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Research article

Rujin Jiedu decoction protects against influenza virus infection by modulating gut microbiota

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ABSTRACT

Background: Rujin Jiedu decoction (RJJDD) is a classical prescription of Traditional Chinese Medicine that has long been applied to treat pneumonia caused by external infection, but whether and how it benefits influenza virus therapy remains largely unclear. The aim of this study was to investigate the anti-inflammatory effect of RJJDD on the mouse model of influenza and to explore its potential mechanism.

Methods: The mice were mock-infected with PBS or infected with PR8 virus followed by treatment with RJJDD or antiviral oseltamivir. The weight loss and morbidity of mice were monitored daily. Network pharmacology is used to explore the potential pathways that RJJDD may modulate. qRT-PCR and ELISA were performed to assess the expression of inflammatory cytokines in the lung tissue and macrophages. The intestinal feces were collected for 16S rDNA sequencing to assess the changes in gut microbiota.

Results: We demonstrate that RJJDD protects against IAV-induced pneumonia. Comprehensive network pharmacology analyses of the Mass Spec-identified components of RJJDD suggest that RJJDD may act through down-regulating key signaling pathways producing inflammatory cytokines, which was experimentally confirmed by cytokine expression analysis in IAV-infected mouse lung tissues and IAV single-strand RNA mimic R837-induced macrophages. Furthermore, gut microbiota analysis indicates that RJJDD prevented IAV-induced dysbiosis of host intestinal flora, thereby offering a mechanistic explanation for RJJDD's efficacy in influenza pneumonia.

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Conclusion: This study defines a previously uncharacterized role for RJJDD in protecting against influenza likely by maintaining homeostasis of gut microbiota, and provides a new therapeutic option for severe influenza.

1. Introduction

Influenza viruses are important respiratory pathogens that cause substantial seasonal and pandemics, leading to high morbidity and mortality across the world [1,2]. Clinically, severely infected patients with influenza often die from acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) [3], both of which can result from a combination of intrinsic viral pathogenicity and the host immune response. Although an early phase of immune response helps to inhibit the replication and dissemination of viruses, an uncontrolled over-activated inflammatory response exacerbates lung injury [4]. It is well-known that the complications or eventual death of flu patients are usually associated with the over-production of inflammatory cytokines, also named "cytokine storm" [5]. Influenza A viruses (IAV) are the most important and frequently mutated viruses of all influenza viruses, accounting for the majority of the pandemic. Antigenic drift and shifts in the IAV genome render vaccines and antiviral drugs less effective in combating influenza [6]. The development of new therapies against influenza virus infection is an urgent need [7,8].

Rujin Jiedu decoction (RJJDD), a traditional Chinese medicine formula consisting of six herbs (Table 1), is based on Huanglian Jiedu decoction which was originally documented in *Elbow Reserve Emergency Prescription* written by Ge Hong in Eastern Jin Dynasty, has the functions of decreasing internal heat and removing the toxin, and is often used to treat pneumonia [9]. A case report showed that RJJDD combined with antibiotics was effective in treating patients with severe shock-type pneumonia caused by bacterial infection [10]. Consistently, a recent mouse model study indicated that RJJDD alleviated LPS-induced acute lung injury by down-regulating cytokine storm [11]. Based on the property and flavor network pharmacology, it was proposed that RJJDD has potential to benefit the therapy of viral pneumonia [12]. However, if RJJDD protects against influenza virus infection remains unclear.

The microbiome plays an important role in the unbalanced transition of the host from homeostasis to disease. Disorders of the gut microbiota have been linked to autoimmunity, inflammatory conditions, and infectious illnesses [13]. A growing number of studies indicate that the gut microbiota can influence the physiology and pathology of the lung through the gut-lung axis [14,15], which is further supported by evidence that gut microbiota protects against respiratory inflammation, allergic asthma, and infections such as IAV [16] and *Klebsiella pneumonia* [17] and that gut dysbiosis exacerbate lung infections by IAV and Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) [18]. The oral administration of a TCM formula allows it to enter the gastrointestinal tract where it contacts and impacts the gut microbiota by regulating the species, quantity, and composition of intestinal flora [19]. Therefore modulation of gut microbiota may be a critical means by which TCM treats diseases including lung diseases. If RJJDD can act through gut microbiota to protect against influenza is unknown.

2. Materials and methods

2.1. Animals

Inbred mice have been widely used for the study of influenza viruses as a mammalian model [20,21]. Besides BALB/c mice, C57BL/6 mice are also commonly used to study the pathogenesis of influenza virus-induced lung injury [22,23]. Female C57BL/6 mice at 5–8 weeks old (16–21 g) were purchased from Joint Ventures Sipper BK Experimental Animal Company (Shanghai, China) and maintained in a specific pathogen-free (SPF) environment.

2.2. Mouse model establishment and grouping

On the day of the experiment, influenza virus A/Puerto Rico/08/1934 (PR8) was diluted to the experimental concentrations (4×10^5 PFU/mL) using sterile PBS and placed on a mixture of ice water for later use. The mice were weighed and their original body weights were recorded. After randomization, the mice were anesthetized with isoflurane and then intranasally infected with 50 μL of PBS or diluted PR8 virus solution in one nostril of a mouse. The C57BL/6 female mice were divided into five groups and treated as follows: control group "PBS", model group "PR8", oseltamivir-treated group "PR8+OSV" and low dose of RJJDD-treated group,

Table 1
Composition information of RJJDD.

Botanical information	Pharmaceutical name	Chinese name	English name	Batch No.	Quality (g)
Rhizome	*Coptis chinensis* Franch.	Huánglián (黄連)	Rhizoma Coptidis	20210902–1	4
Dried roots	*Scutellaria baicalensis* Georgi	Huángqín (黄芩)	Radix Scutellariae	20210910–1	4
Bark	*Phellodendron chinense* Schneid.	Huángbò (黄柏)	Cortex Phellodendri	20210629–1	4
Dried ripe fruit	*Gardenia jasminoides* J. Ellis	Zhīzǐ (梔子)	Fructus Gardeniae	20210830–1	4
Dried roots	*Platycodon grandiflorum A. DC.*	Jiégěng (桔梗)	Radix Platycodi	20211014–1	6
Dried roots and rhizomes	*Glycyrrhiza uralensis* Fisch.	Gāncǎo (甘草)	Radix Glycyrrhizae	20210825–1	9

"PR8+RJJDD(L)", 4 g/kg and high dose of RJJDD-treated group, "PR8+RJJDD(H)", 16 g/kg. Each group of mice was sacrificed by cervical dislocation at 2 h after the last dose. Serum and lung samples were collected for cytokine analysis, and lung samples were collected for hematoxylin-eosin staining.

2.3. RJJDD formula preparation

Quality control for the RJJDD herbal medicine was performed according to the Chinese Pharmacopoeia [24]. These crude ingredients were acquired from Shanghai Wanshicheng Pharmaceutical Co., Ltd. (Shanghai, China), and further details regarding RJJDD, such as the botanical information and batch no., are shown in Table 1. Prepared the medicinal materials and added them to 800 mL water. After immersing for 30 min, decoct for 45 min in the first time, strain out the medicinal liquid, and the second decocting as above. The solution obtained from the two times was mixed and concentrated to 0.9 g/mL, and the impurities were removed by double gauze filtration.

