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# Paeonol alleviates dextran sodium sulfate induced colitis involving *Candida albicans*-associated dysbiosis

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## Abstract

Inflammatory bowel disease (IBD), which consists of ulcerative colitis (UC) and Crohn's disease (CD), is a chronic inflammatory disorder of the gastrointestinal tract. Occurrence and development of UC have been associated with multiple potential causative factors, which include fungal dysbiosis. Growing evidence reveals that *Candida albicans*-associated dysbiosis is correlated with clinical deterioration in UC. Paeonol (PAE) is a commonly used traditional medicine with multiple reported properties including effective alleviation of UC. In this study, a murine UC model was established by colonizing mice with additional *C. albicans* via gavage prior to dextran sodium sulfate (DSS) administration. Effects of PAE treatment were also assessed at initiation and in preestablished *C. albicans*-associated colitis. The results showed that *C. albicans* supplementation could aggravate disease activity index (DAI), compromise mucosal integrity, exacerbate fecal and tissue fungal burdens, increase serum $\beta$-glucan and anti-*Saccharomyces cerevisiae* antibody (ASCA) levels, promote serum and colonic tissue pro-inflammatory cytokine secretion (tumor necrosis factor (TNF)-$\alpha$, interleukin (IL)-1$\beta$, IL-6, and IL-8) and decrease the anti-inflammatory cytokine IL-10 level. It also stimulated Dectin-1, TLR2 and TLR4 as well as expression of their downstream effector NF-$\kappa$B in colonic tissue. After PAE treatment, the adverse impacts of *C. albicans* on colitis were relieved, via decreased receptor-associated local and systemic inflammation. Our study suggests that PAE should be a candidate for treatment of fungal dysbiosis-associated UC and may act through the Dectin-1/NF-$\kappa$B pathway in collaboration with TLR2 and TLR4.

## Lay Summary

*Candida albicans* is believed to be an important stimulator in ulcerative colitice (UC) development. Suppressing the growth of intestinal *C. albicans* can be contributory to the amelioration of UC. Paeonol (PAE) is a commonly used traditional medicine with multiple biological functions. In this study, we observed that PAE could alleviate symptoms in mice UC model accompanying with burden reduction of *C. albicans*. Therefore, we suppose that PAE can be a candidate in the treatment of *C. albicans*-associated UC.

**Key words:** *Candida albicans*, ulcerative colitis, mycobiota, paeonol, Dectin-1.

## Introduction

Ulcerative colitis (UC), a common type of inflammatory bowel disease (IBD), is characterized by persistent and nonspecific intestinal inflammation, with typical clinical manifestations including abdominal pain, diarrhea and hematochezia.[1] Recently, the morbidity and mortality of UC have unfortunately increased around the world.[2] Without timely effective treatment, colorectal cancer (CRC) is likely to occur, as the overall risk of CRC in UC patients is reportedly up to 1.4%.[3] The etiology of UC is largely unclear at present. However, several factors are considered to play a role in development of UC, including genetics, host immune response, environmental triggers and gut microbiota.[4,5]

Although constituting only 0.1% of the whole gut microbiota in healthy individuals,[6] gut commensal fungi are critically important in maintaining host homeostasis by acting synergistically with other microbial communities. *Candida albicans*, one of the most common opportunistic polymorphic fungi, is responsible for a series of pathologies ranging from mucous infections to systemic infections.[7] *C. albicans* is nevertheless a normal commensal in the human gastrointestinal tract (GT), but it can transform into a pathogen in response to external environmental factors. Growing evidence reveals that *C. albicans*-associated dysbiosis is correlated with high UC occurrence, and *C. albicans* colonization is capable of promoting UC, with increased inflammatory cytokine levels.[8–11]

Traditional Chinese medicine (TCM) is a valuable resource and several TCMs have demonstrated satisfactory efficacy in UC treatment.[12–15] Paeonol (PAE, 2′-hydroxy-4′-methoxyacetophenone; $C_9H_{10}O_3$) is a natural phenolic compound from *Moutan Cortex*, the root bark of *Paeonia moutan Sims,* and has diverse biological effects including analgesic,[16] neuroprotective,[17] anti-allergic,[18] anti-atherosclerosis,[19] anti-osteoclastogenesis and anti-inflammatory activities.[20,21] Moreover, there are several lines of evidence that support anti-colitic effects for PAE.[22–24] Therefore, it is pertinent to explore the potential of PAE and its associated mechanisms in the treatment of UC involving *C. albicans*-associated dysbiosis.

In this study, a DSS-induced UC model with *C. albicans* precolonization in mice was established. Post PAE treatment, it was found that fecal and organ fungal burdens were reduced, local and systemic infections were ameliorated, the activity of tissue macrophages was suppressed, and the expression of Dectin-1, TLR2, TLR4 and NF-$\kappa$B were decreased.

## Methods

### Strains and cultivation

*C. albicans* SC5314 was a gift from Professor Yuanying Jiang, School of Pharmacy, Second Military Medical University, Shanghai, China. The isolate was routinely maintained in liquid sabouraud medium (Hope Biotechology, Qingdao, Shangdong, China) at 37°C for 12–16 hours to reach exponential phase (Invitrogen, Carlsbad, CA, USA).

### Animals

Fifty mice (C57BL6/J, female, 8–10 weeks) were purchased from Pengyue Experimental Animal Breeding Co. Ltd (License: SCXK20190003, Jinan, China) and fed with sterilized chow and water *ad libitum*. They were maintained in the Animal Center of College of Integrated Chinese and Western Medicine (College of Life Science), Anhui University of Chinese Medicine, and acclimated for 7–10 days prior to initiation of the UC model. All animal protocols were approved by the Experimental Animal Ethics Committee of the Anhui University of Chinese Medicine.

