presented as means ± SD. * $P < 0.05$, ** $P < 0.01$ vs. Ctrl serum group; # $P < 0.05$, ## $P < 0.01$ vs. LPS/ATP + Ctrl serum group.

We conducted a network pharmacology analysis to identify the potential pathways associated with these components. By intersecting the 627 targets related to blood-entry components with 426 CKD targets, we identified 102 common targets (see Fig. 7B and Supplementary Tables 7 and 8). After excluding free targets, the protein-protein interaction (PPI) network for the common targets comprised 89 nodes and 275 edges. As shown in Fig. 7C, the targets with the highest degree values included IL6, TP53, IL1B, TNF, and RELA. We performed Gene Ontology (GO) enrichment analysis of the common targets using the DAVID database, resulting in 807 identified terms. This included 640 biological process (BP) terms, 55 cellular component (CC) terms, and 112 molecular function (MF) terms. The top 10 representative GO terms have been visualized in Fig. 7D. Additionally, a KEGG pathway enrichment analysis yielded 156 signaling pathways, with the top 20 presented after removing low-correlation items (see Fig. 7E). The results suggest that the PSAE-mediated anti-CKD effects may be linked to several pathways, including nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), IL-17, and Th17 cell differentiation.

Subsequently, we constructed a “component-target-pathway” network that included 15 components found in PASE-containing serum, their action targets, and the top 20 significant KEGG pathways (see Fig. 7F). Within this network, we identified NLRP3, IL6, ACHE, BCL2, and CCL2 as common targets for most of the PASE components. Given that NLRP3 is a typical gene involved in forming inflammasomes in kidney tissues, we decided to focus our experiments on the role of the NLRP3 inflammasome pathway in elucidating the molecular mechanism of PASE's effects against CKD.

3.9 PASE-containing serum inhibited NLRP3 inflammasome activation in THP-1 macrophages

The NLRP3 inflammasome is primarily activated in immune cells [33]. To investigate the effect of PSAE-containing serum on the NLRP3 inflammasome in THP-1 cells, we conducted specific experiments (see Fig. 7G-7H and Supplementary Fig. 7). Following stimulation with LPS and ATP, we observed an increase in the protein levels of regulators associated with the NLRP3 inflammasome in cell lysates ($P < 0.05$ or 0.01 , vs. Ctrl serum group). These included NLRP3, the GSDMD N-terminal fragment (GSDMD-N), cleaved caspase-1, cleaved IL-1 β , and COX-2. Additionally, the levels of cleaved caspase-1 and cleaved IL-1 β in the cell culture supernatants were significantly elevated ($P < 0.01$, vs. Ctrl serum group). However, there were no statistically significant changes in the protein level of ASC among the groups.

When the PSAE-containing serum were provided by rats with a normal gut microbiota, the protein changes in LPS/ATP-induced THP-1 cells were considerably reversed ($P < 0.05$ or 0.01 , vs. LPS/ATP + Ctrl serum group). In contrast, the suppressive effect on the NLRP3 inflammasome pathway was lost when the serum came from rats with depleted gut microbiota (Fig. 7G-7H). Based on these findings, we propose that the metabolism of PSAE by gut microbiota before absorption is critical for its suppression of the NLRP3 inflammasome and its subsequent therapeutic effects on CKD.

