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Pulsatillae radix extract alleviates DSS-induced colitis via modulating gut microbiota and inflammatory signaling pathway in mice

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A R T I C L E I N F O

Keywords:
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DSS
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RNA sequencing
NOD-Like receptor signaling pathway

A B S T R A C T

Ethnopharmacological relevance: Ulcerative colitis (UC) is a chronic relapsing intestinal disease with complex pathogenesis. The increasing morbidity and mortality of UC become a global public health threat. Baitouweng decoction (BD), a formulated prescription of Traditional Chinese Medicine, has been applied to cure UC for many centuries. However, the therapeutic efficacy and working mechanisms of this medicine are not well studied.

Aim of study: In this study we determined whether *Pulsatillae radix*, one of four ingredients in BD, had a therapeutic effect on colitis. And explore the underlying mechanism of *Pulsatilla chinensis* (Bunge) Regel radix in the improvement of DSS-induced colitis in mice model.

Methods: The active compounds of *Pulsatilla chinensis* was identified by UPLC. The composition of the mice's cecum microbiota was determined by 16S rRNA sequencing. And gene expression profile of colon was detected by transcriptome.

Results: The results showed that *Pulsatillae radix* significantly improved the clinical symptom, prevented the shorten of colon length, and decreased the diseased activity index (DAI) in an 3 % DSS-induced ulcerative colitis mouse model. We found that *Pulsatillae radix* reversed the dysbiosis of gut microbiota as evidenced by increase in the relative abundance of Bacteroidetes, Deferribacteres, and Proteobacteria phyla and decrease in Firmicutes, as well as by decrease in the genera levels of *Bacteroides*, *Parabacteroides*, *Prevotella*, *Mucispirillum*, *Coprococcus*, *Oscillospira*, and *Escherichia*. The results of transcriptome showed *Pulsatillae radix* administration led to 128 genes up-regulation, and 122 genes down-regulation, up-regulate NOD-like receptor signaling

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pathway, down-regulate Cytokine-cytokine receptor interaction, and TNF and IL-17 signaling pathways.
Conclusion: in this study, we demonstrate *Pulsatillae radix* alleviates DSS-induced colitis probably via modulating gut microbiota and inflammatory signaling pathway in DSS-induced colitis mouse model.

1. Introduction

Ulcerative colitis (UC) is a persistently recurring inflammatory disorder targeting the colon and rectum. Common symptoms encompass abdominal discomfort, frequent diarrhea, blood in stools, and mucus-filled feces. Globally, UC cases are on the rise, with roughly 20 cases per 100,000 individuals observed in advanced nations. [1], and 1.5-fold to almost 20-fold increase has been seen in the Asian countries in the last few decades [2]. The growing UC prevalence has caused a global public health concern and economy burden. It was estimated that annual costs were approximately 20 billion Euros in Europe and 10 billion dollars in the United States [1]. The origins of UC are intricate, stemming from a combination of factors such as genetic tendencies, imbalances in immune reactions, disruptions in the epithelial barrier, and disturbances in the gut microbiome [3–5]. Mesalamine is mainstay of treatment for UC patients, however, long-term administration and variability among ulcerative colitis have led to poor outcomes [6]. Traditional Chinese Medicine (TCM), characterized by complex compounds with multi targets of UC, has been suggested to be a potential alternative therapeutic option.

Baitouweng tang decoction, a Chinese traditional medicine prescription invented in the Han dynasty, has been used to treat UC for centuries [7]. Baitouweng tang decoction is composed of four traditional Chinese herbal ingredients: *Pulsatilla chinensis* (Bunge) Regel (baitouweng) radix, *Phellodendron chinense* C.K.Schneid (huangbai) cortex, *Coptis chinensis* Franch. (huanglian) rhizoma, and *Fraxinus chinensis* Roxb. (qinpi) cortex (The plant name has been checked with http://www.theplantlist.org). Recently, three studies demonstrated that baitouweng decoction alleviated the ulcerative colitis induced by the dextran sulfate sodium (DSS) by various mechanism, such as regulating intestinal microbiota and the IL-6/STAT3 signaling pathway [8], modulating gut microbiota and bile acids with FXR and TGR5 signaling pathways [9], and improving Th17/Treg balance and intestinal epithelial barrier [10]. However, as far as we know, there is no data available regarding the protective effect of Pulsatilla radix on colitis.

There was also a study that reported that Triterpenoid saponins, a ingredient of *Pulsatilla chinensis*, ameliorated the ulcerative colitis of the rat by restoring the gut microbiota, which, however, did not provided an reasonable explanation for some important biological activitis of *Pulsatilla chinensis* except modulating intestinal flora, e.g., regulating excessive inflammation reponse [11,12]. So the pharmacological mechanism of *Pulsatilla radix* remained less clear and needed further research.

Thus, we conducted the current study in which we tested effect of *Pulsatilla chinensis* (Bunge) Regel radix (Pul) extract on DSS-induced colitis and related inflammatory signaling pathways in the model mice. Since UC was reported to be associated with dysbiosis of microbiota [13], and drugs digested interact with commensal bacteria in cecum, we analyzed the composition of microbiota in cecum content. To clarify the underlying mechanism, we also analyzed colon transcriptome. Our data showed Pul administration reversed the dysbiosis of gut microbiota and regulated the inflammatory signaling pathway in DSS. Our work indicates *Pulsatilla chinensis* radix can be considered as an alternative therapeutic option.

2. Materials and methods

2.1. Ethics statement

All protocols of this study were assessed and granted by the Ethic Review Committee of the National Institute for Communicable Disease Control and Prevention at the Chinese Center for Disease Control and Prevention (No.2017002, Beijing, China).

2.2. Plant material

Pulsatilla chinensis (Fig. S1) used in this study grew in the TCM Herb Planting Base in Longhua county, Chengde city, Hebei province. There it is a mountain area with temperate monsoon climate in northern China. The extract of *Pulsatilla chinensis radix* was produced by Beijing Kangrentang Pharmaceutical Company (http://www.tcmages.com/) according to good manufacturing practice.

2.3. Identification of active compounds of Pulsatilla chinensis radix

Pulsatilla chinensis radix was purchased from Beijing Kangrentang Pharmaceutical Co., Ltd. The plant as raw material grown in Longhua County, Chengde City, Hebei Province (117.72°E 41.32°N) A total of 150 mg Pul was grinded in liquid nitrogen. The supernatant after pyrolysate and centrifugation was separated on ACQUITY UPLC platform Waters HSS T3 column by Biomsomics Biotechnology Co., Ltd. The liquid phase consisted of A and B phase, operation conditions were the followings: column temperature 40 °C, sample temperature 4 °C, and liquid flow rate 2 μL/min. The separated substances were detected on mass spectrometer (TripleTOF 5600). Negative and positive ion acquisition modes were conducted, ION source parameters were set as the followings: The gas sheath velocity at 20, gas 1 velocity at 20, gas 2 velocity at 0, temperature at 150 °C, and the spray voltage at 2300. The scanning time

Table 1
The criteria of DAI.

Score	Body weight	Stool consistency	Bleeding
0	No weight loss	Normal	No bleeding
1	1–5% weight loss		
2	5–10 % weight loss	Faecal pellets wet and	Faecal occult blood
3	10–20 % weight loss	loose consistency	
4	>20 % weight loss	Diarrhea	Evident bleeding

was 25 min. Twelve second-order scanning was followed by each first-order scanning [14,15].