### Establishment of UC model

The procedures for establishment of the UC model were modified from several reported protocols.[25,26] Briefly, animals were first gavaged for 4 consecutive days with *C. albicans* SC5314 ($1 \times 10^8$ cfu/mouse/day) suspended in sterile phosphate-buffered saline (PBS) and then given 3% dextran sodium sulfate (DSS; MP Biomedicals, LLC, Aurora, OH, USA) in distilled water *ad libitum* for 7 days continuously to induce colitis. Some groups were treated with PAE (400 mg/kg/d) or mesalazine (MES, 200 mg/kg/d) alongside DSS administration. Vehicle control mice were given physiological saline. Feces were collected at day 1, 3, 5, 7, 9, and 11 for fungal enumeration. All mice were sacrificed by cervical dislocation at day 12 to harvest blood and organs (colon, liver, spleen, kidney and stomach). Tissue samples were stored at −20°C until use. Colitis severity was assessed using DAI, calculated based on weight loss, stool consistency and occult blood, as previously described.[27]

### Fungal burden assay

*C. albicans* enumeration was performed according to previously reported methods with minor modifications.[28] Feces and organs were weighed, suspended, or homogenized in sterile PBS (pH 7.4, Boster Biological Technology, Wuhan, China) and then plated on CHROMagar (cat. no. K08A, Shanghai Central Bio-engineering, Shanghai, China) containing 100 U/ml penicillin and 0.1 mg/ml streptomycin (Beyotime Biotechnology, Shanghai, China) prior to incubation for 48 hours at 37°C. Green colonies of *C. albicans* were counted and recorded for each group.

### F4/80 staining

Staining for macrophages was carried out according to reported techniques with minor modifications.[29] Briefly, colonic tissue was sliced and fixed in acetone for 20 minutes. After washing 3 times for 10 min in PBS, the slices were blocked with 10% goat serum at 37°C for 45 min. After three rinses with PBS, the tissues

were incubated with anti-F4/80 antibodies (0.2 mg/mL, cat. no. 123 110, BioLegend, San Diego, CA, USA) at 4°C overnight. After a further 3 washes with PBS, DAPI (100 ng/ml, cat. no. C1005, Beyotime, Shanghai, China) was added for a 4 min incubation. Staining was observed and recorded using a fluorescent microscope (Olympus IX81, Japan).

### Assays for cytokines, $\beta$-glucan, and anti-*Saccharomyces cerevisiae* antibodies (ASCA)

Levels of tumor necrosis factor (TNF)-$\alpha$ (cat. no. CK-E20852), interleukin (IL)-1$\beta$ (cat. no. CK-E20174), IL-6 (cat. no. CK-E20188), IL-8 (cat. no. CK-E20191), and IL-10 (cat. no. CK-E20162) were measured using ELISA kits (Ruixin Biotechnology, Quanzhou, Fujian, China). Serum $\beta$-glucan content was determined by a commercial ELISA kit (Associates of Cape Cod Inc, Beijing, China). Serum ASCA levels were also measured by a commercial ELISA kit (cat. no. CK-E22222, Ruixin Biotechnology, Quanzhou, Fujian, China) according to the manufacturer's instructions.

### Hematoxylin-eosin (HE) staining

HE staining was performed in line with described methods with minor adjustments.[30] In brief, colonic tissue was fixed in 10% neutral formalin (cat. no. ZP1101, Zhenwo Bicmedical, Guangzhou, Guangdong, China), then embedded in paraffin and cut into 4 $\mu$m-thick sections. Sections were then stained with hematoxylin and eosin (cat. no. DH006, Leagene, Beijing, China) and pathological changes observed using an optical microscope (Olympus BX51, Japan).

### Immunohistochemistry

Colonic tissue was sectioned for immunohistochemical analysis using reported procedures with minor modulation.[31] Briefly, slices were deparaffinized by treatment in xylene (cat. no.20190610JN, Shanghai Richjoint Chemical Reagents, Shanghai, China) 3 times for 15 min each time and then hydrated with 100%, 95%, 90%, 80%, and 75% gradient ethanol (Guanhua Chemical, Suzhou, Anhui, China). After rinsing with PBS for 2 min, sections were boiled in sodium citrate buffer for 20 min (0.01 M, pH 6.0, Leagene, Beijing, China) for antigen repair. After cooling to room temperature, sections were incubated in 3% $H_2O_2$ in methanol (cat. no. 13L26C08, Boster Biological Technology,Wuhan, China) for 15 min to inhibit endogenous peroxide activity. Following rinsing with PBS, sections were incubated with anti-TLR2 (cat. no.10q2471, Affinity Bioscience, OH, USA), anti-TLR4 (cat. no. 16c5074, Affinity Bioscience, OH, USA) or anti-Dectin-1 (cat. no. ab140039, Abcam, USA) antibodies at 4°C overnight. After a further three washes in PBS (5 min/time), goat anti-mouse immunoglobulin G (IgG) secondary antibody (cat. no. 1919D0919, Zhong Shan-Golden Bridge Biological Technology, Beijing, China) was added for 30 min at 37°C. Finally, 3, 3-diaminobenzidin (DAB, cat. no. K186621D, Zhong Shan-Golden Bridge Biological Technology, Beijing, China) was added at room temperature in the dark for 5 min. Colorization was stopped with distilled water. Sections were then dehydrated with an ethanol gradient (75%, 80%, 90%, 95%, and 100%) and xylene to transparency, sealed with neutral resin (Shanghai Specimen and Model Factory, Shanghai, China), and observed with an optical microscope (Olympus BX51, Japan), with data calculated using Image J (National Institutes of Health).