3.10 PSAE inhibited activation of NLRP3 inflammasome pathway in CKD mice

To validate the crucial role of the NLRP3 inflammasome pathway in the PASE-mediated reduction of inflammatory responses, renal tubule injury, and renal interstitial fibrosis, we conducted a complete blood count for each experimental group. The results showed that PASE significantly decreased the number of white blood cells (WBC), lymphocytes (LYM), monocytes (MON), and granulocytes (GRA) in CKD mice ($P < 0.05$ or 0.01 , vs. Adenine group) (Fig. 8A). Given that NLRP3 is primarily expressed in immune cells, we examined the changes in F4/80⁺ macrophages in kidney tissues using immunohistochemical staining. As illustrated in Fig. 8B, the number of F4/80⁺ cells increased in the Adenine group, but this increase was reversed by all three doses of PASE. Additionally, PASE reduced the mRNA levels of inflammatory mediators in the kidney tissues of CKD mice, including *Nlrp3*, *Il-1 β* , *Il-6*, tumor necrosis factor- α (*Tnf- α*), and matrix metalloproteinase 9 (*Mmp9*) ($P < 0.05$ or 0.01 , vs. Adenine group) (Fig. 8C). Notably, PASE significantly inhibited the activation of NLRP3 inflammasome-related proteins in the kidneys of CKD mice. This included proteins such as COX-2, NLRP3, ASC, GSDMD-N, cleaved caspase-1, and cleaved IL-1 β ($P < 0.01$, vs. Adenine group) (Fig. 8D and 8E).

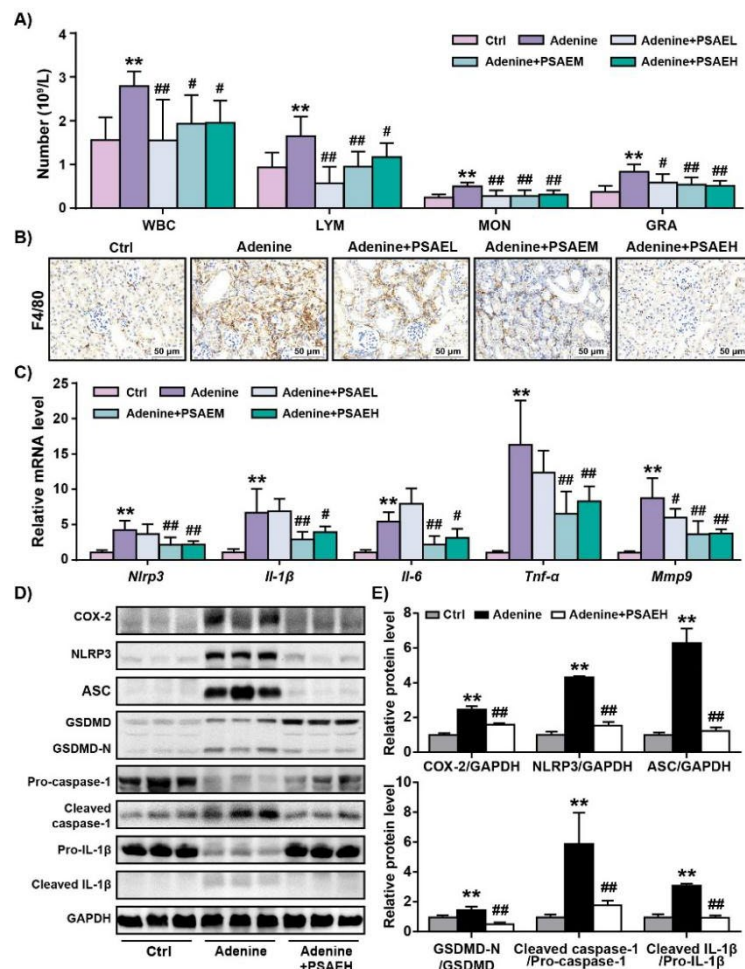


Fig. 8. PASE inhibited activation of NLRP3 inflammasome pathway in CKD mice. (A) Blood analysis of hematological parameters, including white blood cell (WBC), lymphocyte (LYM), monocyte (MON), and granulocyte (GRA) counts ($n = 8$). (B) Changes in F4/80⁺ macrophages in kidney tissues as observed through immunohistochemical staining (400 $\times$ magnification). (C) Relative mRNA levels of *Nlrp3*, *Il-1 β* , *Il-6*, *Tnf- α* , and *Mmp9* in kidney tissues by RT-PCR ($n = 8$). (D) Protein expression levels of COX-2, NLRP3, ASC, GSDMD, caspase-1, and IL-1 β in kidney tissues by WB ($n = 3$). (E) Quantification of relative levels of target proteins ($n = 3$). Data are presented as means $\pm$ SD. ** $P < 0.01$ vs. Ctrl group; # $P < 0.05$, ## $P < 0.01$ vs. Adenine group. WBC, white blood cell; LYM, lymphocyte; MON, monocytes; GRA, granulocytes.