2.4. DSS-induced colitis mouse model and intervention

Thirty female C57/BL mice, 7 weeks old and weighing (16–18)g, were acquired from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). They were kept at the Animal Center of China CDC, with unrestricted access to water and food, following a 12-h light/dark routine. After a 7-day acclimation phase, the mice were randomly segregated into three distinct groups (n = 10 per group): control, PBS, and Pul. The PBS and Pul groups were provided with drinking water infused with 3 % DSS for a straight 7 days, while the control group received sterile water. The Pul group mice received a daily oral dose of 300 μL PBS with 0.5 g of Pul extract, whereas both the control and PBS groups were administered 300 μL of plain PBS. Daily observations included body weight measurements, testing for fecal occult blood, and monitoring stool consistency. The disease activity index (DAI) was graded by using these three indicators and the following criteria (Table 1) [16].

2.5. Histological analysis of the colon

On day 8 after DSS treatment, mice were euthanized and the colons were removed, photographed, and colon length were recorded. A segment of 1 cm colon was treated with 10 % formalin solution for 24 h and then embedded using paraffin.4 mm-sliced sections were stained with Hematoxylin and Eosin. Histologic score was blindly examined by two experimental pathologists according to previously described criteria: normal (0 score), moderate inflammatory response without erosion and ulcer (1 score), severe inflammatory response with erosion (2 score), severe inflammatory response inflammatory response with <1 mm ulcer (3 score), severe mucosal inflammation with 1–3 mm ulcer (4 score), and severe mucosal inflammation with >3 mm (5 score) [17].

2.6. 16S rRNA sequencing and data analysis

DNA from the cecum content of 18 mice was individually isolated using the QIAamp DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany) following the provided instructions. The integrity of the extracted DNA was verified using the Qubit dsDNA HS assay kit and 1 % agarose electrophoresis. Suitable DNA samples were then amplified, focusing on the V3 and V4 regions of 16S rRNA with universal primers. The amplified sequences were then purified using Agencourt AMPure XP magnetic beads. An appropriate DNA library was generated and sequenced on the Illumina Hiseq 2500 system by the Beijing Genomics Institute Co., Ltd. (Beijing, China). After removing low-quality sequences, clean sequences were assembled using FLASH [18]. Qualified sequences were grouped into operational taxonomic units (OTUs) clustered by USEARCH (v7.0.1090) with a threshold of 97 % [19]. α-diversity including Chao, Shannon, and Simpson index was analyzed by mothur software based on OTU abundance table [20]. The principal components analysis (PCA) was performed using the unweighted unifrac analysis (FastTree version 2.1.3, http://www.microbesonline.org/fasttree/). We performed the Linear discriminant analysis effect size (LEfSe) analysis online via the platform (http://huttenhower.sph.harvard.edu/galaxy/), utilizing the nonparametric Kruskal–Wallis and Wilcoxon rank-sum tests, setting a threshold at 2.0. All primary data was uploaded to the NCBI SRA database under the accession number PRJNA765473.

2.7. Transcriptome analysis of colon tissue

Total RNAs from colon samples were isolated using Trizol, adhering to the manufacturer's guidelines. The integrity of the RNA was first verified using the Qubit RNA HS assay kit. Suitable RNA samples were then utilized to construct a DNA Nano Ball (DNB) library. This DNB library, once deemed appropriate, was sequenced on the BGISEQ-500 system by Beijing Genomics Institute Co., Ltd. Subsequent to sequencing, raw data underwent a cleanup process to remove adapters and substandard reads. The refined reads were then aligned to the reference genome employing the HISAT software to ensure alignment quality [21]. The matched clean data were normalized to FPKM by RSEM software with threshold at $|\log2FC| \geq 1$, $P \leq 0.01$ [22]. The KEGG pathway analysis and GSEA for the differential genes were carried out on the Dr. Tom platform from Beijing Genomics Institute Co., Ltd. Differential gene heatmaps were crafted using the R software tool. For original datasets, refer to the NCBI SRA database with the accession number PRJNA765487.

2.8. Validation of quantitative real-time PCR

Quantitative real-time PCR (RTq-PCR) was employed to measure the gene expression levels of Il6, Cxcl2, Ccl2, S100a8, Gsdmc2,

Table 2
Primer sequences.

Genes	Forward primer (5'→3')	Reverse primer (5'→3')
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Il6	ACAAGTCGGAGGCTTAATTACACAT	TTGCCATTGCACAACTCTTTTC
Cxcl2	CCAACCACCAGGCTACAGG	GCGTCACACTCAAGCTCTG
Ccl2	AGGTCCCTATGGTGCCAATGT	CGGCAGGATTTTGAGGTCCA
S100a8	AAATCACCATGCCCTCTACAAG	CCCACTTTTATCACCATCGCAA
Gsdmc	ATGTCCTACACATTTGACTGGC	TTAAACAGGCAGAATTTGGTTGC
Muc4	CCTCCTCTTGCTACCTGATGC	GGAACTTGGAGTATCCCTTGTTG
Lcn2	ATGTATGGCCGGTACACTCAG	AACAAATGCGACATCTGGCAC
Saa3	TGCCATCATTCTTTGCATCTTGA	CCGTGAACTTCTGAACAGCCT

Table 3
Chemical compositions of *Pulsatillae radix*.

Name	MW	Adduct	Intensity	*m/z*	RT(min)
Genistein	270.05283	+H	557845	271.0601	12.6
Isoferulic acid	194.05791	+H	166472	195.06518	10.29
L(+)-Arginine	174.11168	+H	137660	175.11896	0.68
Adenosine	267.09674	+H	114994	268.10402	4.87
Adenine	135.05449	+H	99843	136.06177	2.05
Hypericin	504.0845	-H	69635	503.07723	11.18
Daidzein	254.05791	+H	46579	255.06518	13.54
L-Tryptophan	204.08987	+H	43911	205.09715	8.48
Caffeic acid	180.04225	+H	40884	181.04953	9.31
Vitamin B2	376.13828	+H	37341	377.14555	9.44
Chlorogenic acid	354.09509	+H	31779	355.10237	9.07
Phenprobamate	165.07898	+H	30777	166.08626	3.48
Proline	115.06333	+H	28468	116.07061	0.7
Esculin hydrate	340.07944	-H	11731	339.07216	1.25
Betaine	117.07898	+H	7527	118.08626	0.77
Leucine	131.09464	+H	7416	132.10191	2.35
Aucubin + HCOOH	392.13187	-H	6701	391.12459	10.2
Atractyloside	802.13452	-H	6133	801.12725	10.82
Schizandriside	492.19955	-H	5954	491.19228	10.64
Coumestrol	268.03717	-H	5769	267.02989	9.01
Ginkgolide B + HCOOH	470.14243	-H	5503	469.13515	6.11
Coptisine	320.09229	-H	5254	319.08501	9.89

Muc2, Lcn2, and Saa3. Using Trizol, total RNA was extracted from colon tissue and subsequently converted to cDNA. The PCR reaction mix, totaling 20 μL, contained 300 nM of the primer, 2 μL cDNA, 0.4 μL ROX Reference Dye II, and 10 μL of TB Green Premix Ex TaqTM II. This reaction was executed on the ABI 7500 Real-Time PCR System. The specific primers utilized for the RT-qPCR are listed in Table 2. Amplification conditions were set at 95 °C for 30s, followed by 40 cycles of 95 °C for 3s and 60 °C for 30s. A dissolution curve was generated with each cycle set at 95 °C for 15s and 60 °C for 1 min. The relative expression of mRNA was calculated using comparative $2^{-\Delta\Delta Ct}$ method, β-actin was also amplified as an internal control.

2.9. Statistical analysis

Continuous variables from various mouse groups (including body weight, DAI index, histological scores, colon length, α diversity, and gene differential expressions) were compared using one-way analysis of variance (ANOVA). For PCoA, a nonparametric MANOVA based on Adonis was applied. A P-value below 0.05 was deemed statistically significant. All statistical evaluations were conducted with GraphPad Prism (version 5.0).