### Immunoblotting

Total protein preparations were mixed with phenylmethanesulfonyl fluoride (PMSF, cat. no. P0100, Solarbio, Beijing, China), prepared by solution in RIPA lysis buffer (cat. no. P10013B, Beyotime, Shanghai, China) to a final concentration of 1 mM (30 min at 4°C). Following centrifugation at 10 000 g for 10 min, the protein concentrations were determined using BCA protein analysis kits (cat. no. 0912A19, Leagene, Beijing, China). Extracted proteins were separated by SDS-PAGE, transferred to PVDF membranes (cat. no. IPVH00010, Merck Millipore Ltd, China) and blocked with 5% skimmed milk powder (Inner Mongolia Yili Industrial Group, Neimeng, China) dissolved in TBST buffer (prepared by mixing 0.05 M TBS (pH 7.4, Biosharp Life Sciences, Hefei, Anhui, China) with 1 ml Tween 20 (Runhua Chemistry, Guangzhou, China)) at room temperature for 2 hours. Membranes were then incubated with anti-Dectin-1, anti-TLR2, anti-TLR4, or anti-NF-$\kappa$Bp65 (cat. no. 26j7543, Affinity Bioscience, OH, USA) antibodies overnight at 4°C. Horseradish peroxidase-conjugated anti-rabbit IgG (cat. no.56j9958, Affinity Bioscience, Cincinatti, OH, USA) as secondary antibody was added to membranes, at room temperature for 2 hours. An enhanced chemiluminescence detection kit (cat. no. P0018S, Beyotime Institute of Biotechnology, Shanghai, China) was used for development of protein bands which were scanned with a protein imaging system (LAS4000, GE, Pittsburgh, PA, USA) and quantified with Image J.[32]

### Statistical analysis

All experiments were performed in triplicate. The results were expressed as mean ± standard deviation and analyzed by SPSS 17.0 (SPSS Inc, Chicago, IL, USA). Groups were compared by one-way ANOVA with the least significance difference (LSD) method; $P < .05$ was considered statistically significant.

## Results

### PAE ameliorates colitis and fungal burden

Establishment of the UC model is summarized in Figure 1A. After precolonization with *C. albicans*, mice showed greater