We examined the effects of PSAE on inflammatory responses and NLRP3 inflammasome activation in the kidneys of CKD mice with depleted gut microbiota. As shown in Fig. 9A, antibiotic treatment exhibited an inhibitory trend on the expression of inflammatory factors. However, with the exception of TNF- α , PSAE did not significantly enhance this effect. Additionally, PSAE did not reduce the levels of NLRP3 inflammasome-associated proteins in the renal tissues of CKD mice whose gut microbiota had been exhausted (see Fig. 9B and 9C).

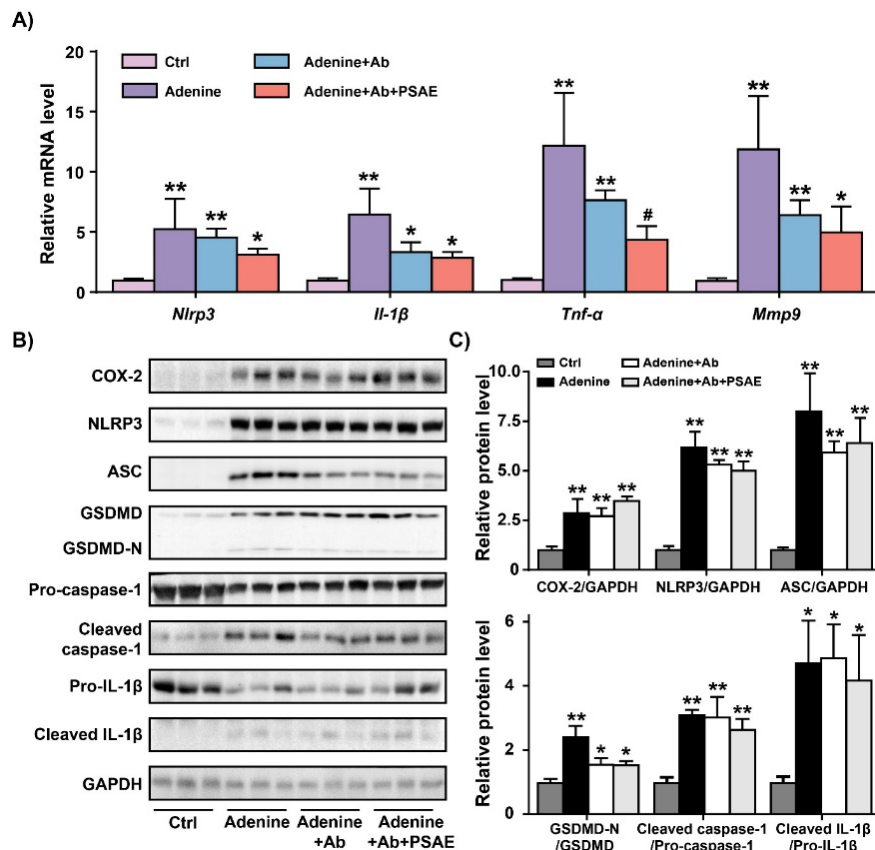


Fig. 9. Effect of PSAE on inflammatory responses and NLRP3 inflammasome activation in CKD mice with gut microbiota depletion. (A) Relative mRNA levels of *Nlrp3*, *Il-1β*, *Tnf-α*, and *Mmp9* in kidney tissues by RT-PCR (n = 8). (B) Protein expression levels of COX-2, NLRP3, ASC, GSDMD, caspase-1, and IL-1β in kidney tissues by WB (n = 3). (C) Quantification of relative levels of target proteins (n = 3). Data are presented as means ± SD. **P* < 0.05, ***P* < 0.01 vs. Ctrl group; #*P* < 0.05 vs. Adenine+Ab group.

