Baicalin ameliorates APEC-induced intestinal injury in chicks by inhibiting the PI3K/AKT-mediated NF-κB signaling pathway

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ABSTRACT Avian pathogenic *Escherichia coli* (**APEC**) is the causative agent of avian colibacillosis. Baicalin (**BA**) possesses multiple pharmacological effects, but the mechanism underlying its activity in APEC-induced intestinal injury remains unknown. This study aims to investigate the protective effects and possible mechanism of BA against APEC-induced intestinal injury. Sixty 1-day-old chicks were randomly divided into 4 groups: the control group (basal diet), *E. coli* group (basal diet), BAI10 group (10 mg/kg BA), and BAI20 group (20 mg/kg BA). After pretreatment with BA for 15 d and subsequent induction of APEC infection by pectoralis injection, the ileum was collected and analyzed. The results showed that BA-pretreatment demonstrated an alleviation of chicks in diarrhea rate, mortality, and histopathological changes in intestinal tissues after APEC infection. Additionally, following APEC infection, BA improved the intestinal barrier by elevating zona occludens (**ZO**)s (ZO-1, 2, 3), Claudins (Claudin1, 2, 3), Occludin, avian β-defensin (**AvBD**)s (AvBD1, 2, 4), lysozyme (**Lyz**) mRNA levels and ZO-1, Claudin1, and Occludin protein levels. Besides, the activities of total superoxide dismutase (**T-SOD**), catalase (**CAT**), and glutathione peroxidase (**GSH-Px**) and the SOD-1 and CAT mRNA levels and SOD-1 protein level were elevated by BA pretreatment. BA pretreatment also decreased the malondialdehyde (**MDA**) content, heme oxygenase-1 (**HO-1**) and NADH quinone oxidoreductase 1 (**NQO1**) mRNA levels, and HO-1 protein level after APEC infection. BA alleviated the APEC-induced inflammatory response, including downregulating the mRNA levels of proinflammatory cytokines (tumor necrosis factor-α (**TNF-α**), interleukin [**IL**]-1β, IL-6, IL-8) and upregulating the mRNA levels of anti-inflammatory cytokines (IL-4, IL-10, IL-13, transforming growth factor-β [**TGF-β**]). Furthermore, BA decreased the mRNA and protein levels of phosphatidylinositol 3 kinase (**PI3K**), protein kinase B (**AKT**), and nuclear factor kappa-B (**NF-κB**) as well as the expression of the phosphorylated forms of these proteins after APEC infection. Collectively, our findings indicate that BA exerts a protective effect against APEC-induced intestinal injury in chicks by inhibiting the PI3K/AKT-mediated NF-κB pathway, suggesting that BA may be a potential therapeutic approach for avian colibacillosis.

Key words: Baicalin, avian pathogenic *Escherichia coli*, intestinal injury, PI3K/AKT/NF-κB, chick

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INTRODUCTION

Avian colibacillosis, a disease caused by avian pathogenic *Escherichia coli* (**APEC**), is one of the principal bacterial diseases that affect birds of all ages, and it is especially severe in the chick phase (Azam et al., 2020). The bacterium causes a substantial economic burden on the poultry industry worldwide by decreasing growth performance and egg production and increasing mortality and condemnation rates (Korf et al., 2020). In addition, Yuan et al. (2021) verified that severe diarrhea was linked with inflammation, leading to rapid electrolyte imbalance and death in chicks and other young animals. A recent study showed that the major pathological phenomenon induced by APEC in chicks was intestinal inflammation, which affects the development of intestinal injury and can lead to diarrhea and even death (Lin et al., 2018). Increasing evidence has shown that the intestinal injury induced by APEC is closely related to the inflammatory response.