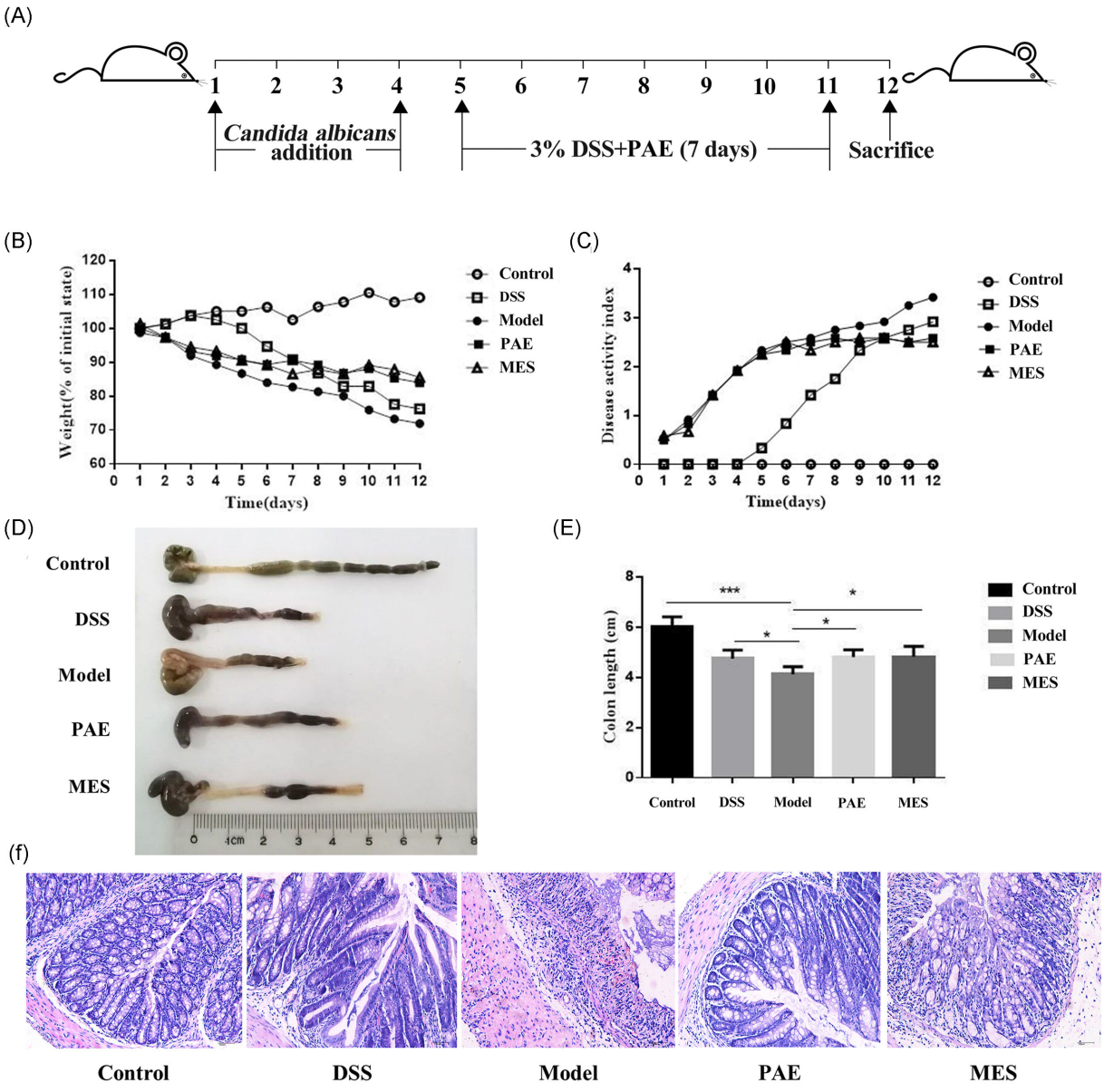


**Figure 1.** Paeonol (PAE) ameliorates *Candida albicans* aggravated colitis. (A) Schema of dextran sodium sulfate (DSS) induced colitis with *C. albicans* supplementation. (B) Body weight assessment. (C) Disease activity index (DAI) calculation based on weight loss, fecal consistency and blood. (D, E) Colon length measurement. (F) Colon tissue H&E staining. The control group was mice fed with normal saline throughout the experiment. The DSS group was mice given 3% DSS orally from day 5 to 11 following normal saline feeding from day 1 to 4. The model group was mice orally administered with *C. albicans* from day 1 to 4 followed by 3% DSS from day 5 to 11. The PAE group included mice treated with 400 mg/kg/d PAE along with the same exposure to *C. albicans* and DSS as the model group. MES group refers to mice treated with mesalazine instead of PAE with the same protocol as the PAE group. * $P < .05$, ** $P < .01$, *** $P < .001$.

body weight loss, higher DAI, shorter colon length and more severe erosions of the colonic mucosal surface, crypts and glands, compared to the DSS only group (Fig. 1B–F). These changes were significantly ameliorated in the PAE group. In the DSS group, no *C. albicans* was isolated from feces and selected organs (Fig. 2A, B). Conversely, fecal *C. albicans* quantities were decreased from $>5 \times 10^5$ cfu at day 1 to <80 cfu per 10 mg feces at day 11 (Fig. 2A). Approximately 10–800 cfu of *C. albicans* per gram of tissue were found in liver, kidney, stomach, and colon but not in the spleen, in the DSS group compared to the model group, where *C. albicans* could only be found in the stomach (Fig. 2B). Compared with the model group, PAE did not cause a significant change in fecal *C. albicans* burden (Fig. 2A), but dramatically suppressed the quantities of *C. albicans* in liver, kidney, and colon (Fig. 2B).

## PAE inhibits local and systematic inflammation

Serum $\beta$-glucan is a strong indicator of fungal infection, and serum ASCA is an important marker of Crohn's disease, another form of IBD.[33] As shown in Figure 3A and B, pretreatment with *C. albicans* aggravated systemic inflammation, as serum

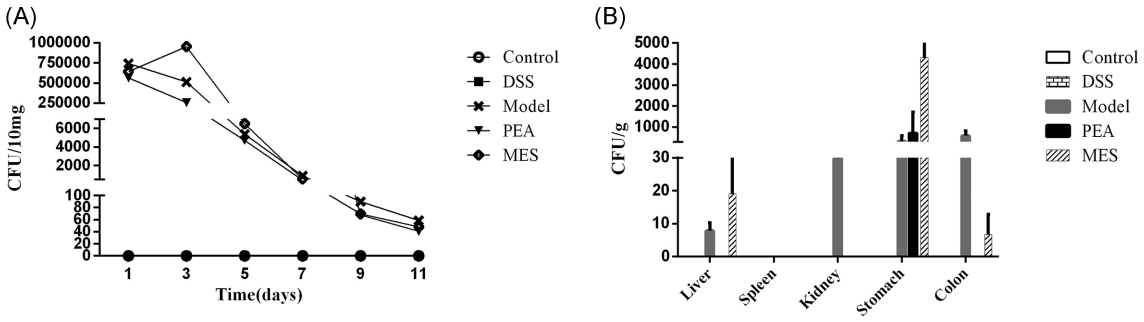


**Figure 2.** *C. albicans* loading measurements (A) per 10 milligram feces at day 1, 3, 5, 7, 9, and 11; (B) per gram of tissue from five organs: liver, spleen, kidney, stomach, and colon after sacrifice at day 12, assessed by agar plating. Definitions of control, DSS, model, PAE, and MES are in the Figure 1 legend.

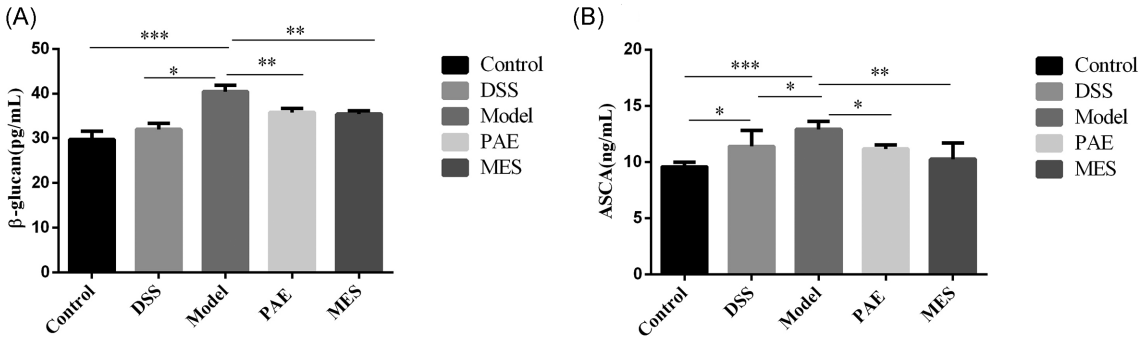


**Figure 3.** Analysis of serum levels of (A) $\beta$-glucan and (B) anti-*Saccharomyces cerevisiae* antibodies (ASCA) by ELISA after euthanasia at day 12. Definitions of control, DSS, model, PAE, and MES are in the Figure 1 legend. * $P < .05$, ** $P < .01$, *** $P < .001$.