3.11 PSAE failed to suppress renal fibrosis in CKD mice with NLRP3 knockout

To confirm the critical role of the NLRP3 inflammasome in the alleviation of renal fibrosis by PASE, we established a NLRP3^{-/-} mouse model for CKD (see Supplementary Fig. 8). As shown in Fig. 10A-10D and Supplementary Fig. 9, PASE treatment significantly reduced plasma urea levels (*P* < 0.01, vs. NLRP3^{-/-} Adenine group). However, it did not lead to improvements in weight loss, plasma creatinine levels, or the elevated expression of AQP2, AQP3, ENaC- α , and ENaC- γ in the renal tissues of the NLRP3^{-/-} CKD mice. Furthermore, in NLRP3^{-/-} CKD mice, PSAE treatment did not ameliorate the histopathological damage. H&E staining still showed evident tubular injury, including dilation and distortion (black arrows) and vacuolar degeneration (yellow arrows). Similarly, PAS and Masson's trichrome staining revealed persistent basement membrane thickening (red and blue arrows) and interstitial collagen deposition (light blue arrows),

respectively, which were not reversed by PSAE (see Fig. 10E). Consistent with the histological findings, PSAE also failed to normalize the expression levels of renal fibrosis-related biomarkers such as TGF- β 1, Fibronectin, Col1 α 1, E-cadherin, and α -SMA in these mice (see Fig. 10E-10H).