2.4. Reagents

Oseltamivir Phosphate Granules (OSV), Imiquimod (R837, Cat: tlrl-imqs) and the influenza virus PR8 were described previously [25]. Enzyme-linked immunosorbent assay (ELISA) kits for mouse IL-6 (cat: DY406; M6000B), mouse IL-10 (cat: DY417; M1000B), mouse TNF-α (cat: DY410; MTA00B), mouse MCP-1 (cat: DY479; MJE00B), mouse MIP-2 (cat: DY452), and mouse IFN-β (cat: DY8234-05) were obtained from R&D System of Bio-Techne Brand (MN, USA). Total RNA was isolated from mouse lung tissues using a RNA extraction kit (RNAfast200, Fastagen, Shanghai, China) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 0.5 μg total RNA using Prime Script RT reagent kit (Takara, Dalian, CHN) following the manufacturer's directions. As previously described [26], we used the SYBR Premix Ex Taq kit (Takara, Dalian, CHN) and the LightCycler (Roche Diagnostics, Indianapolis, IN) to conduct quantitative real-time PCR (qRT-PCR) analyses. Cells were cultured in high-glucose DMEM medium (Hyclone, USA) containing 10 % of fetal bovine serum (Biowest, France). The viability of the cells was determined with the CCK-8 assay using Cell counting kit-8 (cat: C6005L) was obtained from US EVERBRIGHT (Sayreville, NJ, USA).

2.5. Identification of chemical constituents and metabolites of RJJDD by UHPLC-Q-exactive orbitrap MS (UHPLC-QE-MS) analysis

2.5.1. Previous chemicals reagents and animal experiments

Methanol and acetonitrile were of HPLC grade from CNW Technologies (Düsseldorf, Germany), and formic acid was of HPLC grade from SIGMA (St. Louis, MO, USA). Deionized water used throughout the experiment was purified by Mingche D24 UV (Merck Millipore, Billerica, MA, USA). Acquity UPLC BEH C18 1.7 μm (2.1 × 100 mm) was provided from Waters™. Moreover, all of the other regents were in accordance with analytical standards, which were easily accessible at the workstation (Shanghai, China).

Mice were randomly divided into two groups (Blank Group, n = 6 and RJJDD Group, n = 9). Feeding conditions were the same as above. Animals were administered RJJDD (16 g/kg) and the blank group was just administered sterile water. Mice were collected 7 days after administration for lung samples.

2.5.2. Biological sample preparation

Weigh all lung samples, add normal saline (0.9 % NaCl aqueous solution), add two small steel balls, homogenate, 35 Hz, 240 s, after 3 times, absorb 800 μL of homogenate, add 80 μL hydrochloric acid (2 mol/L), vortex 1 min, 4 °C static 15 min, repeat vortex static 4 times, add 3.2 mL acetonitrile, vortex 5 min, then the mixed sample solvents were centrifuged at 13800 (× g) with a radius of 8.6 cm, 4 °C, after 10 min, nitrogen was used to blow the supernatant dry. The residue was redissolved in 100 μL 80 % methanol (internal standard concentration was 10 μg/mL) solution. Then the mixed sample solvents were centrifuged at 13800 (× g) with a radius of 8.6 cm, 4 °C, for 5 min. In the final step, further instrumental analysis was performed on the collected supernatant.

2.5.3. Instruments and analytical conditions

Liquid chromatography system and mass spectrometry and data processing were performed as described previously [27].

2.6. Cell culture

The mouse macrophage cell line Raw264.7 was from American Type Culture Collection. Primary mouse peritoneal macrophages were isolated from female C57BL/6J mice intraperitoneally injected with thioglycolate as described previously [25]. Cells were cultured in high-glucose DMEM medium containing 10 % of fetal bovine serum in a humidified incubator maintained at 37 °C and 5 % CO_2.

2.7. CCK-8 assay was used to calculate cell viability

Raw264.7 cells were pretreated with the indicated concentrations of RJJDD (1, 50, 100, 200, and 400 μg/mL) for 24 h. The viability of the cells was determined with the CCK-8 assay using a 96-well culture plate. 10 μL CCK-8 solution was added to each well. Incubation at 37 °C for 2 h was followed by absorbance readings at 450 nm using an Epoch 2 microplate reader (BioTek, USA).

2.8. Enzyme-linked immunosorbent assay (ELISA)

As directed by the manufacturer (R&D Systems, MN, USA), IL-6, TNF-α, IL-10, MCP-1, MIP-2, and IFN-β concentrations in the supernatants of cell cultures were measured using ELISA kits.

2.9. RNA extraction and quantitative real-time PCR

Data were normalized to β-actin in each sample. The primers used for qRT-PCR analysis are described in Table S1.

2.10. Organ indexes

We weighed each mouse separately after removing its lung and spleen and weighing their bodies on the day of sacrifice. The organ index was calculated by dividing the weight of the organ by the weight of the body.

2.11. Histology

A hematoxylin-eosin (H&E) staining was performed to assess the pathological damage in mouse tissues. To produce standard H&E stainings, lung tissues were fixed in 4 % formaldehyde and paraffin-embedded after being fixed in formaldehyde for 24h. After then, Axio Imager M2 microscope (Carl Zeiss Micro Imaging, Goettingen, Germany) was used to observe the pathological alterations. The lung histological scores were calculated based on a 0 to 12-point scale, which included alveolar infiltration, pulmonary vasculitis, and lung edema [28].

2.12. Network pharmacology-based analysis

2.12.1. RJJDD target prediction for absorbed ingredients of lung tissues

LC-MS was used to identify the herbal ingredients absorbed into the lung of the RJJDD administrated mice. The herbal source of the identified ingredients was determined by conducting a search on the SymMap and TCMSP databases. The SMILES structure files for these ingredients were downloaded from the PubChem, and deposited into the SwissTargetPrediction to determine their predicted targets. The websites of the databases for network pharmacology are listed in Table S2.

2.12.2. The targets collection of influenza

Using “influenza” as the keyword, relevant influenza genes were obtained by searching online disease databases such as OMIM, GeneCards, PharmGKB, DrugBank, Polysearch2, CTD, Phenopedia, DisGeNET, HERB, TTD.

2.12.3. The intersection targets of RJJDD and influenza

The online program Draw Venny Diagram was used to predict the common targets of the active ingredient and the disease. This intersection set is the potential targets of the RJJDD for the treatment of influenza.

2.12.4. “RJJDD-Targets-Influenza” network

The above-mentioned intersecting targets and their mapping ingredients were utilized to construct the “RJJDD-Targets-Influenza” network via the use of STRING and cytoscape software. And the “Main ingredients-Herb distribution and Target number” graph was constructed through Bioinformatics platform.

2.12.5. The core PPI network extraction of RJJDD against influenza

The above intersection targets were submitted to STRING to generate the primary PPI network. Then the network was imported into Cytoscape software. The CytoNCA plug-in was used to calculate the network topology parameters such as Betweenness centrality (BC), Closeness centrality (CC), Degree centrality (DC), Eigenvector centrality (EC), Local average connectivity based method (LAC) and Network centrality (NC). Each node with topological parameters exceeding the median was extracted to construct the secondary PPI network. Following two rounds of screening, we obtained the core PPI network for RJJDD against influenza.

2.12.6. GO and KEGG analysis of RJJDD against influenza

R 3.6.2 software and Bioconductor packages including org. Hs. eg. db, colorspace, stringi, DOSE, clusterProfile, pathview, ggplot2 were employed to conduct the GO and KEGG enrichment analysis on the 115 common targets. Among them, functional analysis for GO mainly includes three categories: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). And the significant results (P value < 0.05) of GO or KEGG analysis were visualized by Bioinformatics platform.