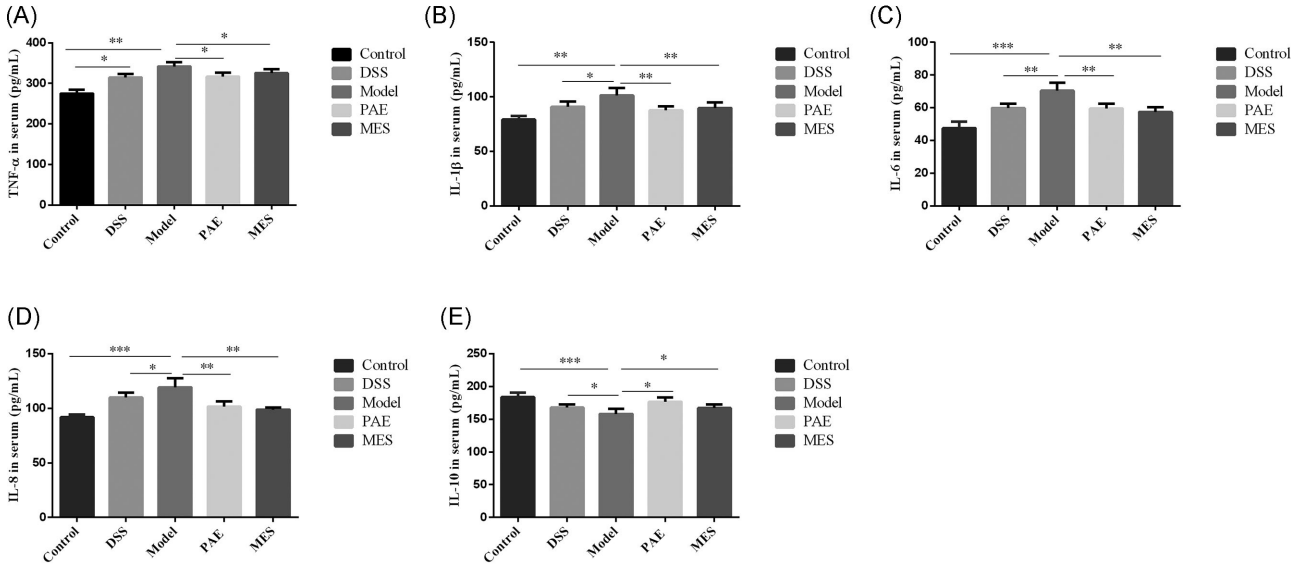


**Figure 4.** Determination of serum pro- and anti-inflammatory cytokines (A) TNF-$\alpha$, (B) IL-1$\beta$, (C) IL-6, (D) IL-8, and (E) IL-10 by ELISA at day 12. Definitions of control, DSS, model, PAE, and MES are in the Figure 1 legend. * $P < .05$, ** $P < .01$, *** $P < .001$.

$\beta$-glucan and ASCA levels were increased markedly compared with the DSS control (). These data are consistent with cytokine results, where pro-inflammatory cytokines TNF-$\alpha$, IL-1$\beta$, IL-6, and IL-8 levels were greatly enhanced, while anti-inflammatory cytokine IL-10 was suppressed in the model group (Fig. 4A–E). Notably, PAE could effectively alleviate systemic inflammation by lowering serum $\beta$-glucan, ASCA and pro-inflammatory cytokines and elevating IL-10 (Figs. 3 and 4). Concurrent exogenous *C. albicans* could significantly worsen local inflammation in the colon compared with the DSS group, as the levels of pro-inflammatory cytokines TNF-$\alpha$, IL-1$\beta$, IL-6, and IL-8 were increased while anti-inflammatory cytokine IL-10 was inhibited

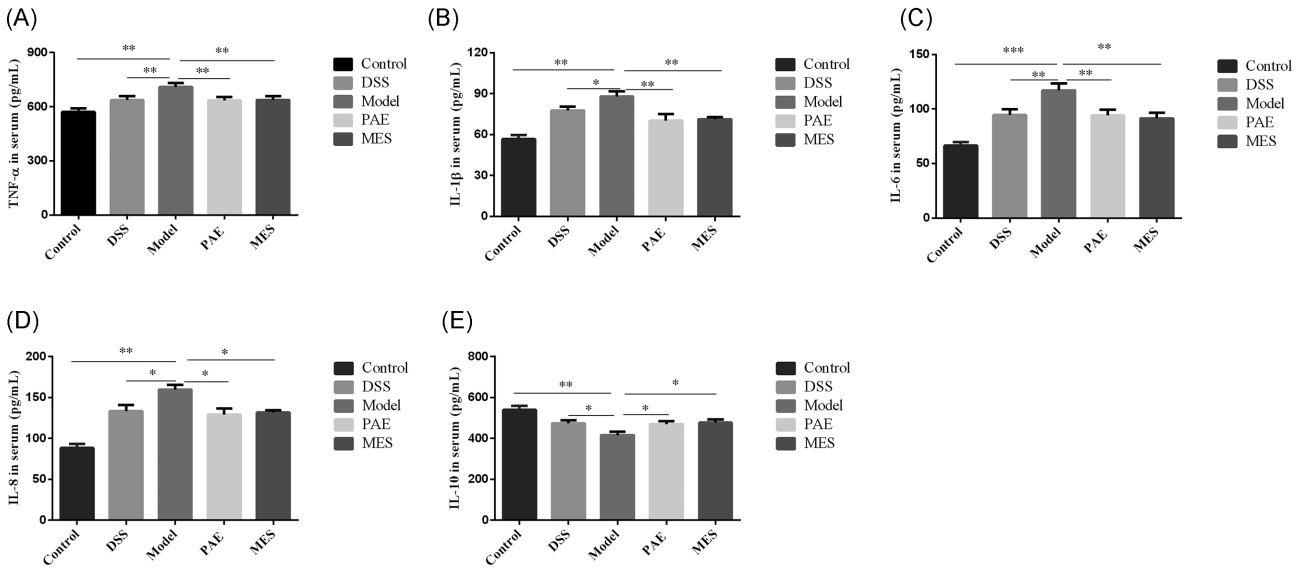


**Figure 5.** Detection of colonic pro- and anti-inflammatory cytokines (A) TNF-$\alpha$, (B) IL-1$\beta$, (C) IL-6, (D) IL-8, and (E) IL-10 by ELISA at day 12. Definitions of control, DSS, model, PAE, and MES are in the Figure 1 legend. * $P < .05$, ** $P < .01$, *** $P < .001$.