3. Results

3.1. Major chemical compounds of Pulsatillae radix

There were 22 chemical compounds detected by UPLC-MSass (Table 3). The top 5 most abundant components were Genistein, Isoferulic acid, L(+)-Arginine, Adenosine, and Adenine in positive mode, and Hypericin had the highest intensity in negative mode.

3.2. Pulsatillae radix extract alleviated clinical symptoms of mice with colitis

Body weight of mice treated with DSS began to decline on the day 4 post treatment and reached to lowest weight on day 7 compared

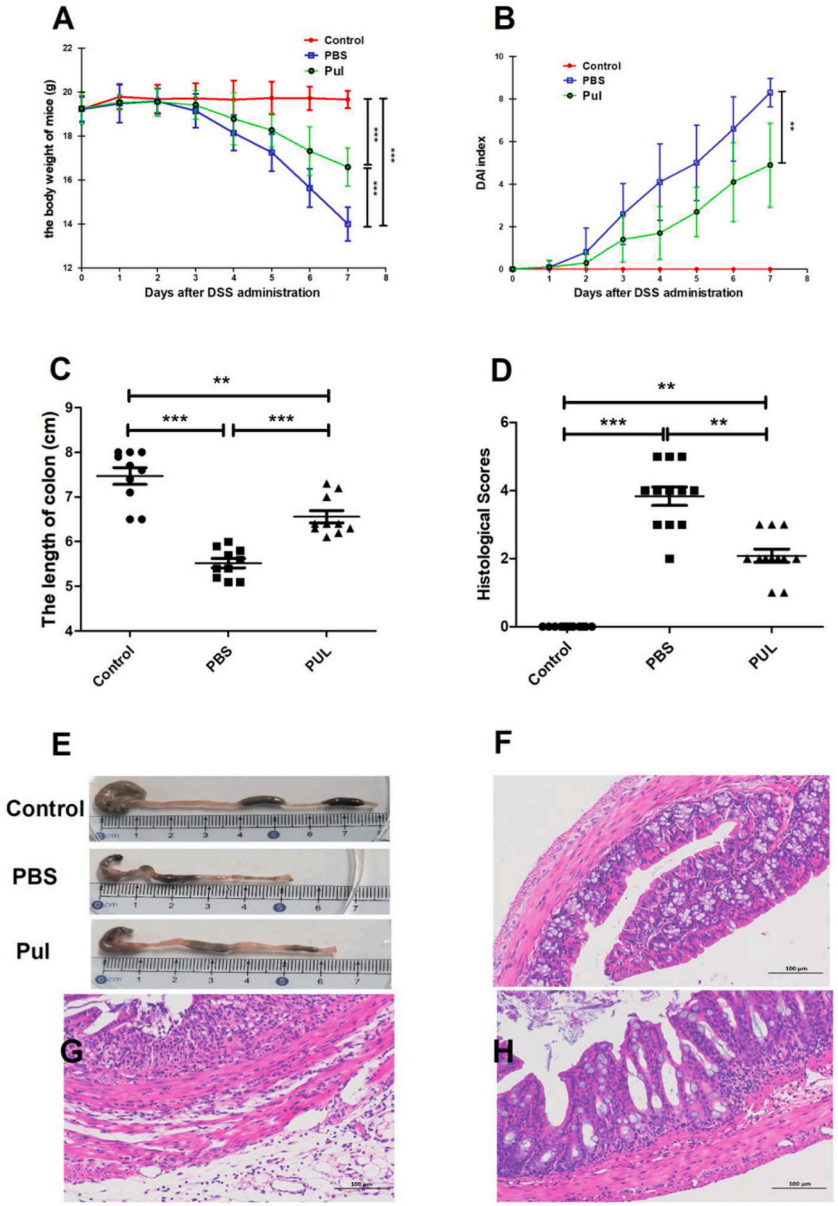

Fig. 1. *Pulsatillae radix* extract alleviated the clinical symptom of mice with colitis induced by DSS. A total of 30 mice were randomized into 3 groups (n = 10 per group): control, PBS, and Pul group. Both PBS and Pul group were given drinking water containing 3 % DSS for consecutive 7 days. A total of 300 μL PBS containing 0.5 g *Pulsatillae radix* extract was orally administrated once daily to mice in Pul group, and 300 μL PBS was administrated to mice in control and PBS groups. The bodyweight of mice (A), DAI index (B) was detected daily for consecutive 7 days. At the seventh days, collected colons were pictured (E), and their length was measured (C). The colon tissue was fixed with 10 % formalin. HE sections were examined and the images (200 ×) acquired under a light microscope (F, G, H). The histological scores were given by an experienced pathologist (D). The data were analyzed by ANOVA. Values at $P < 0.05$ considered significantly different. $^{**}P < 0.01$, $^{***}P < 0.001$.

with the control mice which had kept stable weight of 19.66 ± 0.39 g. Body weight of mice in *Pulsatillae radix* group (16.59 ± 0.87 g) was significantly higher than that in PBS group (14.00 ± 0.77 g) on day 7 although not reaching the level in control mice (Fig. 1A). DAI, which was based on weight loss, stool consistency, and bleeding, began to rise at day 3 post treatment. At the end of experiment, there were significantly differences among three groups. *Pulsatillae radix* administration significantly lowered the DAI (Fig. 1B). *Pulsatillae radix* prevented the shortening of colon caused by DSS administration, and alleviated inflammation (Fig. 1C and E). HE staining showed heavily damaged mucosa, almost disappeared goblet cells, edema in muscularis, and inflammatory cell infiltration across mucosa, submucosa, and muscularis (Fig. 1G). *Pulsatillae radix* treatment alleviated the inflammatory cell infiltration (confined to mucosa), improved the edema, and increased the number of glands and goblets (Figure H), but not to the extent comprable to the control group (Fig. 1F). Histological scores were also significantly lower in PBS group compared with Pul group (Fig. 1D).