2.13. 16S rRNA gene sequencing and analysis

Six cecum samples were collected and frozen at −80 °C until 16S rRNA sequencing from each group on the 7th day after model establishment. Total genomic DNA was purified with a MagPure Soil DNA LQ Kit following the manufacturer’s instructions. DNA concentration was determined with NanoDrop 2000 (Thermo Fisher Scientific, USA) and and DNA integrity was evaluated by agarose

gel electrophoresis. DNA samples were used as the template to PCR-amplify bacterial 16S rRNA genes with barcoded primers and a Taq polymerase (Takara, Dalian, CHN). For bacterial diversity analysis, V3–V4 variable regions of 16S rRNA genes were amplified with universal primers 343F (5′-TACGGRAGGCAGCAG-3′) and 798R (5′-AGGGTATCTAATCCT-3′) [29]. The PCR products were analyzed by agarose gel electrophoresis, purified, and quantified using Qubit dsDNA Assay Kit (Thermo Fisher Scientific, USA). The 16S rRNA gene

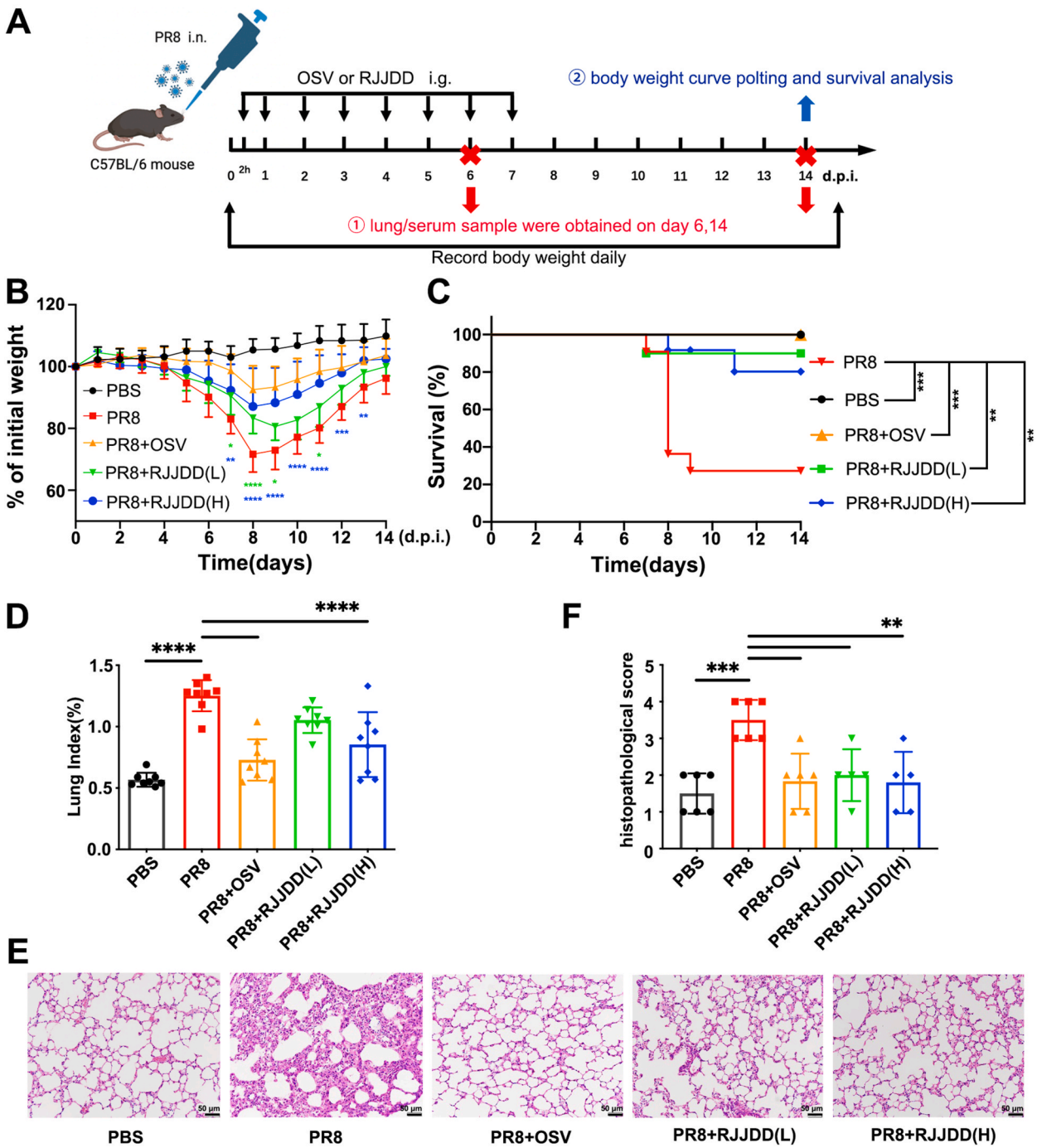
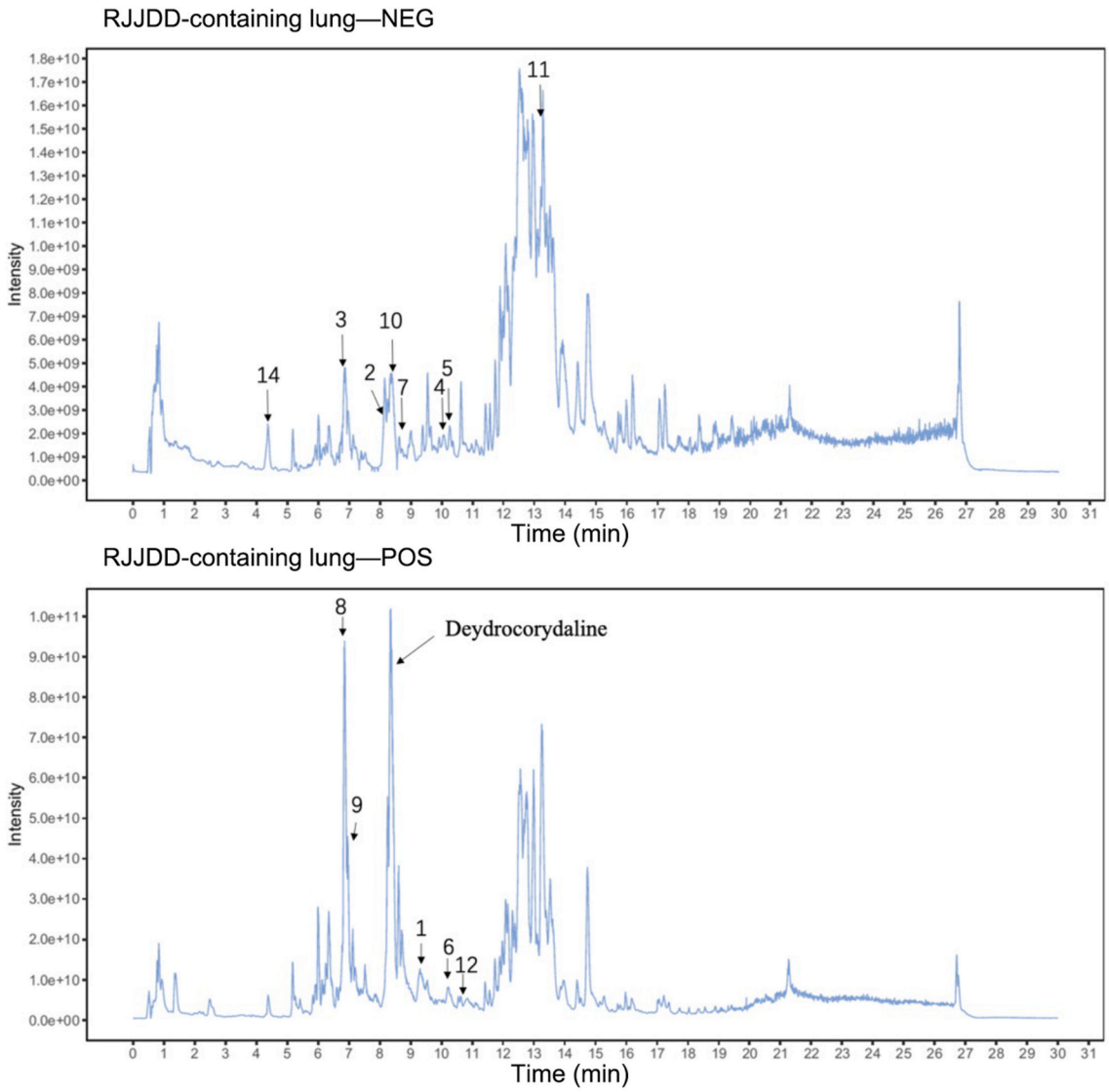


Fig. 1. RJJDD reduced weight loss and mortality of IAV-infected mice. (A) Illustration of the general scheme of the experimental design of *in vivo* study. (B) Body weight for 14 days (n = 8). (C) Survival rate of infective mice for 14 days post infection (n = 10). (D) Lung index (lung weight/body weight × 100 %) in uninfected or infected mice following the administration of RJJDD or OSV at 14 dpi. (E and F) Histopathological injury of representative lung sections in each group at 14 dpi with 400× magnification (Bar = 50 μm). Survival data were analyzed by Log-Rank test and survival curves were generated by GraphPad Prism 8 software. Data are shown as mean ± standard deviations (SD) for each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, *vs.* model group.