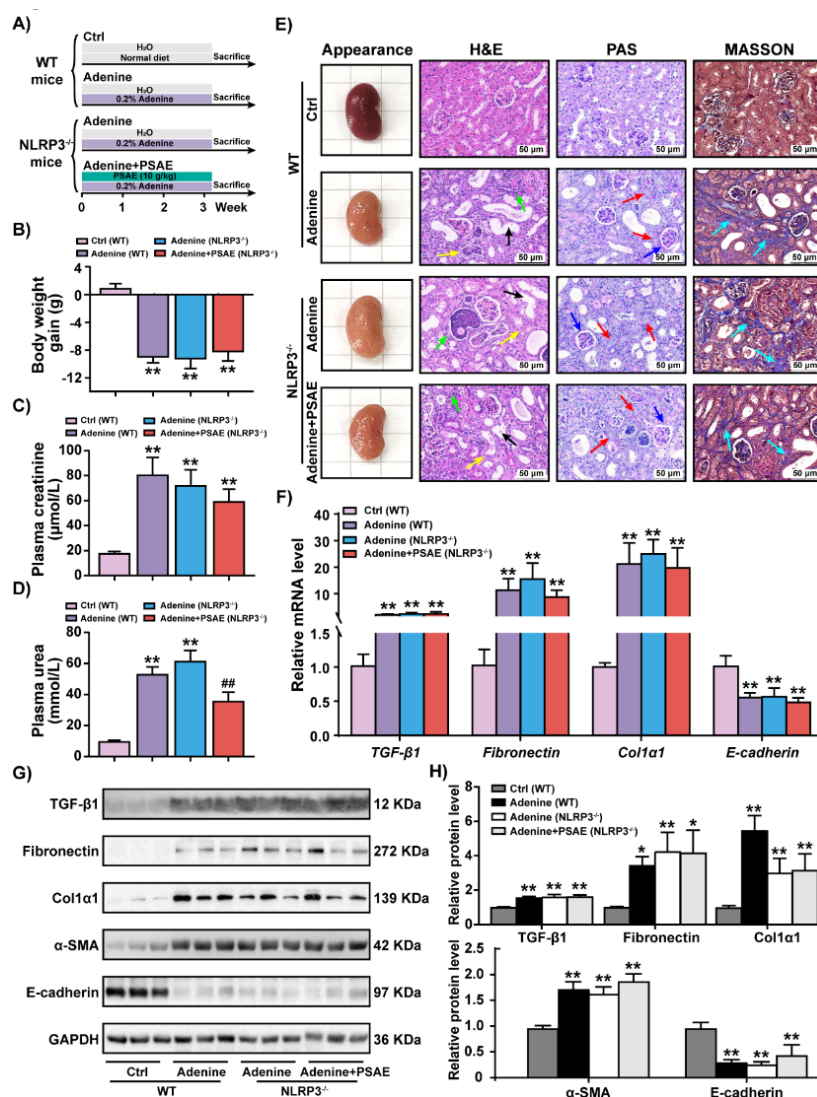


Fig. 10. Ameliorative effects of PSAE on renal fibrosis in NLRP3^{-/-} CKD mice. (A) Schedule of animal experiments. (B) Body weight gain (n = 8). (C) Plasma creatinine levels (n = 8). (D) Plasma urea levels (n = 8). (E) Renal appearance and morphological changes of kidneys assessed using H&E staining (400 ×), PAS staining (400 ×), and Masson's trichrome staining (400 ×). Specific pathological alterations are indicated by arrows: yellow arrows, vacuolar degeneration of tubular epithelium; black arrows, tubular dilation and distortion; green arrows, interstitial inflammatory cell infiltration; red arrows, thickening of the tubular basement membrane; blue arrows, expansion of the glomerular mesangial matrix; light blue arrows, interstitial collagen deposition. (F) Relative mRNA levels of renal fibrosis-related factors in kidney tissues including Tgf- β 1, Fibronectin, Col1 α 1, and E-cadherin by RT-PCR (n = 8). (G) Protein expression of renal fibrosis-related biomarkers in kidney tissues, including TGF- β 1, Fibronectin, Col1 α 1, α -SMA, and E-cadherin by WB (n = 3). (H) Quantification of relative protein levels of target proteins (n = 3). Data are expressed as the means $\pm$ SD. * P < 0.05, ** P < 0.01 vs. Ctrl group; ## P < 0.01 vs. NLRP3^{-/-} Adenine group.

4. Discussion

Polygonatum sibiricum (PS) has been widely used for kidney protection in countries such as China, the United States, and Canada^[20-29]. However, there is a lack of in-depth studies investigating the pharmacodynamic mechanisms of PS in CKD. This study demonstrated that aqueous extract of Polygonatum sibiricum (PSAE) could reduce plasma creatinine and urea levels while ameliorating renal injury in CKD mice

(Fig. 1). These benefits may be attributed to the presence of various active components in PSAE, including carbohydrates (notably polysaccharides), flavonoids, saponins, and alkaloids (Supplementary Table 3-5), which possess anti-inflammatory, antioxidant, anti-aging, immune-enhancing, and kidney-protecting properties [19, 20, 26-28]. The bioactivity of these components, particularly the polysaccharides, is consistent with the reported health benefits of similar compounds from a wide range of natural sources, including other Chinese herbs [28] and marine organisms [34]. Our findings, which pinpoint a gut microbiota-dependent inhibition of the NLRP3 inflammasome as a key mechanism, provide a novel and concrete pharmacological basis for the traditional use of PS. In contrast to the single-target effects of current clinical drugs for CKD, such as renin-angiotensin blockers and mineralocorticoid receptor antagonists, PSAE offers a multi-target advantage for addressing renal injury. This multi-target mechanism underscores the value of investigating whole extracts from “medicine and food homology” substances [21-34] like PS, as their complex composition can orchestrate synergistic effects.