(Fig. 5A–E). In addition, *C. albicans* inoculation could stimulate excessive macrophage activity (Fig. 6). This abnormal local inflammation was reversed, to a large extent, by treatment with PAE (Figs. 5 and 6).

### PAE ameliorates colitis involving *C. albicans*-associated dysbiosis via effects on key proteins

Three receptors Dectin-1, TLR2, and TLR4 play a pivotal role in recognition and clearance of *C. albicans* via NF-$\kappa$B activation.[34] Expression of Dectin-1 (Fig. 7), TLR2 and TLR4 (Figs. 7 and 8) was dramatically increased in the model group compared with the DSS group ($P < .05$). Although expression of NF-$\kappa$B seemed unaffected in the model group compared to the DSS group, it was clearly upregulated compared to the control group ($P < .01$, Fig. 8). Aberrant expressions of Dectin-1, TLR2, TLR4 and NF-$\kappa$Bp65 could be corrected by PAE ($P < .05$, $P < .01$, Figs. 7 and 8). These results correlated with the findings for $\beta$-glucan, ASCA, pro/anti-inflammatory cytokine levels as well as macrophage activity, as described above (Figs. 3–6).

## Discussion

Although *C. albicans* is regarded as an unusual resident in the mouse GT, effective colonization with exogenous *C. albicans* and promotion of associated colitis, required the presence of DSS or drug-induced bacterial dysbiosis, since *C. albicans* cannot survive in immunocompetent mice without intestinal mucosal damage.[25,35] In this study, a *C. albicans*-associated UC model was established using a relatively high inoculum of *C. albicans* ($1 \times 10^8$ cfu/ml) by continuous gavage for four days (once a day, Fig. 1A).

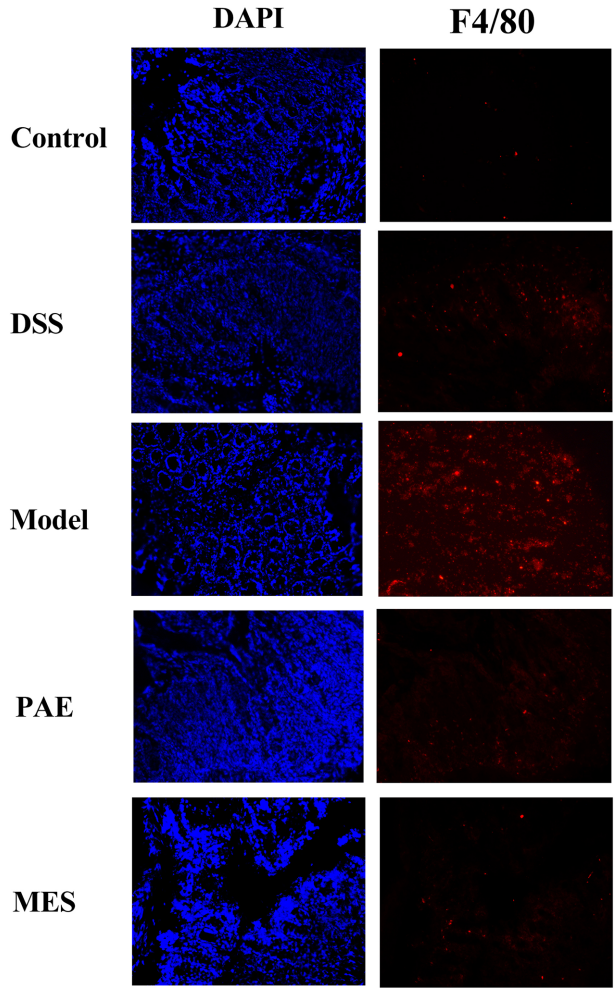


**Figure 6.** Immunofluorescent staining of colonic tissue with DAPI and F4/80 following euthanasia at day 12. Definitions of control, DSS, model, PAE, and MES are in the Figure 1 legend. Magnification: × 200.