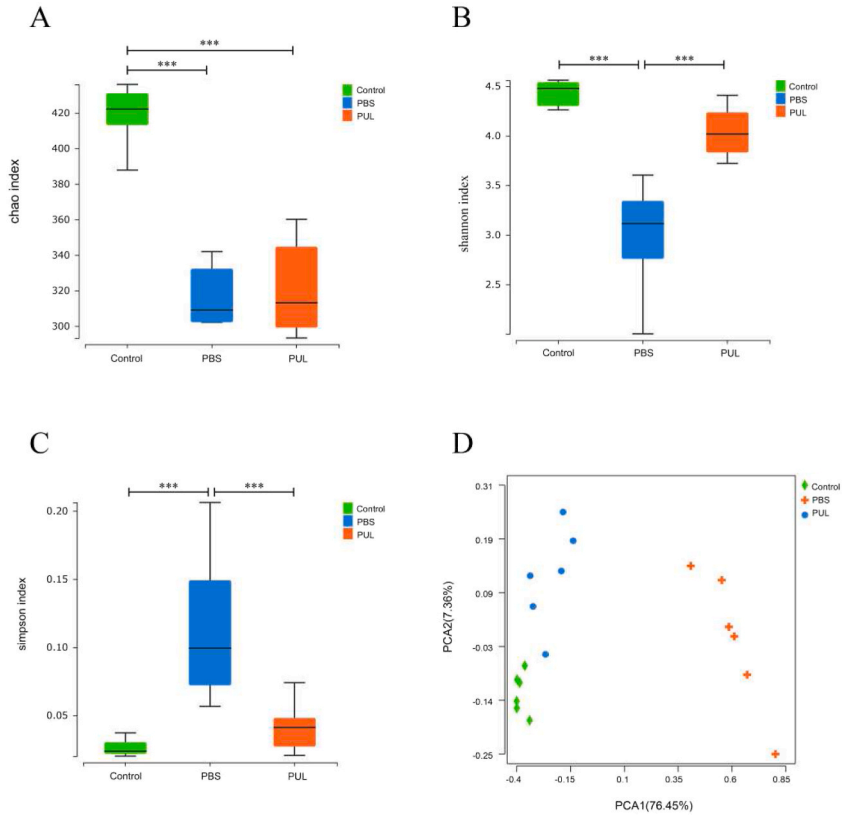

Fig. 2. *Pulsatillae radix* extract improved diversity of gut microbiota. Cecal content of mice was sequenced on the Illumina Hiseq 2500 platform. Chao (A), Shannon (B), simpson index (C), which represent the α-adversity, are shown. The PCA based on the OTU abundances is shown in D. The data of Chao (A), Shannon (B), Simpson index were analyzed by ANOVA, the PCA was analyzed using the unweighted unifrac analysis. Values at $P < 0.05$ are considered significantly different. ***$P < 0.001$.

3.3. *Pulsatillae radix extract improved gut microbiota of cecum content*

16 S rRNA sequencing showed that *Pulsatillae radix* did not restore DSS-induced reduction of chao index caused by DSS, one of indicators of α diversity (Fig. 2A). However, Pulsatillae radix restored Shannon, and Simpson index, two other indicators of α diversity, to the normal levels (Fig. 2B and C). PCA, which represents β diversity, showed three independent, separated groups, however there was no significant difference in PCA1 axis (accounting for 76.45 % of PCA) between control and PBS group (Fig. 2D). The clustering stack diagram at phylum level also indicated two independent, separated groups which were divided; PBS group was an independent group, and control and pul group can't be separated (Fig. 3B). LEfSe results showed that *Pulsatillae radix* improved the levels of four major phyla, i.e., Bacteroidetes, Deferribacteres, Firmicutes, and Proteobacteria (Fig. 3A, C), decreased the levels of *Bacteroides*, *Parabacteroides*, *Prevotella*, *Mucispirillum*, *Coprococcus*, *Oscillospira*, and *Escherichia* at the genus, and also reduced the relative abundance of species of *Parabacteroides_distasonis*, *Mucispirillum_schaedleri*, *Bacteroides_ovatus*, and *Escherichia_coli*.

3.4. *The effect of Pulsatillae radix extract on the transcriptome of colon*

To investigate the underlying mechanism for protective effect of *Pulsatillae radix* on DSS-induced colitis, we analyzed the colon transcriptome. The results showed that 128 genes were up-regulated, and 122 genes were down-regulated by *Pulsatillae radix* (Fig. 4A). The differentially regulated genes were mainly those related to signal transduction and immune system at the level2 in Kegg_pathway (Fig. 4B), such as NOD-like receptor signaling pathway, TNF signaling pathway, cytokine-cytokine receptor interaction, IL-17 signaling pathway, and chemokine signaling pathway (Fig. 4C).

To more clearly show the differential genes, the heat-map was generated (Fig. 5A) and it demonstrated the down-regulated differential family genes which were increased in DSS-induced colitis mice, such as *Acot*, *Ccl*, *Clec*, *IL*, *Serpina*, and *Slc* gene family. The up-regulated gene families were shown in Fig. 5B, such as *Apol*, *Gbp*, *Gsdmc*, *Ifit*, *Oas*, *Slfn*, and *Trim* gene family. The highly expressed differential genes were listed in Fig. 5C, including *Isg15*, *Muc4*, *Ace2*, *Lcn2*. To verify the reliability of RNA sequencing, RTq-PCR was performed. The data indicated that the relative gene expressions of *IL-6*, *CXCL-2*, *CCL-2*, *S100a8*, *Lcn2*, and *Saa3* were significantly upregulated in the colon of PBS-treated colitis model group compared with control group, and *Pulsatillae radix* administration restored the higher expression of genes to the levels similar to the control group (Fig. 6A, B, C, D, G, H). DSS-induced-downregulation of genes,

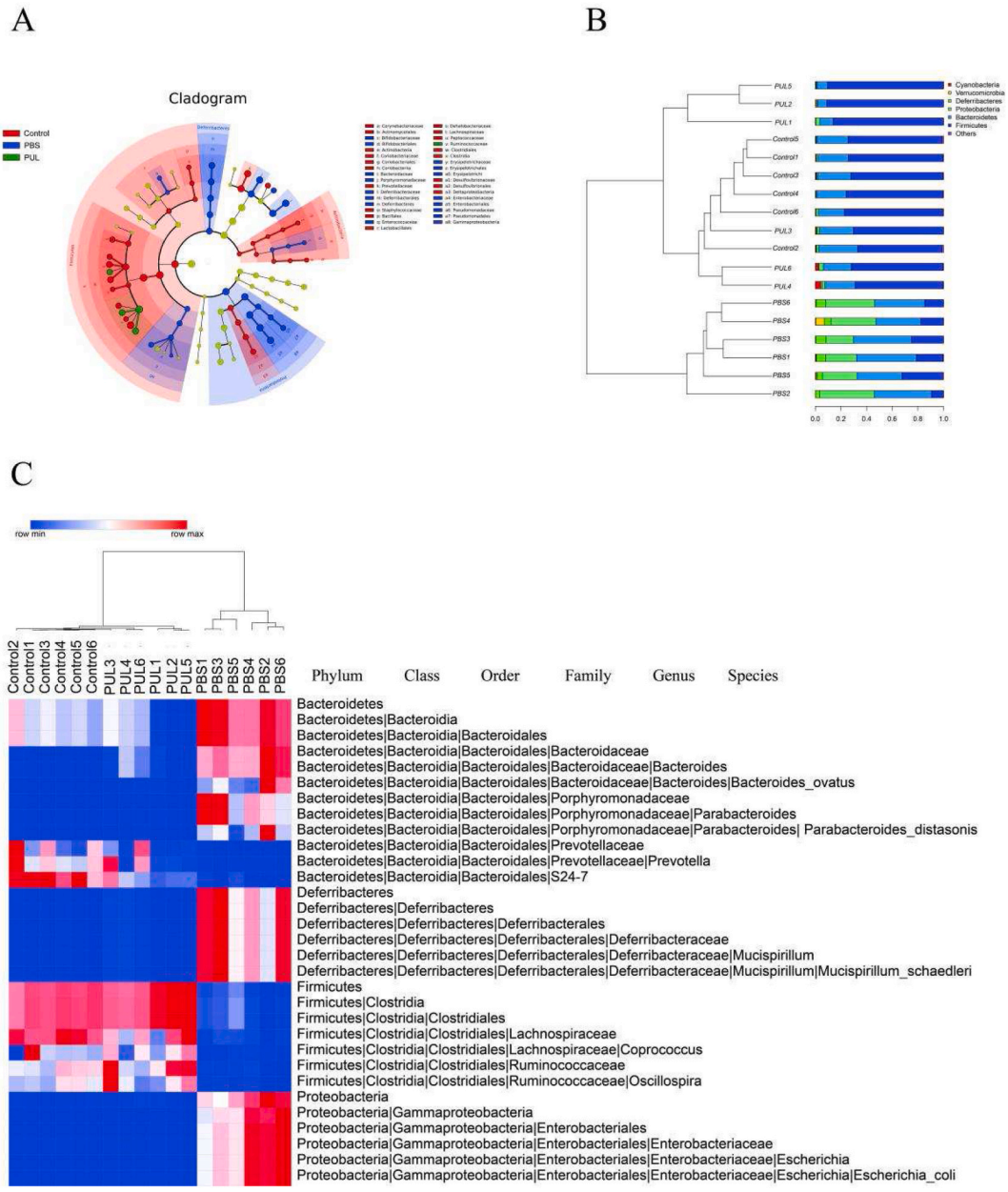

Fig. 3. *Pulsatillae radix* extract restored the relative abundance of some OTUs. LEfSe, the clustering stack diagram of cecum content at the phylum level, and clustering heatmap of differential OTUs are presented in A, B, and C, respectively.

such as *Gsdmc2* and *Muc2*, was also improved by *Pulsatillae radix* (Fig. 6 E, F). The results of up- and down-regulated genes verified by RTq-PCR were consistent with the results of RNA sequence.