A



B

NO.		NEG	POS	NO.		NEG	POS
1	Limonin	/	9.75	8	Jatrorrhizine	/	6.73
2	Kaempferol	8.1	/	9	Epiberberine	/	6.75
3	Scutellarein	6.99	/	10	Naringenin	8.45	/
4	Isorhamnetin	10.16	/	11	Enoxolone	13.37	/
5	Baicalein	10.23	/	12	Ursolic acid	/	10.03
6	Chrysin	/	10.2	13	Protopine	/	/
7	Genistein	8.85	/	14	Verbenalin	4.31	/

Fig. 2. Total ion chromatogram (TIC) in positive ion mode and negative ion mode by UHPLC-QE-MS. (A) Total positive ion and negative ion chromatograms of samples illustrated the main bioactive compounds of RJJDD contained in the lung. (B) Totally fourteen components of RJJDD were identified from the lung.

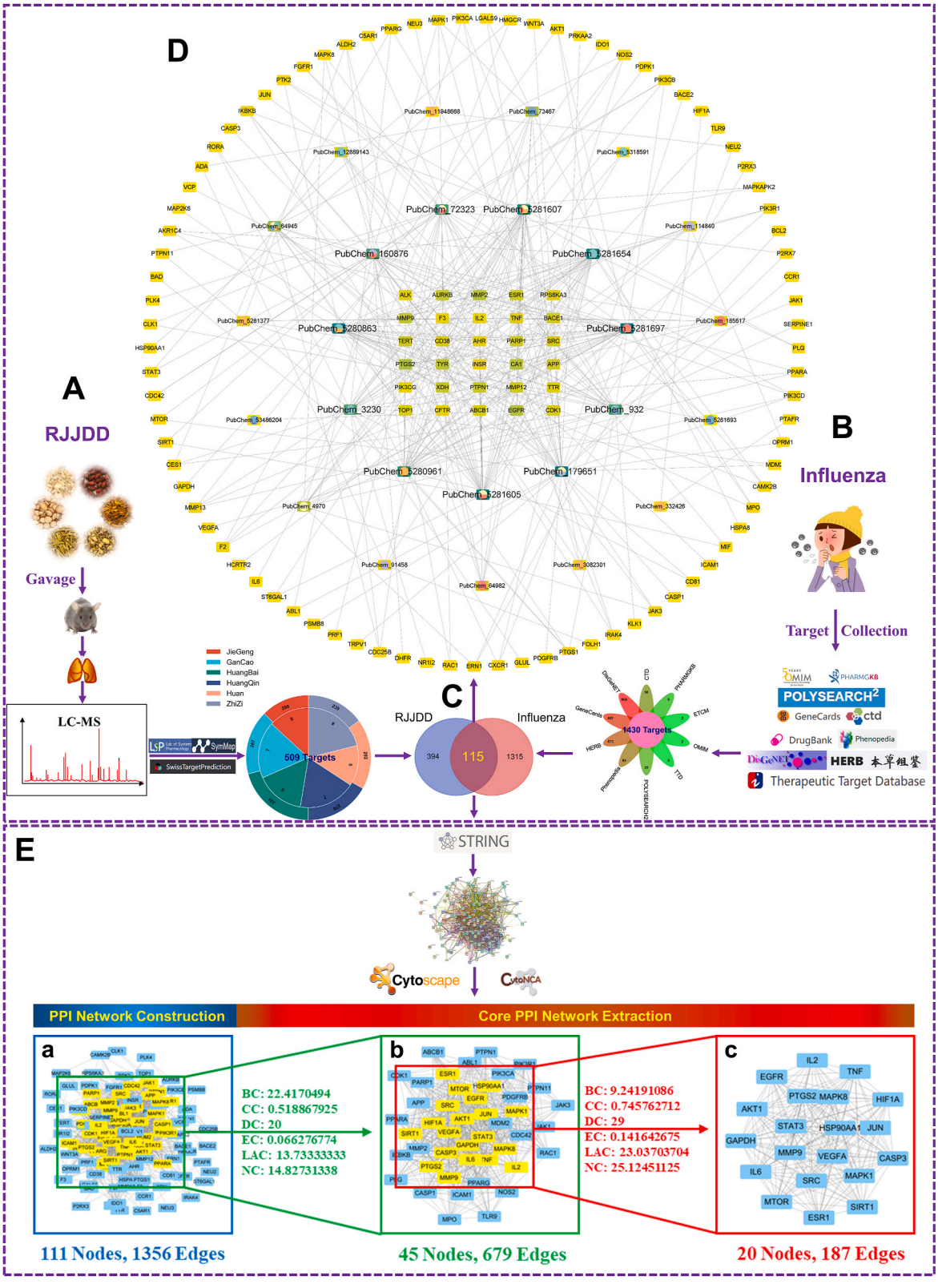


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Fig. 3. The construction of "RJJDD-Targets-Influenza" network and core PPI network extraction of RJJDD against influenza. (A)The target prediction of ingredients of RJJDD into the lungs. (B)The target collection of influenza. (C)The intersection targets of RJJDD and influenza. (D) "RJJDD-Targets-Influenza" network. (E)The core PPI extraction of RJJDD against influenza. (a) The primary PPI network of RJJDD against influenza; (b) the secondary PPI network of RJJDD against influenza; (c) the core PPI network of RJJDD against influenza.

sequencing was conducted by Shanghai OE Biotech. Co., Ltd. (Shanghai, China) following the established method [30].

2.14. Statistical analysis

The data were analyzed using Prism GraphPad 8 software. The survival rate was analyzed by the Log-Rank test. Comparison analyses were performed using the *t*-test and Kruskal-Wallis test and presented as mean ± standard deviation (SD). Statistical significance was determined as *, $P < 0.05$; **, $P < 0.01$ or ***, $P < 0.001$.

3. Results

3.1. RJJDD reduced weight loss and mortality of IAV-infected mice

To investigate the potential therapeutic effect of RJJDD on IAV infection *in vivo*, influenza virus PR8 was used to infect mice intranasally to establish a mouse model of severe IAV infection. The infected mice were then intragastrically administrated with PBS, RJJDD, and a control drug OSV which is a specific anti-influenza viral agent (Fig. 1A). The body weight and survival of mice were monitored every day for consecutive 14 days. As shown in Fig. 1B and C, PR8 infection resulted in significant weight loss and mortality of mice over time, and eventually only 27 % of infected mice survived by day 14. As expected, an obvious protective effect was observed for the administration of OSV which protected against the infected mice, leading to much less weight loss and 100 % survival. Interestingly, both low and high doses of RJJDD significantly alleviated the weight loss of infected mice, and increased the survival rate of mice to 83.33 % and 90 %, respectively, suggesting that RJJDD has a critical role in protecting mice against IAV infection *in vivo*.