Renal fibrosis is considered a key outcome of chronic kidney disease (CKD) [3]. Despite its significance, there is currently no recognized anti-fibrotic therapy for the kidneys due to a limited understanding of renal fibrosis [35]. The main cellular processes involved in renal fibrosis include the infiltration of inflammatory cells, activation and proliferation of fibroblasts, apoptosis of tubular cells, and epithelial-mesenchymal transition (EMT) [36]. In this study, PSAE demonstrated a suppressive effect on these cellular events associated with renal fibrosis. For instance, PSAE inhibited the expression of α -SMA and TGF- β in CKD mice (Fig. 2). α -SMA is typically expressed by fibroblasts after being activated by pro-fibrotic cytokines such as TGF- β and fibroblast growth factor. The activation of fibroblasts leads to increased production of extracellular matrix (ECM), as indicated by the reduced expression of ECM components, including Fibronectin and Colla1 (Fig. 2) [37]. Additionally, the down-regulation of the epithelial marker E-cadherin, coupled with the upregulation of the mesenchymal marker vimentin, suggests that EMT may have occurred in the renal tubular epithelial cells of CKD mice, a process that was reversed by PSAE, highlighting its protective effect on EMT. Moreover, the suppressive effect of PSAE on the key EMT-transcriptional repressor Snail1 (Fig. 2) indicates that it acts, at least in part, by targeting the upstream regulatory program that drives EMT [38].

Chronic inflammation is a key factor in the development of fibrogenesis, creating a harmful cycle of inflammation, tissue damage, and fibrosis. In line with earlier research [39], our findings indicate that the kidneys of mice with chronic kidney disease (CKD) exhibit significant inflammatory changes, particularly marked by macrophage infiltration (see Fig. 8). Previous studies have shown that the number of macrophages in the kidneys increases in various kidney diseases. These activated macrophages contribute to kidney injury and renal fibrosis by producing pro-inflammatory cytokines such as IL-1, IL-6, and TNF, as well as pro-fibrotic factors like TGF- β [40-41]. Our results suggest that the suppression of these inflammatory responses by PSAE may effectively prevent the activation of the fibrotic program and the subsequent development of renal fibrosis (see Fig. 8).

Receptors such as toll-like receptors (TLRs) and NOD-like receptors (NLRs) are present on the surface of macrophages. These receptors can be activated by harmful stimuli, leading to the promotion of inflammatory cytokines and chemokines. In this study, network pharmacology identified the NLRP3 signaling pathway as a potential target of PSAE, which was confirmed through both *in vitro* and *in vivo* experiments (see Fig. 7 and Fig. 8). NLRP3 plays a crucial role in mediating renal tubule injury, macrophage infiltration, and renal interstitial fibrosis^[42]. Once activated, the NLRP3 inflammasome induces the cleavage of Gasdermin D (GSDMD) to GSDMD-N. This fragment binds to the cellular membrane, forming pores that allow the release of cytoplasmic contents, ultimately leading to a form of cell death known as pyroptosis^[43]. Moreover, NLRP3 activation enhances the maturation of caspase-1 and IL-1 β , which triggers the recruitment of immune cells and initiates interstitial fibrosis. In our study, PSAE intervention reduced the activation of the renal NLRP3 inflammasome in CKD mice, alongside inhibiting the cleavage of pro-caspase-1 via ASC (see Fig. 8). However, this effect was lost in mice with an *Nlrp3* gene knockout (see Fig. 10). This finding aligns with a previous study that showed NLRP3 inflammasome inhibitors can mitigate fibrosis during the early stages^[44]. These data suggest that PSAE may alleviate inflammation by inhibiting the NLRP3 inflammasome, thereby reducing renal fibrosis in CKD mice.