To verify the signaling pathways regulated by *Pulsatillae radix* was up- or down-regulated, GSEA analysis was conducted. The data showed that NOD-like receptor signaling pathway was up-regulated including 46 up-regulated genes (Fig. 7A–D, Fig. S2). IL-17 signaling pathway, TNF signaling pathway, and Cytokine-cytokine receptor interaction were down-regulated, including 21, 50, 19 genes, respectively. We also showed 46 up-regulated genes of NOD-like receptor signaling pathway. The heat map showed that the expression of genes, such as *nlrp1b*, *nlrp6*, *nlrp12*, and *NOD2*, were higher in *Pulsatillae radix* group than in PBS group (Fig. S2).

3.5. Spearman's correlation of clinical symptoms, OTUs, and differential genes

To determince the correlation between clinical symptoms, differential OTUs, and differential genes, Spearman's correlation analysis was conducted based on the spearman's rank correlation coefficient. The results showed that disease activity index (DAI)

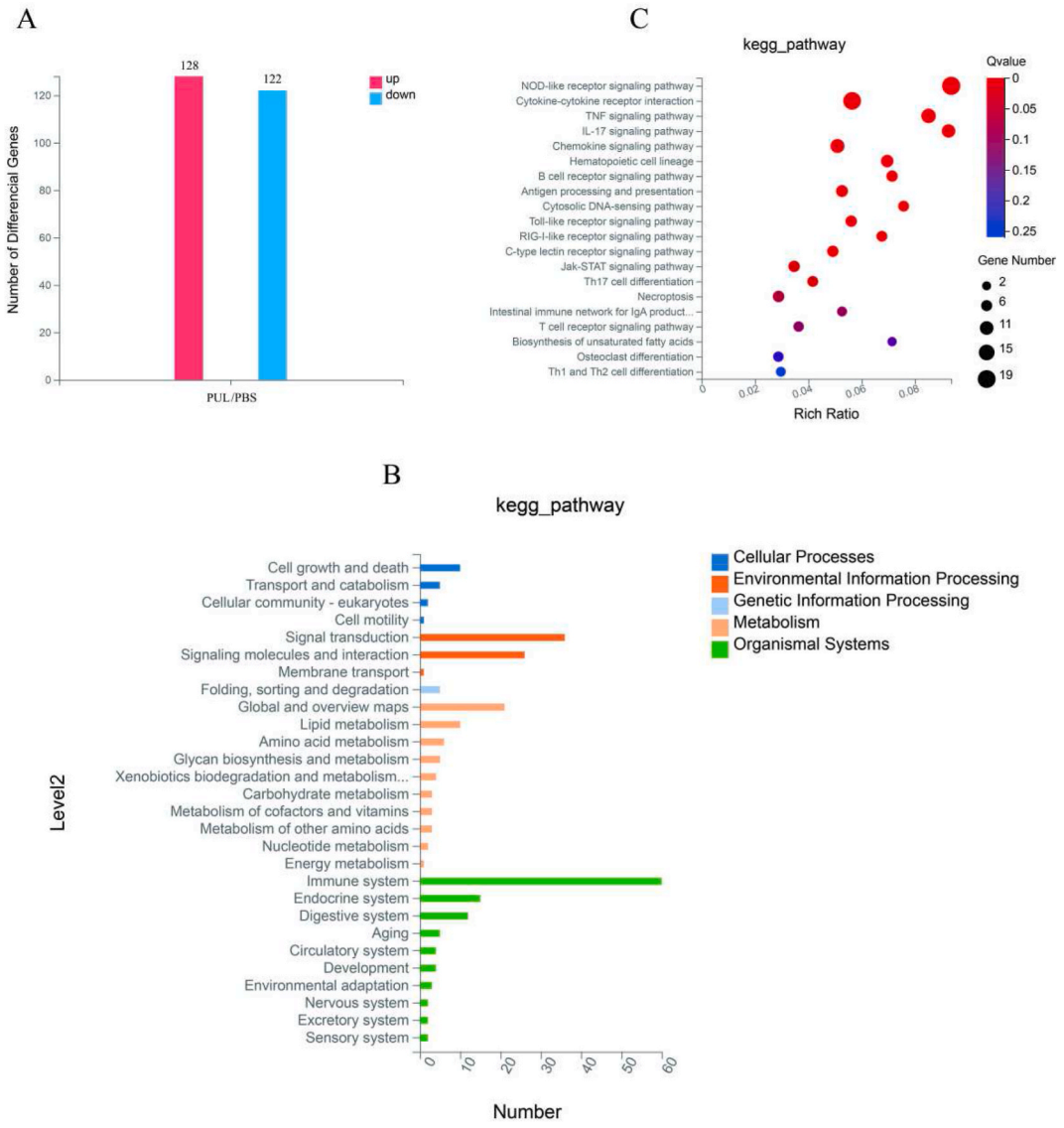

Fig. 4. Effect of *Pulsatillae radix* extract on the colon transcriptome. A: the number of up and down-regulated genes. B: differential genes distribution at the level2 in Kegg_pathway. C: differential signaling pathway between the PBS and Pul groups.

was positively correlated with the relative abundance of phylum of Bacteroidetes, Deferribacteres, and Proteobacteria, and negatively correlated with Firmicutes phylum. At the level of species, *Parabacteroides_distasonis*, *Mucispirillum_schaedleri*, *Bacteroides_ovatus*, and *Escherichia_coli* were significantly positively correlated with DAI. DAI was positively correlated with inflammatory factors and chemokines, such as *IL6*, *CCL2*, *CXCL2*, and negatively correlated with *Muc4*, *Gsdmc*, which is in favor of the intestinal homeostasis (Fig. 8).

4. Discussion

Pulsatillae radix is a Chinese traditional medicine, and it is also one of ingredients of famous Baitouweng tang decoction, a formulated prescription which has been used to treat UC for centuries. However, the working mechanism for its therapeutic effect is still unclear. In the current study, we identified the active compounds of *Pulsatillae radix* by UPLC-MS. The top six chemical components were Genistein, Isoferulic acid, L(+)-Arginine, Adenosine, Adenine, and Hypericin. Genistein was reported to alleviate DSS-induced colitis via multiple mechanisms, such as inducing macrophage polarization and systemic cytokine production [23], inhibiting activation of TLR4/NF-κB signaling [24], and repressing Nlrp3 inflammasome via TGR5-cAMP signaling [25]. Dietary Arginine or arginine supplementation attenuated symptom and improved responses to injury and inflammation in DSS-induced colitis [26,27]. Adenosine and its receptor are highly related to the colitis, and adenosine 2B receptor has been targeted for inhibiting colitis [28,29].