3.2. RJJDD alleviated tissue damage in the lung of IAV-infected mice

Influenza is an infectious disease of the upper and lower respiratory tract. Viral infection can cause destructive changes to the lung structure, such as diffuse alveolar injury, idiopathic pulmonary fibrosis, and varying degrees of pulmonary hemorrhage, which represent pathological characteristics of influenza [1]. To examine the effect of RJJDD on these characteristics, mice were sacrificed on day 14 after infection and the lung was collected for lung index calculation and hematoxylin-eosin (H&E) staining. Both OSV and RJJDD treatments reduced the lung index caused by infection (Fig. 1D). As shown in Fig. 1E and F, PR8 infection significantly increased the lung index of mice, accompanied by the thickening of the pulmonary interstitium, connective tissue hyperplasia, and the infiltration of a large number of inflammatory cells. Consistently, both treatments dramatically improved the integrity of the alveoli structure and down-regulated the interstitial infiltration of inflammatory cells. These data indicate that RJJDD can alleviate the lung injury caused by PR8 infection in mice.

3.3. Identification of main compounds of RJJDD from the medicated lung

To gain insight into the mechanism underlying the protective role of RJJDD in combating influenza, we first took advantage of systems pharmacology tools to predict the potential pathways that RJJDD may regulate during IAV infection. We employed UHPLC-QE-MS to identify the main components of RJJDD from the lung of RJJDD-administrated mice. After intragastric administration of RJJDD or boiled water for 7 consecutive days, the lung was collected to be taken into samples, the samples were analyzed by Vanquish (Thermo Fisher Scientific) ultrahigh performance liquid chromatography. Totally fourteen components of RJJDD were identified from the lung (Fig. 2A–B), some of which such as Limonin [31], Scutellarein [32], and Isorhamnetin [33] have been reported to inhibit LPS-induced inflammatory response in Raw264.7 cells.

3.4. Network pharmacology analysis results of RJJDD against influenza

3.4.1. "RJJDD-Targets-Influenza" network construction and core PPI network extraction

Through LC-MS, combined with SymMap and TCMSP databases, 26 ingredients that are absorbed into the lungs were identified for RJJDD. There are 509 non-redundant targets of these ingredients were predicted by SwissTargetPrediction (Fig. 3A). After conducting a search on the online disease gene databases, a total of 1430 genes associated with influenza were identified (Fig. 3B). There are 115 intersection targets between the two sets that were considered potential targets for RJJDD against influenza (Fig. 3C). Through the STRING platform and Cytoscape software, the "RJJDD-Targets-Influenza" network was constructed (Fig. 3D). The intersection targets were imported into the STRING platform and generated a primary PPI network consisting of 111 nodes and 1356 edges. The average Node Degree of the network was 19.8 (P value $< 1.0\ e{-}16$). After two steps of topological parameters screening, a core PPI network consisting of 20 nodes and 187 edges was ultimately obtained (Fig. 3E).

3.4.2. GO and KEGG analysis results of RJJDD against influenza

The analysis of gene intersection resulted in a total of 2723 entries that were statistically significant in the GO analysis. Among them, 2459 entries were classified as BP, including peptidyl-serine phosphorylation, response to oxidative stress, regulation of cell-cell adhesion, etc. The CC classification comprises 94 entries, including membrane raft, membrane microdomain, membrane region, and others. The MF classification comprises primarily 170 entries, such as phosphatase binding, protein phosphatase binding, nuclear receptor activity, and various others. The top 10 entries with the smallest corrected *P* values for each classification are displayed in Fig. 4A. The results of the KEGG enrichment analysis indicate that a grand total of 156 signaling pathways were significantly enriched and the 20 significantly enriched pathways are shown in Fig. 4B, including the Toll-like receptor signaling pathway, RIG-I-like receptor signaling pathway, TNF signaling pathway, NF-κB signaling pathway, and MAPK signaling pathway. These results indicate that modulating cytokine production signaling pathways to regulate IAV-induced inflammatory responses may be the mechanism for the anti-influenza effects observed for RJJDD.

3.5. RJJDD down-regulated the expression of inflammatory cytokines upon IAV infection

Following the network pharmacology analysis, we next tested if RJJDD administration had an effect on the cytokine levels in the PR8-infected mice. The high dose of RJJDD-treated group mice were sacrificed at 6 dpi and the lung and peripheral blood were collected for the examination of inflammatory cytokines or chemokines by qRT-PCR and ELISA, respectively. As shown in Fig. 5A–H, RJJDD inhibited the expression of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α, and IFN-β), and chemokines (MCP-1 and IP-10), but increased the expression of anti-inflammatory cytokines (IL-10 and TGF-β) in the lung. As shown in Supplementary Fig. 1, RJJDD inhibited the expression of 8 viral genes in the lung of influenza mice. Consistently, the levels of IL-6, TNF-α, and MCP-1 in the serum were down-regulated by RJJDD (Fig. 5I-L). These results suggest that RJJDD may protect against PR8-induced excessive inflammatory response in the lungs by balancing the production of pro-inflammatory and anti-inflammatory cytokines.

3.6. RJJDD reduced the expression of inflammatory cytokines in macrophages stimulated by R837

Excessive activation of innate immunity plays a key role in the pathology of IAV infection. To verify the anti-inflammatory effect of RJJDD *in vitro*, we isolated primary peritoneal macrophages from WT mice and stimulated them with TLR7 agonist R837, a mimic of single-stranded RNA, to mimic IAV infection. Treatment with a series of concentrations RJJDD (0–400 μg/mL) significantly down-regulated R837-induced production of pro-inflammatory cytokines, including IL-6, TNF-α, IL-1β and IFN-β (Fig. 6A–D). But the expression of anti-inflammatory factor IL-10 (Fig. 6E) also decreased by RJJDD. By the way, the expression of chemokines including MCP-1 and MIP-2 (Fig. 6F–G) was also down-regulated by RJJDD. We further used a mouse macrophage cell line Raw264.7 to verify the effect of RJJDD on the R837-induced expression of inflammatory cytokines and observed that non-toxic concentrations of RJJDD (Fig. 6H) dramatically down-regulated the transcripts of IL-6, TNF-α, and IL-1β (Fig. 6I–K). As shown in Supplementary Fig. 2, RJJDD treatment decreased the expression of M1, NA and PB2 genes of influenza virus in A549 cells. By the way, the expression of inflammatory cytokines and chemokines, including IL-6, IL-1β, and MCP-1 (Supplementary Fig. 3) was also down-regulated in A549 cells that were infected by PR8 and treated by RJJDD. Together, these data confirm that RJJDD can suppress TLR7-mediated inflammatory response in macrophages by decreasing the production of inflammatory cytokines.

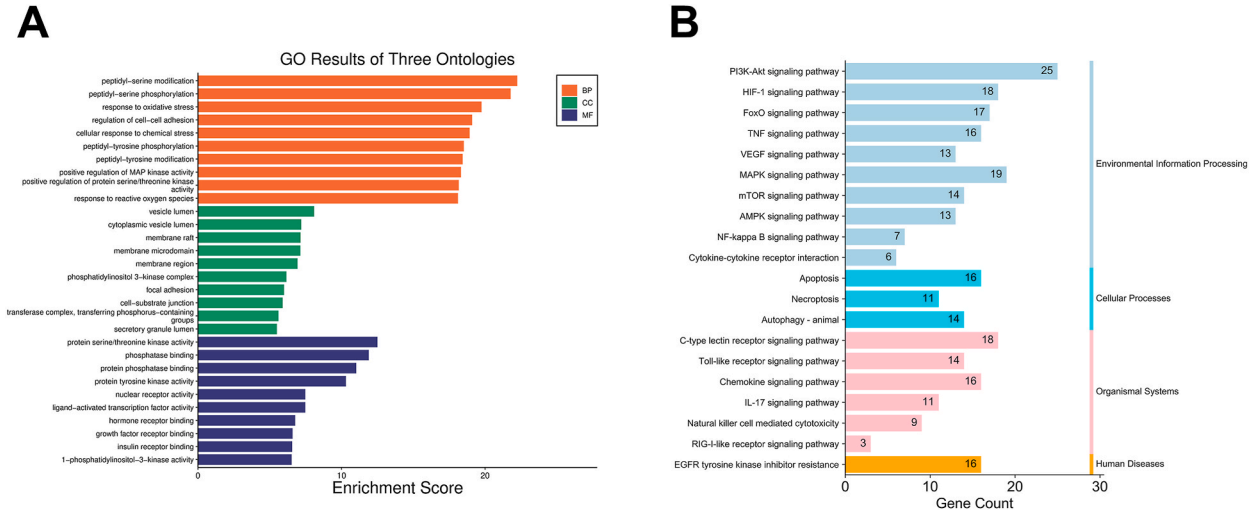

Fig. 4. Gene ontology and KEGG enrichment of candidate targets of RJJDD against influenza. (A) Gene ontology terms of candidate targets of RJJDD against influenza. The top 10 terms in each GO category with *P* adjust value < 0.05 were selected. (B) KEGG pathway enrichment of candidate targets of RJJDD against influenza. Ten terms with *P* adjust value < 0.05 that we interest were visualized.