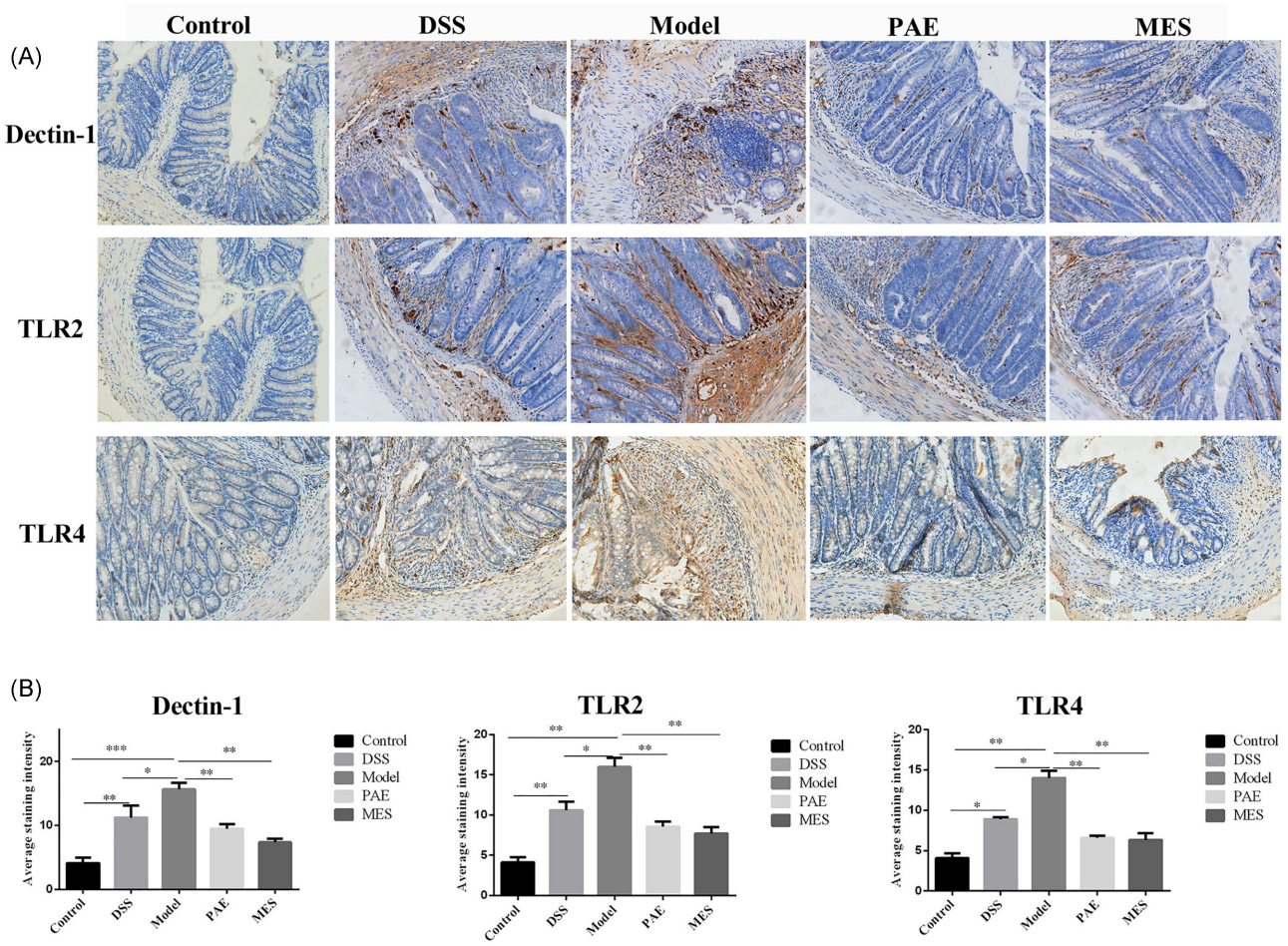


**Figure 7.** (A) Microscopic observations and (B) abundance calculations for Dectin-1, TLR2, and TLR4 by immunohistochemistry in colonic tissues at day 12. Definitions of control, DSS, model, PAE, and MES are in the Figure 1 legend. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

This strategy has been employed elsewhere.[28] In our model, we found that DSS-induced colitis was further aggravated by *C. albicans* introduction, with local and systemic inflammation significantly higher than in the DSS group (Figs. 1B–F, 2B, 3–6). Therefore, our model could reflect clinical IBD for the purpose of our study.

Although there have been several reports that PAE can attenuate colitis,[22–24] it has not been shown to be effective in treatment of colitis with *C. albicans* precolonization. This may be due to the fact that PAE has not been considered an antifungal agent, especially against *C. albicans*. However, the antibacterial effect of PAE, for example, against *Escherichia coli,* a common commensal in the mammalian GT, has been described in several papers.[36,37] It has been demonstrated that exogenous *C. albicans* promotes the growth of *E. coli*,[26] which is believed to be an essential factor in the severity of *C. albicans*-associated colitis.[25] Based on this, we speculate that removal or inhibition of *E. coli* by PAE could weaken or abrogate the negative effect of *C. albicans* in development of colitis.[26] Nevertheless, more evidence is required to link the effect of PAE on *E. coli* with its attenuation of *C. albicans*-associated colitis.

Besides its potential role in the modulation of the intestinal microbiota, PAE may suppress LPS-induced pro-inflammatory cytokines TNF-$\alpha$, IL-1$\beta$, and IL-6 and elevate the anti-inflammatory cytokine IL-10 in macrophages.[38] It is well established that excessive pro-inflammatory and inadequate anti-inflammatory factors play a crucial role in IBD progression.[39] In this study, marked suppression of TNF-$\alpha$, IL-1$\beta$, IL-6, and IL-8 and enhancement of IL-10 was mediated by PAE, accompanied by attenuation of colitis (Figs. 1, 4, 5). Our results are consistent with several previous reports which indicate a role for PAE in modulation of inflammatory factors during progression of colitis.[23,24]

Due to its ability to regulate cytokines, it could be supposed that the immuno-modulatory effect of PAE was responsible for not only relief of colitis, but also elimination of *C. albicans*. Regulation of cytokines by PAE was reflected by pro-inflammatory alleviation (such as TNF-$\alpha$) during the process of *C. albicans* removal.[40] The host innate immune response to *C. albicans* is controlled by several receptor-mediated signaling pathways, whereby synergy between Dectin-1, TLR2, and TLR4 is critical, since activation of the Dectin-1 pathway alone is insufficient