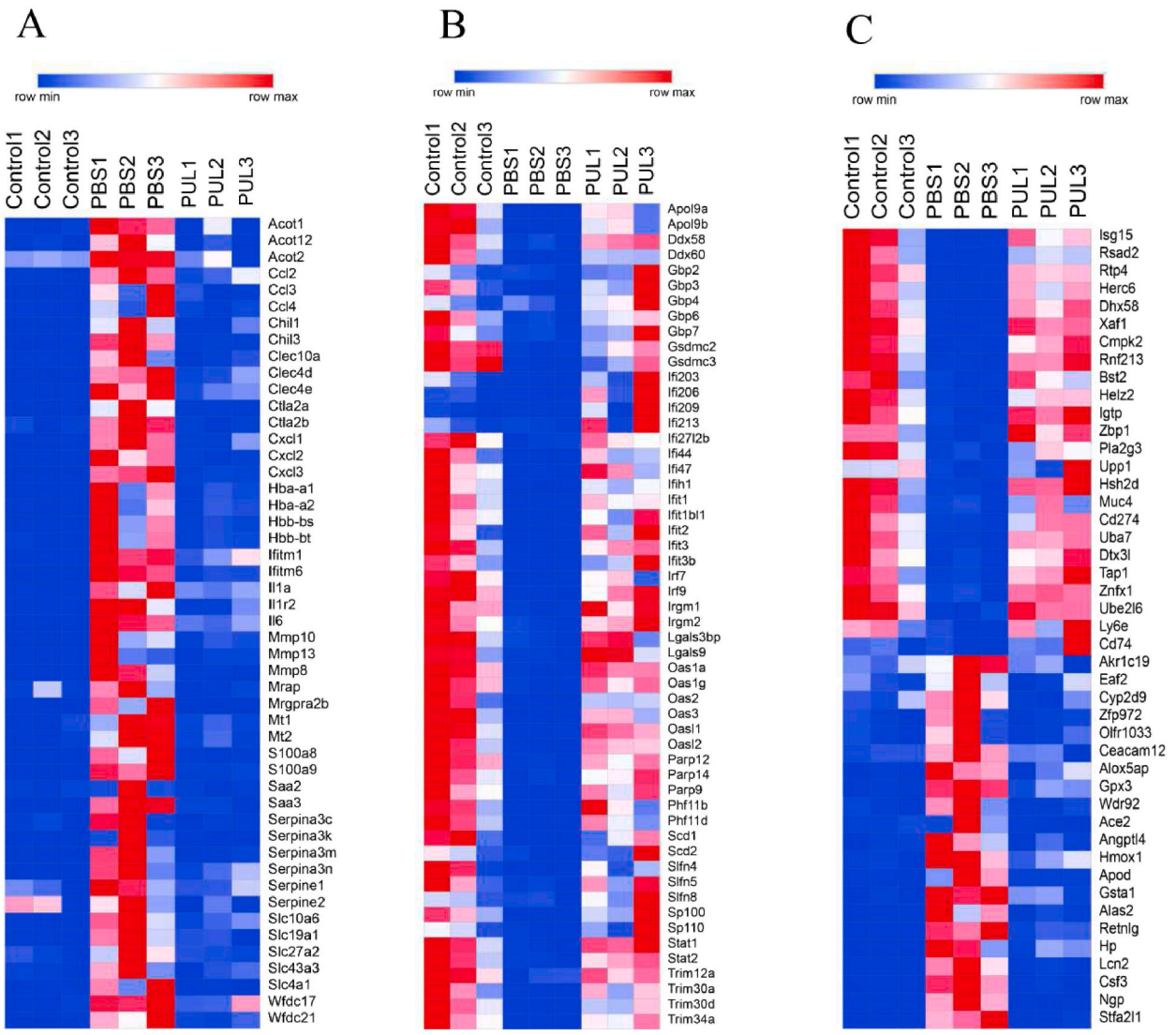

Fig. 5. The heatmaps of differential genes of colon tissue. A and B are the down- and up-regulated differential family genes in Pul group compared to PBS group, respectively. C presents differential genes among three groups.

Adenine alleviated the DSS-induced colitis through inhibition of TNF-α Signaling [30]. These studies suggest that the effect of *Pulsatillae radix* on colitis may be attributed to the concerted action of multi-compounds. Moreover, multi-compounds which targeted multi-targets can effectively avoid drug resistance.

Growing data indicate that intestinal microbiota plays key role in the illness course of colitis [31,32]. The relative abundance of Firmicutes was significantly reduced, while Bacteroides and Proteobacteria were markedly increased in DSS-induced colitis mouse models [8,33,34]. In our study, *Parabacteroides_distasonis* and *Bacteroides_ovatus* which belong to Bacteroides phylum were positively related to the colitis and *Pulsatillae radix* administraiton reversed the change in Firmicutes, Bacteroides, and Proteobacteria induced by DSS-colitis. This result was consistent with two studies which reported that *Parabacteroides distasonis* could enhance DSS-induced colitis [35], and *Bacteroides ovatus* were increased during colorectal carcinogenesis [36]. However, there are also reports showing different results. For example, it has been shown that membranous fraction of *Parabacteroides distasonis* attenuates colitis in mouse via immunmodulation and intestinal microbiota [37], and *Bacteroides ovatus* protects immunodeficient SCID mice against chronic intestinal inflammation [38]. These discrepancies may be due to the different strains used in different studies. It has been reported that relative abundance of Proteobacteria phylum is remarkedly increased in colitis and both scutellaria baicalensis georgi polysaccharide and angiogenin can reverse the enhancement of Proteobacteria and attenuate DSS-colitis [33,39]. Our results showed that *Pulsatillae radix* significantly reduced the level of Proteobacteria, mainly *Escherichia_coli*, which was positively correlated with colitis. This indicates that modulation of targeted microbiota may be an effective way to alleviate colitis.

Colitis is a complicated disease involving many inflammatory signaling pathways. Many intervention factors alleviated colitis via