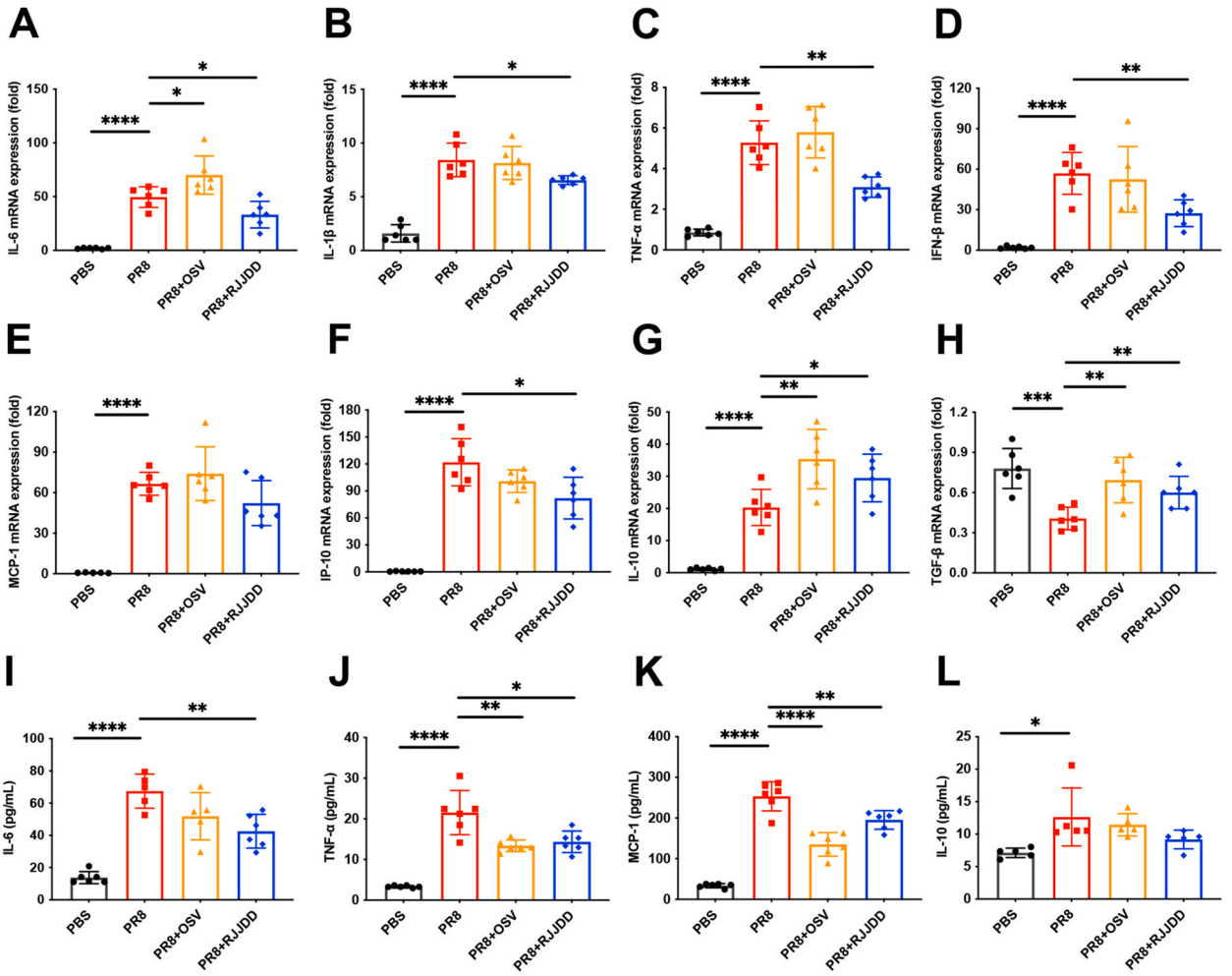

Fig. 5. RJJDD down-regulated the secretion of inflammatory cytokines upon IAV infection. The mRNA expression of (A) interleukin 6 (IL-6), (B) interleukin 1β (IL-1β), (C) tumor necrosis factor α (TNF-α), (D) interferon beta (IFN-β), (E) monocyte chemotactic protein 1(MCP-1), (F) interferon gamma inducible protein of 10 kDa (IP-10), (G) interleukin 10 (IL-10), (H) transforming growth factor β (TGF-β) in lung tissues was detected by qRT-PCR. The serum samples were collected for the examination of (I) IL-6, (J) TNF-α, (K) MCP-1 and (L) IL-10 by ELISA. All mice of the indicated groups were sacrificed on day 6. Data are shown as mean ± standard deviations (SD) for each group (n = 6 per group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, *vs.* model group.

3.7. RJJDD ameliorated gut microbiota dysbiosis in influenza mice

It has been demonstrated that TCM can intervene in lung diseases by modulating gut microbiota [34]. To explore if this was the case for RJJDD protection against IAV infection, we collected feces from all mice for the 16S rDNA sequencing to assess the changes in gut microbiota using an Illumina MiSeq platform. In the alpha diversity analysis, community richness and diversity were revealed by Chao1 and Simpson indices. Compared with the PBS group, the other three groups showed a significantly lower Chao1 index, but no significant differences were found among the three groups (Fig. 7A). In addition, the Simpson index of the four groups showed that the value of PR8 group was markedly lower than PBS group, while groups PR8_RJJDD and PR8_OSV reversed the up-regulation of bacterial diversity. Importantly, similar to OSV treatment, RJJDD treatment restored virus-induced alterations of microbial community diversity levels to nearly these of the PBS group (Fig. 7B). Based on the β-diversity analysis, the results of Principal coordinate analysis (PCoA) revealed four distinct clusters in gut microbiota among groups (Fig. 7C), especially between PR8 and PBS groups, the PR8 group was deviated from that of the PBS group, whereas RJJDD and Oseltamivir treatment groups more closely resembled those of the PBS group. Overall, RJJDD or Oseltamivir administration significantly improved the structural microbial diversity of the intestine in PR8-infected mice.