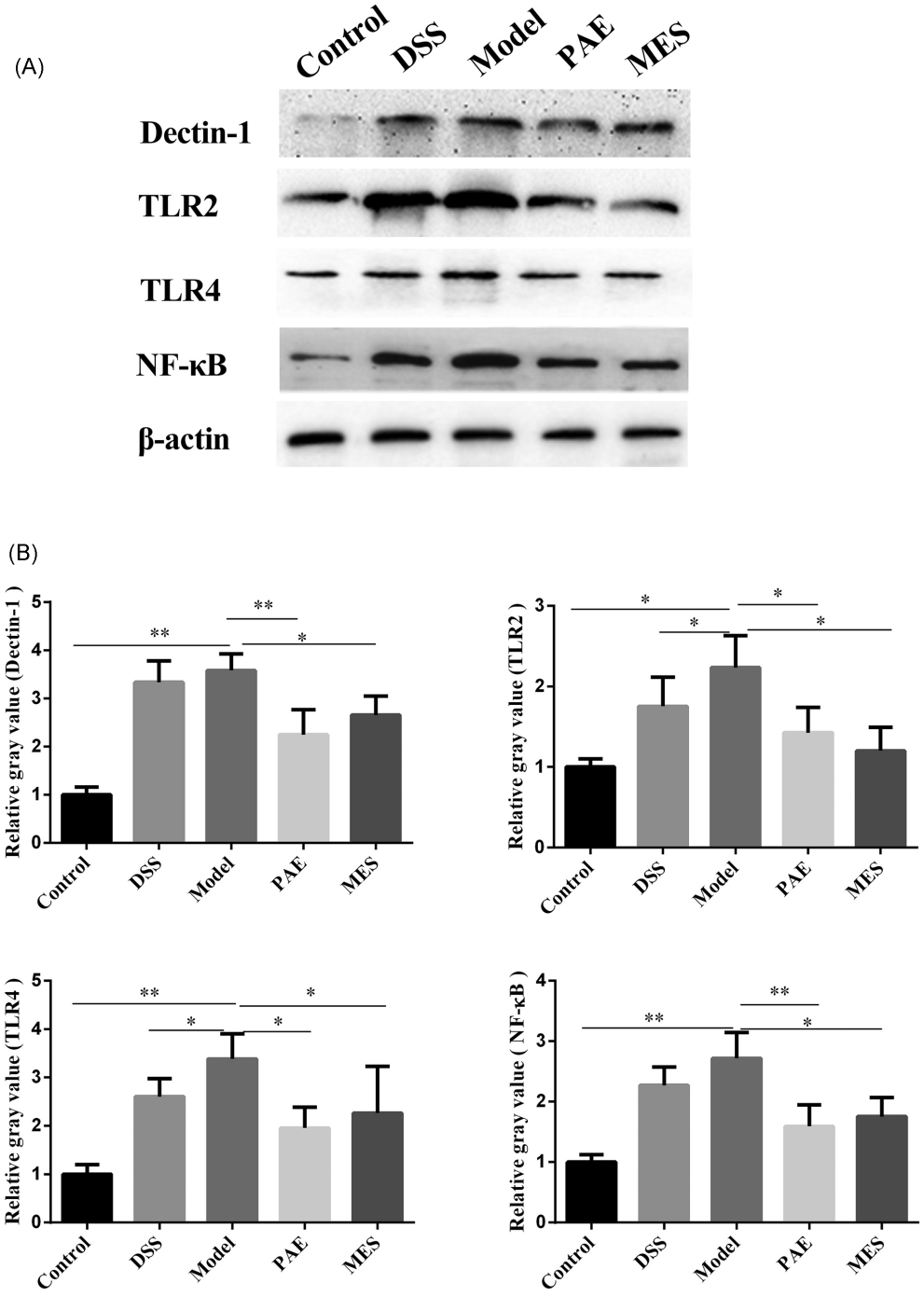


**Figure 8.** (A) Protein analysis and (B) expression of Dectin-1, TLR-2, TLR-4, and NF-$\kappa$B assessed by Western blot in colonic tissue homogenates at day 12. Definitions of control, DSS, model, PAE, and MES are in the Figure 1 legend. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.

for production of pro- and anti-inflammatory cytokines.[34,41,42] In addition, loss of Dectin-1 is detrimental to *Candida* removal, increasing risk of tissue damage during *Candida*-associated colitis,[43] and TLR2 dysfunction leads to worsened clinical symptoms and increased mortality rates.[44] In our study, expression of Dectin-1, TLR2 and TLR4 was significantly upregulated in the model group relative to the DSS group, but this could be subdued by PAE (Figs. 7 and 8), indicating a tight association between cytokine levels modulated by PAE and expression of the three receptors.

Several lines of evidence indicate that the underlying anti-colitic mechanism of PAE could involve inhibition of the MAPK/ERK/p38 pathway and NF-$\kappa$B/STAT1 transactivation.[22,23] As stated, the innate immune system could utilize the Dectin-1-associated pathway to modulate cytokine secretion in response to *C. albicans* invasion, via the downstream effectors

ERK and NF-$\kappa$B.[45,46] In this investigation, although there was no significant difference in NF-$\kappa$B in between the DSS and model groups, its level in the model group was slightly increased compared with the DSS group. The high level of NF-$\kappa$B was notably suppressed by PAE (Fig. 8).

In conclusion, PAE ameliorates the adverse impact of *C. albicans* on progression of DSS-induced colitis, lowering local and systemic inflammation, relieving intestinal mucosal damage, decreasing fecal and organ fungal burdens, and strengthening recognition and clearance of *C. albicans*. This study sheds light on the mechanisms of PAE, implicating activation of the Dectin-1/NF-$\kappa$B pathway in collaboration with TLR2 and TLR4, and supports the possibility of using PAE in prevention and treatment of UC with *C. albicans* dysbiosis.

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### Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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