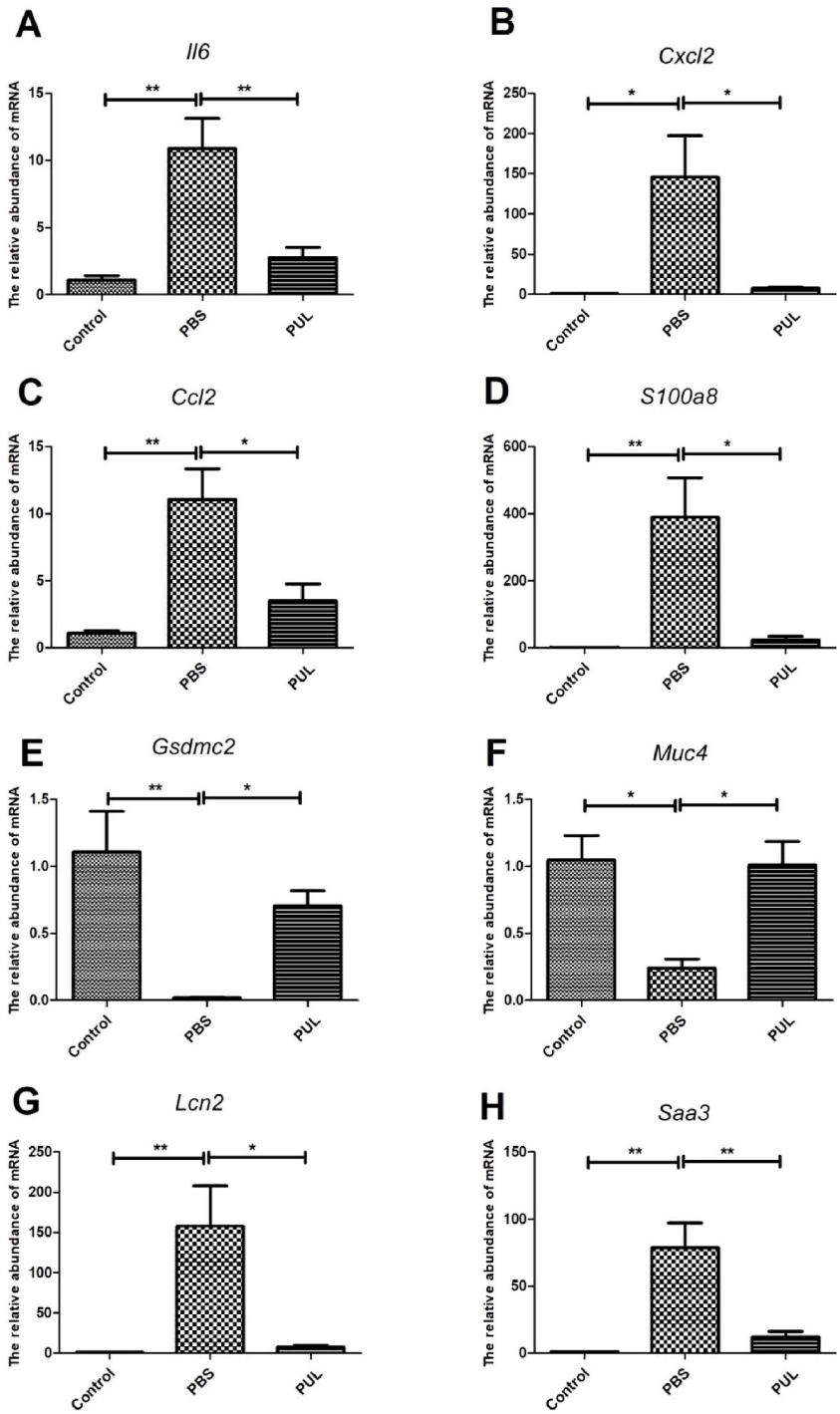

Fig. 6. Verification of differential genes by Rt-qPCR. A, B, C, D, E, F, G, and H represent the relative abundance of *IL-6*, *CXCL-2*, *CCL-2*, *S100a8*, *Gsdmc2*, *Muc4*, *Lcn2*, and *Saa3*, respectively. The data were analyzed by ANOVA. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

modulation of inflammatory pathways. For example, astragalin, luteolin, and *lactobacillus plantarum L15* attenuated acute experimental colitis by inhibiting NF-κB activation [32,40,41]. Qing-Chang suppository modulated IL-17 signal pathway and relieved the colitis in mouse model [42]. NOD-like receptor (NLR) family members are central regulators of host immunity and inflammation, and they play a key role in maintaining gut homeostasis [43–45]. The studies on the relations between NLR family members and inflammatory bowel disease (IBD) had drawn various conclusions. One study reported that Nlrp1 inflammasome activation could attenuate both colitis and colitis-associated tumorigenesis [46], while Tye H et al. reported that Nlrp1 exacerbated IBD via inhibiting

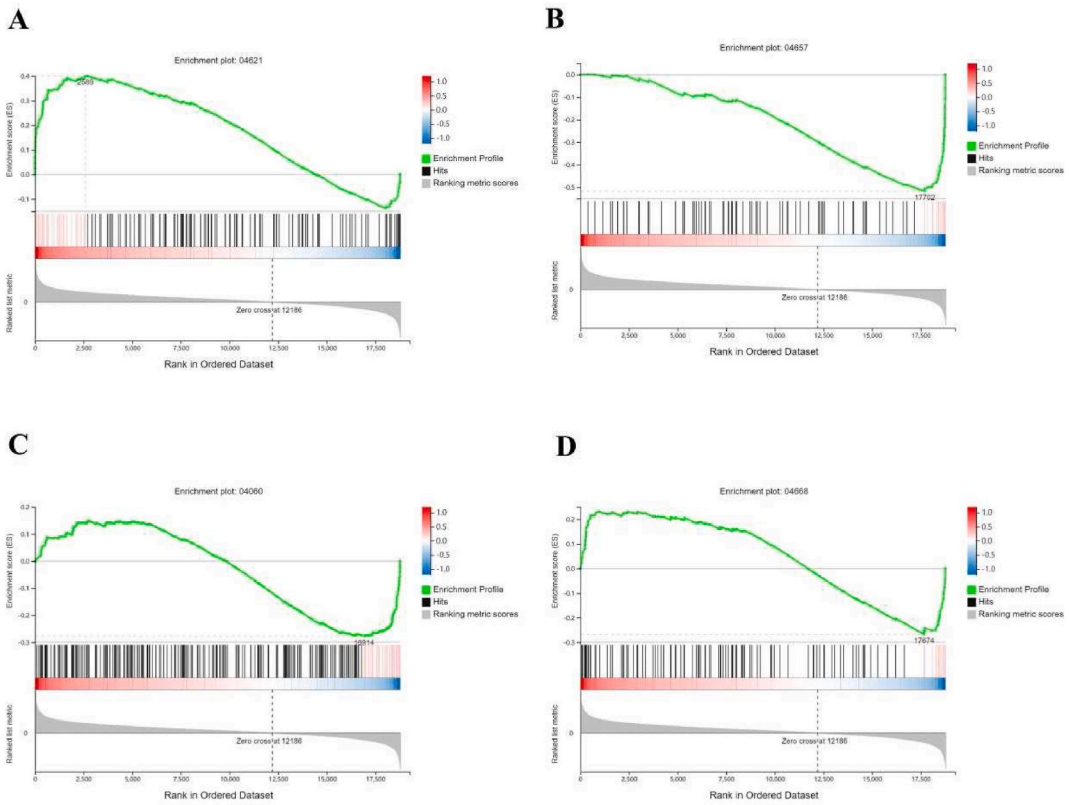

Fig. 7. Signaling pathway analysis by Gene Set Enrichment Analysis (GSEA). A, B, C, and D represent NOD-like receptor signaling pathway, IL-17 signaling pathway, Cytokine-cytokine receptor interaction, and TNF signaling pathway, respectively.

butyrate producing commensals [47]. Curcumin, Kui-Jie-Ling decoction, and L-Fucose alleviated DSS-induced colitis via inhibiting activation of Nlrp3 inflammasome [48–50], while Nlrp3(−/−) mice showed an increase in acute and recurring colitis, which implies that the Nlrp3 inflammasome may function as a negative regulator in colitis [51]. Nlrp3 inflammasome has been shown to be protective effect against epithelial integrity and mortality in colitis mice [52], and play a key role in maintaining and regulating intestinal homeostasis [53]. Nlrp6 was showed to regulate the production of IL-18, maintain the intestinal homeostasis, and protect epithelial integrity and promote barrier function, thus contributing to alleviating DSS-induced colitis [54–56]. Nlrp12 attenuated colon inflammation via maintaining colonic microbial diversity, inhibiting the activity of NF-κB signaling, and maintaining intestinal homeostasis [57–59]. NOD2−/− mice showed aggravated colitis activity induced by DSS compared to wild type mice, suggesting that NOD2 is a protective factor for colitis [60]. In our study, the expressions of *NOD2*, *Nlrp1b*, *Nlrp6*, *Nlrp12* were all significantly up-regulated in the mice of Pul group compared to those of model group,. The inhibitory effect of *Pulsatillae radix* on colitis may be partially attributed to the up-regulation of NOD-like signaling pathway. Transcriptome analysis of colon also showed that *Pulsatillae radix* down-regulated expressions of some important chemokines (*Ccl1*, *Ccl2*, *Ccl3*, *Cxcl2*, *Cxcl3*, *Cxcl4)* which were highly induced by DSS.