An "dysbiosis" of the intestinal flora, can be the cause of several diseases [35]. To determine the genus of bacteria that is correlated with the PR8 infection, we performed a bacterial composition analysis to quantify the relative abundances of the four experimental groups. As indicated by the heat map analysis, among the genera detected *Actinobacteria* was the only one that showed a significant difference between the four groups at phylum levels. PR8 infection dramatically down-regulated the relative abundance of

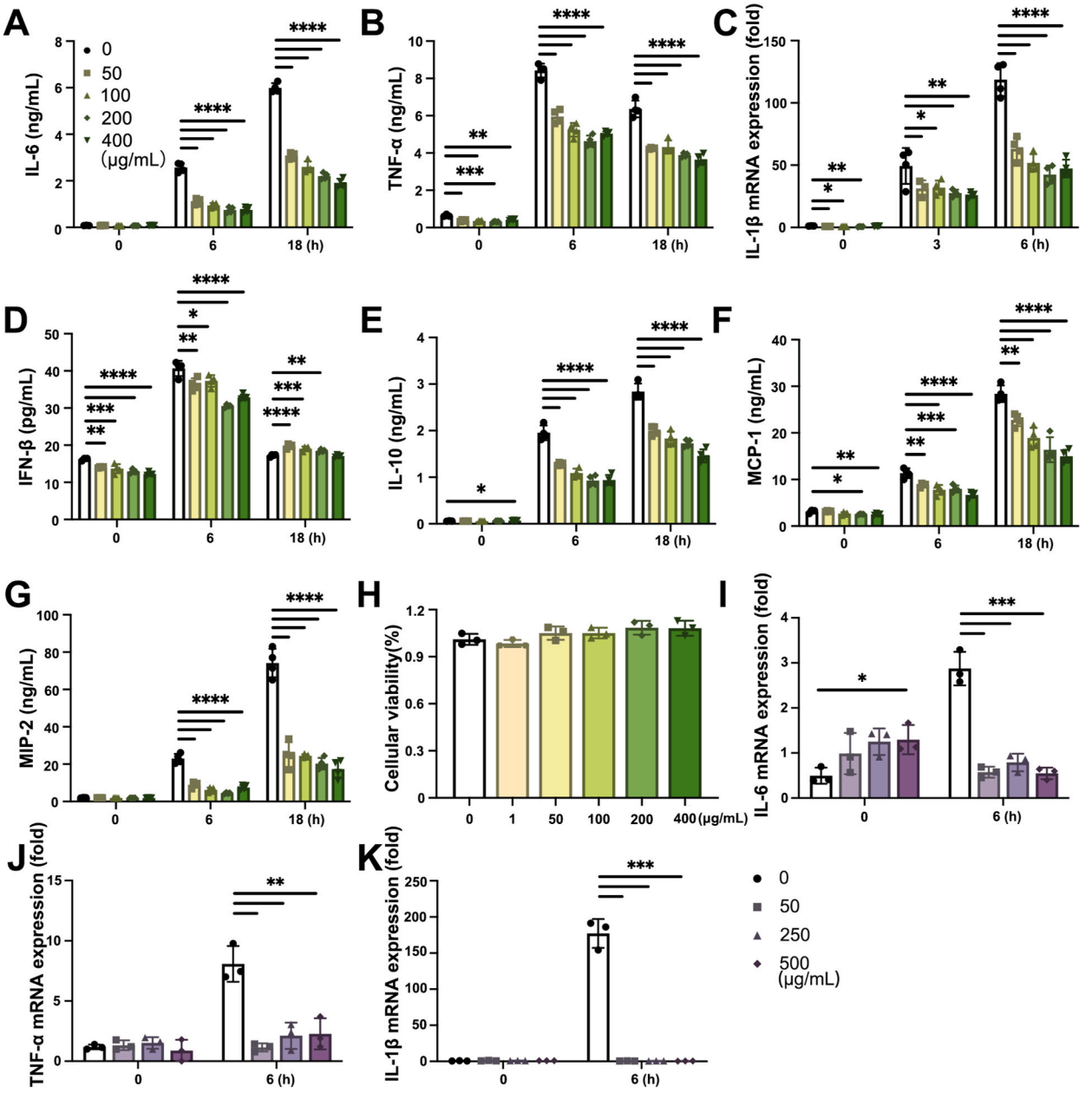

Fig. 6. RJJDD reduced the expression of inflammatory cytokines in macrophages stimulated by R837. Primary peritoneal macrophages were stimulated by R837 (5 μg/mL) with RJJDD (0–400 μg/mL) for 3 h, 6 h and 18 h. Cell culture supernatants were collected and the concentration of (A) IL-6, (B) TNF-α, (D) IFN-β, (E) IL-10, (F) MCP-1, (G) MIP-2 were detected by ELISA. The mRNA expression of (C)IL-1β were detected by qRT-PCR (n = 4 per group). RJJDD reduced the expression of inflammatory cytokines in Raw264.7 stimulated by R837. (H) RJJDD's inhibition time (24 h) and concentration (0–400 μg/mL) on Raw264.7 cells was screened using the CCK8 assay. Cell culture supernatants were collected and the concentration of (I) IL-6, (J) TNF-α, (K) IL-1β were detected by qRT-PCR (n = 3 per group). Data are shown as mean ± standard deviations (SD) for each group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, *vs.* model group.

Actinobacteria, while RJJDD significantly revesed this downregulation (Fig. 7D–E). At the genus level, bacterial community composition was significantly altered after PR8 infection, with the proportions of short-chain fatty acids (SCFAs)-producing bacterial genera including *Bifidobacteriaceae*, *Lachnoclostridium*, and *Lachnospiraceae_UCG−006* down-regulated and that of *Blautia* up-regulated. Interestingly, the relative abundance of all three SCFAs-producing bacterial genera was restored to a range close to their respective normal levels in the RJJDD group (Fig. 7F–J), suggesting a strong ability of RJJDD to maintain the homeostasis of gut microbiota during PR8 infection, particularly those producing anti-inflammatory SCFAs [36].