Integrated metabolomic and gut microbiome analyses confirmed that gut microbiota plays a crucial role in PSAE-mediated inhibition of renal fibrosis. The plasma untargeted metabolomics results revealed that the key mechanism by which PSAE ameliorates CKD is its regulatory effect on a suite of host-microbial co-metabolism pathways, primarily centered on the phenylalanine metabolism pathway (Fig. 3). Gut microbiota can convert phenylalanine into uremic toxins such as phenylacetylglutamine, hippuric acid, and phenylacetylglutamine, which induce renal oxidative stress and macrophage infiltration, and activate the NLRP3 inflammasome, thereby accelerating renal injury and fibrosis^[45-47]. PSAE intervention significantly reduced plasma levels of L-phenylalanine, hippuric acid, and phenylacetylglutamine in CKD mice (Fig. 3F), suggesting that its renal protective effects are mediated through suppression of the phenylalanine metabolism pathway. Based on these findings, we further conducted 16S rDNA sequencing of gut microbiota, which showed that PSAE effectively remodeled the composition and structural balance of gut microbiota in mice with CKD (Fig. 4). Notably, PSAE significantly reduced the abundance of specific bacteria—Lachnospiraceae, Ruminococcus, and the Lachnospiraceae NK4A136 group—in CKD mice (Fig. 4). These three bacterial communities are potential sources of uremic toxins that can induce renal interstitial inflammation and fibrosis^[48]. In contrast, PSAE increased the levels of *Butyrivibrio* and *Faecalibaculum* in CKD mice (Fig. 4). Both of these bacteria are capable of fermenting carbohydrates to produce short-chain fatty acids (SCFAs), which help mitigate renal injury by reducing inflammation and oxidative stress^[49]. Based on these findings, the reduction of uremic toxin-producing bacteria, coupled with the increase of SCFA-producing microbes, thereby regulating host-microbiota co-metabolism (particularly phenylalanine metabolism), appears to be responsible for the inhibitory effect of PSAE on renal fibrosis in CKD mice. This conclusion is further supported by the correlation analysis between CKD-related indicators and gut microbiota (Supplementary Fig. 4).

While PSAE-containing serum repressed the activation of the NLRP3 inflammasome in THP-1 cells, we did not observe this effect in the serum of rats with exhausted gut microbiota (Fig. 7) or in gut microbiota-depleted mice (Figs. 5, 6 and 9). This indicates that the transformation of PSAE by gut microbiota is a prerequisite for its therapeutic effect on renal fibrosis. Due to the complex composition of PSAE, we were unable to determine how the intestinal bacteria transformed its prototype components before absorption, and this needs to be investigated further in future research.

Gut microbiota can express various enzymes, such as β -glucosidase, dioxygenase, and CoA ligase, which facilitate a range of biochemical reactions, including hydrolysis, oxidation, reduction, and esterification [50–51]. Our study confirmed that gut microbiota can influence the metabolism and absorption of key components in PSAE. By UPLC-MS/MS analysis, we identified 15 new compounds in the PASE-containing serum of mice with gut microbiota (Supplementary Table 6). Among these compounds, 3 were also present in the PS decoction and the PASE-containing serum of mice with depleted gut microbiota, suggesting that they are the original components of PS that are directly absorbed into the bloodstream. There are 2 compounds that were present in the PS decoction but could not be detected in the PASE-containing serum with depleted gut microbiota, indicating that the original components of PS might first be metabolized by the gut microbiota into new products, which are then converted back into these compounds by the liver after entering the bloodstream. The remaining 10 compounds were absent in both the PS decoction and the PASE-containing serum of mice with depleted gut microbiota, suggesting that the original components of PS are first metabolized by the microbiota into new products, which are then further metabolized by the liver into these compounds after entering the bloodstream (Supplementary Table 6).

5. Conclusion

In conclusion, this study demonstrated that PSAE can reduce levels of creatinine and urea, improve renal injury, and inhibit renal fibrosis in mice with chronic kidney disease (CKD). Multi-omics analyses revealed that the key mechanism by which PSAE exerts its renoprotective effects is mediated through the remodeling of the disordered gut microbiota and the driving of host-microbial co-metabolic pathways, including phenylalanine metabolism, in a manner that is dependent on the gut microbiota. Further investigation demonstrated that the gut microbiota-dependent circulating components derived from PSAE can further inhibit the activation of the NLRP3 inflammasome, a key downstream signaling pathway in combating CKD. In conclusion, PSAE ameliorates renal fibrosis by multi-targeting the “gut microbiota-metabolism-NLRP3 inflammasome” axis, thus providing a theoretical and experimental foundation for its development as a functional food or therapeutic agent for CKD management.

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Conflict of interest

The authors state that they have no known financial interests or personal relationships that could influence the work presented in this paper.

Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version.

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