Calcium-activated chloride channel regulator 1 secreted by the goblet cell can down-modulate inflammatory responses by decreasing expressions of *Cxcl1* and IL-17 during DSS-induced colitis [61]. In this study, the observed increasing of some chemokines may be partially attributed to the lack of goblet cells in colitis. Therefore, the recovery of goblet cells in *Pulsatillae radix*-treated mice may play an important role in inhibiting chemokines and alleviating colitis. *Pulsatillae radix* administration inhibited high production of inflammatory cytokines (such as IL-1β and IL-6) in colitis in the current study, which is consistent with previous reports that Kui-Jie-Yuan decoction and San-Huang-Shu-Ai decoction reduced the expression of IL-1β and IL-6 and alleviated the symptom of colitis [62,63]. S100A8/A9 in serum is considered as an indicator of the severity of colitis [64], and anti-S100A9 antibody was shown to suppress colitis in mice [65]. S100A8/A9 were also significantly reduced when receiving *Pulsatillae radix* administration compared to model group. *Lcn2* is a multifunctional innate immune protein and disease activity marker in IBD [66]. We observed approximately 150 folds increase of *Lcn2* in PBS group compared with control group, and *Pulsatillae radix* administration restored it down to 7 folds.

Pulsatillae radix also up-regulated several genes whose expression have been reduced in mice with colitis. Gasdermin family is known to mediate pyroptosis, a form of lytic programmed cell death, which is crucial for immune defense and diseases [67,68]. It was reported that Gasdermin D could aggravate colitis [69], and Gasdermin-E-mediated pyroptosis promoted the development of colitis-associated colorectal cancer [70]. However, in the present study, Gasdermin C expression in model mice was significantly lower than that in control group, while *Pulsatillae radix* administration reverted this reducing trend. Spearman's correlation analysis also

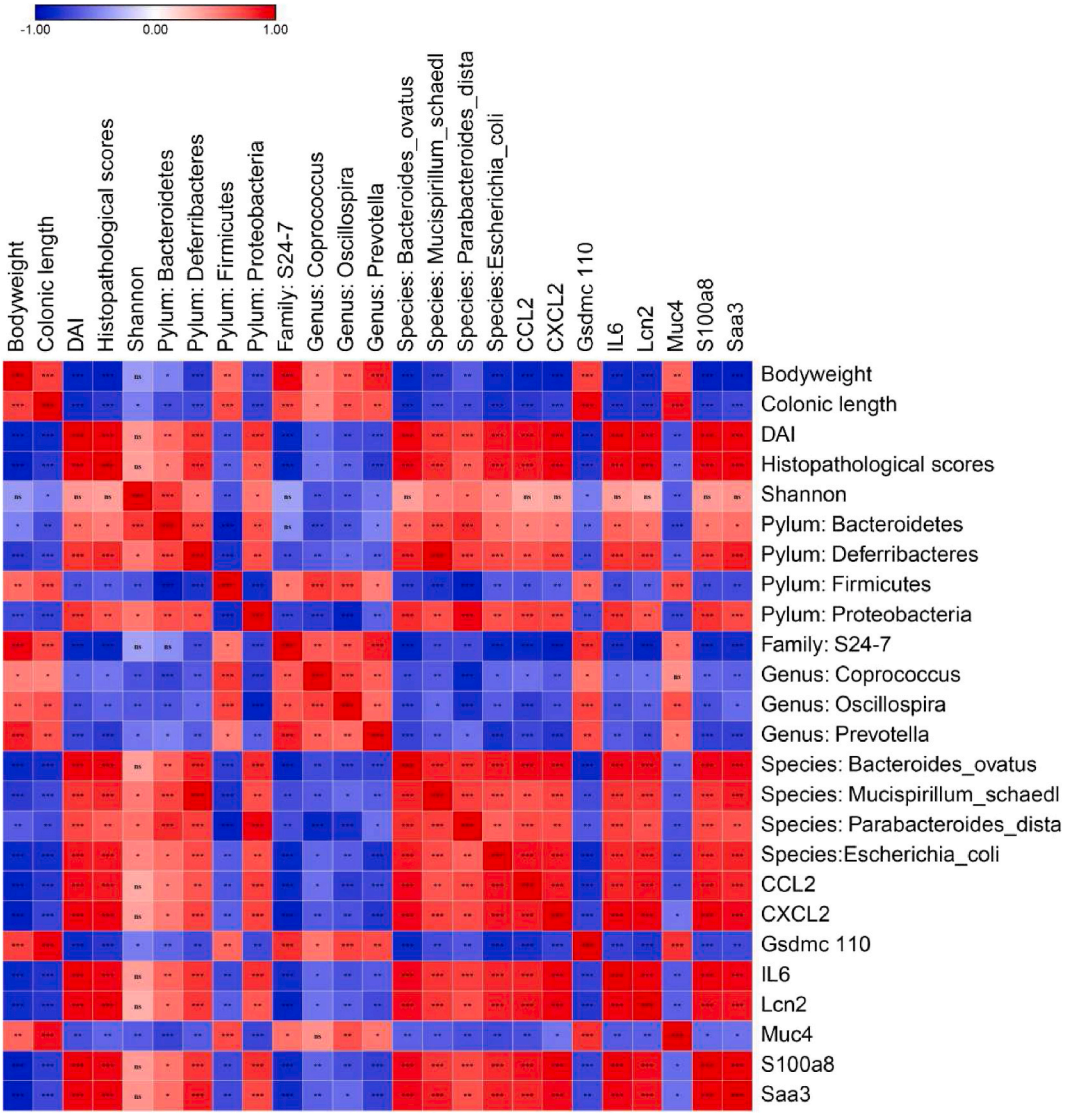

Fig. 8. Spearman's correlation of clinical symptoms, relative abundance of OTUs, and expression levels of differential genes. The R values were indicated by graded red or blue colors, which represent positive or negative correlation. ns, non-significance; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

showed that colitis severity was strongly negatively correlated with Gasdermin C. Muc4 is an important member of mucin family which are important for maintaining intestinal barrier. The reduced Muc4 in colitis mice were also observed to restored significantly after receiving *Pulsatillae radix* administration.

In conclusion, we demonstrated *Pulsatillae radix* alleviated DSS-induced colitis probably via modulating gut microbiota and inflammatory response in DSS-induced colitis mouse model using its multiple active ingredients acting on multiple targets. However, the exact mechanisms as for how *Pulsatillae radix* alleviates colitis by modulating inflammatory pathways, changing gene expressions of colon and composition of gut microbiota, remain to be clarified in the future using gene deficient mice and germ-free mice.

Data availability statement

All data included in this study are available upon request, and can be obtained by contact with the corresponding author. The data of 16rRNA sequencing (accession number: PRJNA765473) and transcriptomic analysis (accession number: PRJNA765487) are now available in the NCBI SRA data base (https://www.ncbi.nlm.nih.gov/sra).

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CRediT authorship contribution statement

Xianping Li: Conceptualization, Data curation, Writing – original draft, Visualization. **Zhihuan Wang:** Data curation, Formal analysis, Software, Visualization, Writing – review & editing. **Hongyuan Gao:** Methodology, Resources. **Yuchun Xiao:** Data curation, Methodology, Resources. **Mengde Li:** Formal analysis, Software. **Yuanming Huang:** Methodology, Software. **Guoxing Liu:** Data curation, Methodology, Resources. **Yanan Guo:** Data curation, Methodology. **Liqiong Song:** Formal analysis, Project administration, Writing – review & editing. **Zhihong Ren:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

All authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21869.

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