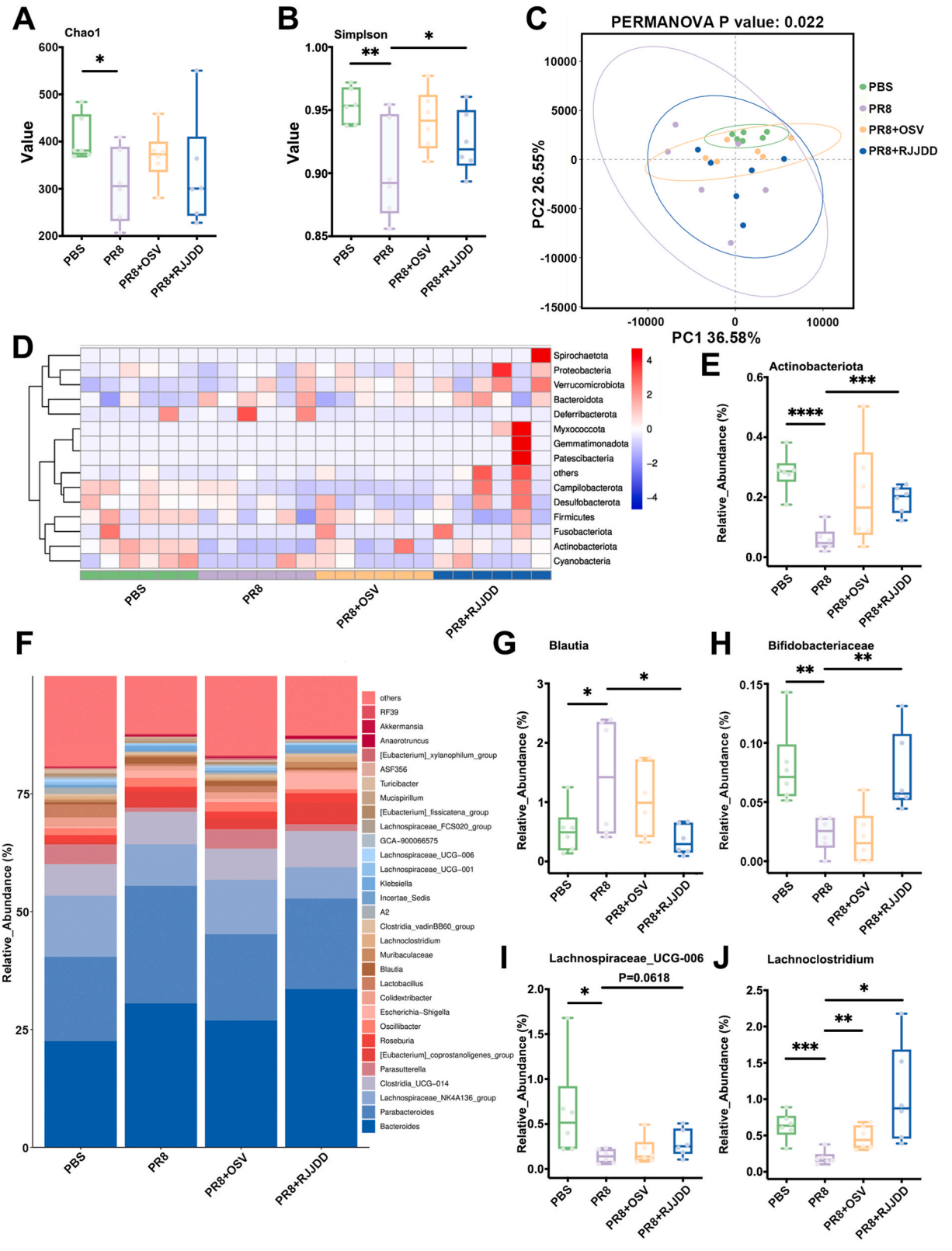

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Fig. 7. RJJDD ameliorated gut microbiota dysbiosis in influenza mice. A α-diversity analysis represented by the (A) chao1 and (B) simpison index. Principal coordinate analysis (PCoA) plots upon oral RJJDD assessed by (C) PERMANOVA. The relative abundance of bacterial (D) phylum level and (F) genus level. (E)Relative abundance of *Actinobacteria*. (G–J) Represent the genus of *Blautia*, *Bifidobacteriaceae*, *Lachnospiraceae_UCG−006*, *Lachnoclostridium*, respectively. Data are shown as mean ± standard deviations (SD) for each group (n = 6 per group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$, *vs.* model group.

4. Discussion

Respiratory infectious diseases, e.g., influenza, COVID-19 and other respiratory virus infections, have long been a huge threat to global health. Uncontrolled host immune response to viral infections is a common cause of these diseases leading to unwanted pathological consequences, such as cytokine storm formation, tissue damage and even death [37]. There is an urgent need to develop new therapeutics for treating these diseases. In this study, we used a mouse model of influenza to show that RJJDD plays a protective role in fighting IAV infection, evidenced by attenuation of IAV-induced acute lung injury, down-regulation of viral load, and balancing of cytokine production. We further show that RJJDD maintains the homeostasis of gut microbiota, particularly those producing SCFAs which are known to limit lung inflammation through the gut-lung axis, providing a potential mechanism for RJJDD's efficacy on influenza.

The main pathogenic mechanism of acute lung injury is cytokine storm caused by the excessive inflammatory response [38]. We found that RJJDD can not only down-regulate the expression of pro-inflammatory factors such as IL-6, TNF-α, and IL-1β, but also up-regulate the expression of anti-inflammatory factor TGF-β and IL-10 [39]. During acute influenza infection, blocking IL-10 signaling can lead to fatal lung inflammation, increase mortality and accelerate death [40]. Therefore, RJJDD can play a dual role in regulating cytokine production to restrict inflammation during IAV infection.

COVID-19 produces similar cytokine storms [41], studies have demonstrated that patients with severe COVID-19 exhibit higher levels of IL-6, IL-10, IP-10, MCP-1, and TNF-α than those with mild and moderate infections [42]. In our study, RJJDD reduced the levels of these inflammatory cytokines or chemokines in the lungs and serum of the PR8-infected mice. This suggests that RJJDD may be used to treat COVID-19.

Emerging evidence has demonstrated a cross-talk between the gut microbiota and the immune homeostasis of the lung, termed the "gut-lung axis" [14]. Interstingly, alterations in gut microbiota, such as their components and metabolites, have been linked to the fluctuation of the immunological state of the lung. As the fundamental class of metabolites produced by gut resident microbes, SCFAs serve as essential mediators of the gut-lung axis that regulate immune responses not only locally in the gut but also remotely in the lung [15]. Particularly, the critical role of SCFAs in shaping immune response to resolve lung inflammation in the context of chronic obstructive pulmonary diseases, asthma, lung cystic fibrosis, and tuberculosis has been well characterized [43]. Therefore, modulating SCFAs or SCFAs-producing gut microbiota holds promise for treating lung diseases.

Our present study shows that PR8 infection dramatically reduced the SCFAs-producing bacterial genera in the gut microbiota, *Bifidobacteriaceae*, *Lachnoclostridium*, and *Lachnospiraceae_UCG-006*, but intragastric administration of RJJDD significantly restored the levels of these genera. Several studies had shown that increasing SCFAs can help reduce lung damage. Wu and colleagues demonstrated that SCFAs can protect the lungs by modulating macrophage responses during *Klebsiella pneumoniae* infection. Moreover, Baicalin can protect against inflammation induced by avian pathogenic *Escherichia coli* (APEC) by boosting SCFA production in the gut [44,45]. Additionally, through their anti-inflammatory properties, SCFAs such as butyrate can inhibit NF-κB signalling, increase levels of anti-inflammatory cytokines such as IL-10, and suppress pro-inflammatory cytokines like IL-12 and IFN-γ produced by dendritic cells [46]. Given the well-defined anti-inflammatory function of SCFAs in attenuating lung inflammation, maintaining the SCFAs-producing components of the gut microbiota could be, at least in part, the mechanism underlying the protective effect of RJJDD on IAV-induced lung injury.

Some studies have shown that the primary constituents of RJJDD absorbed into the blood could alter the composition and functions of the gut microbiota. For instance, it is reported that the gut microbiota was significantly altered, species richness was increased and predicted microbial function was altered in response to limonin treatment [47]. Various studies have suggested that genistein can decrease inflammation, modify the gut microbiota, and enhance epithelial barrier function in numerous animal models of intestinal disease [48–50]. Given the critical role of the gut-lung axis, modulating intestinal microbiota and their metabolites by various TCM formulas would represent a promising strategy for treating lung diseases.

Gut microbiota at least those probiotic microbes are an important part of the host. Therapies acting via gut microbiota rather than directly targeting viruses themselves represent a type of host-directed therapies (HDTs) [51] that can bypass the challenge of viral mutation-induced drug resistance faced by antivirals and thereby serve as an alternative strategy to antivirals. More importantly, this feature of HDTs has a great advantage in dealing with emerging infectious diseases caused by previously unknown viruses, such as SARS-CoV2 and new subtypes of influenza viruses.

5. Conclusion

In this study, the mouse influenza model was used to prove that RJJDD has a protective effect against IAV infection in mice, can reduce acute lung injury induced by IAV, and down-regulate the expression of inflammatory factors. Comprehensive network pharmacology analyses of RJJDD suggest that RJJDD may act through down-regulating key signaling pathways producing inflammatory cytokines, which was experimentally confirmed by cytokine expression analysis in IAV-infected mouse lung tissues and IAV single

strand RNA mimic R837-induced macrophages cells. We further found that RJJDD maintains the homeostasis of gut microbiota, especially those that produce SCFAs which have been shown to limit pulmonary inflammation through the gut-lung axis, providing a potential mechanism for the efficacy of RJJDD in the treatment of influenza.

Ethics approval and consent to participate

All the animal experiments were in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals with the approval of Shanghai Public Health Clinical Center Laboratory Animal Welfare & Ethics Committee, and ethics committee with the number of 2019-4007-01 (Shanghai, China).

Data availability statement

Data included in article/supplementary material/referenced in article.

CRediT authorship contribution statement

Qilin Huang: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Guizhen Yang:** Validation, Supervision, Project administration, Methodology. **Chenchen Tang:** Validation, Software, Funding acquisition. **Biao Dou:** Writing – original draft, Visualization, Software. **You Hu:** Writing – original draft, Validation, Data curation. **Hui Liu:** Validation, Conceptualization. **Xiao Wu:** Visualization, Validation. **Huan Zhang:** Supervision. **Haikun Wang:** Supervision. **Lirong Xu:** Funding acquisition. **Xiao-Dong Yang:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Yanwu Xu:** Writing – review & editing, Validation, Software. **Yuejuan Zheng:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34055.

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