The inflammatory response is normally identified as the main line of defense against pathogenic invasion, but the inflammatory response also serves as the major

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pathological feature of APEC invasion (Peng et al., 2019). Thus, inhibition of inflammation plays an active role in APEC prevention. Nuclear factor kappa-B (**NF-κB**) is considered to be one of the most important regulators of the inflammatory process and is a widely expressed nuclear transcription factor (Lai et al., 2017). Activation of NF-κB can exacerbate the early immune response and the inflammatory reaction by releasing many inflammatory mediators (Kannian et al., 2020). Peng et al. (2019) showed that APEC can significantly increase the protein level of p-NF-κB and that baicalin (**BA**) can alleviate APEC-induced lung injury by inhibiting the activation of NF-κB. A recent study showed that the phosphatidylinositol 3-kinase (**PI3K**)/protein kinase B (**AKT**) pathway was considered to have a remarkable role in the inflammatory response, and when the PI3K/AKT pathway was inhibited with pharmacological compounds, the cellular inflammatory response was attenuated due to downregulation of inflammatory genes (Erasalo et al., 2018). Interestingly, PI3K and AKT were deemed to play a pivotal role in APEC onset and development (Lv et al., 2019; Peng et al., 2019). In addition, PI3K and AKT are involved in the regulation of the NF-κB pathway by activating the phosphorylation of NF-κBα (**IκBα**; Wang et al., 2016; Sun et al., 2018). Therefore, to alleviate inflammatory status, restraining the activation of the PI3K/AKT-mediated NF-κB pathway may have potential application value.

In recent years, the application of functional additives in intestinal health care and disease control has been a research focus worldwide. BA is a flavonoid compound isolated from medicinal plants such as *Scutellaria baicalensis* (Shi et al., 2020). Numerous studies have shown that BA has a variety of pharmacological effects, such as anti-inflammatory, antioxidant, and anticancer effects on the lung, uterus, and other organs (Zhang et al., 2015; Liu et al., 2020). Cui et al. (2014) reported that BA could alleviate colitis through blockade of the toll-like receptor (**TLR**)4/NF-κB pathway in mice. Moreover, BA also possesses strong antioxidant activity, which inhibits the activities of superoxide dismutase (**SOD**), total antioxidant capacity (**T-AOC**), and glutathione (**GSH**; Jia et al., 2021). However, the effects of BA on APEC-induced intestinal injury have not been fully elucidated. In this regard, we speculated that dietary inclusion of BA could alleviate APEC-induced intestinal injury and the inflammatory response by improving the intestinal morphology and intestinal barrier and increasing whole-body antioxidant levels in chicks.

MATERIALS AND METHODS

Experimental Animals

All animals in this experiment were approved by the Committee of Animal Welfare of Jiangxi Agricultural University. Animal care and experimental procedures also complied with the criteria of the Ethics Committee of Jiangxi Agricultural University. In this experiment, 60 healthy 1-day-old Hy-line brown laying hens were selected to establish the animal model.

Experimental Design and Treatment

BA was provided by Sigma−Aldrich Company (Darmstadt, Germany). *Escherichia coli* APAP-O78 was purchased from the China Institute of Veterinary Drug Control (#CVCC1418). All chickens were reared for 1 d under standard chick-rearing conditions (12-h light/dark cycle, relative humidity 60 ± 5%, and relative temperature 37 ± 2°C). Afterward, the chickens were randomly divided into 4 groups: the control group (basic diet), *E. coli* group (basic diet), BAI10 group (basic diet + 10 mg/kg BA), and BAI20 group (basic diet + 20 mg/kg BA; n = 15 per group). The basic diet is based on a chick diet developed by the National Research Council, and all chicks were provided with the same access to water and food. The compositions of the diets are shown in Table 1. At 15 d, the chicks in the *E. coli*, BAI10 and BAI20 groups were injected with 3.39×10^9 CFU/mL *E. coli* liquid by pectoralis injection at a volume of 0.5 mL, and the chicks in the control group were injected with the same dose of saline. Two days later, the chicks were anesthetized with an overdose of sodium pentobarbital through intravenous injection. Then, the ileum was collected on ice and stored at −80°C for the subsequent analyses.

Table 1. Composition and nutrients levels of diets %.

Dietary composition %	Control group	*E. coli* group	BAI10 group	BAI20 group
Corn	66.00	66.00	66.00	66.00
Soybean meal	29.00	29.00	29.00	29.00
Premix*	5.00	5.00	5.00	5.00
Total	100.00	100.00	100.00	100.00
Nutrient level				
DL-Methionine %	0.26	0.26	0.26	0.26
Lysine %	0.88	0.88	0.88	0.88
Metabolic energy (MJ/kg)	11.92	11.92	11.92	11.92
Crude protein %	17.61	17.61	17.61	17.61

*Premix: Copper: 8 g; Iron: 67 g; Zinc: 87 g; Cobalt: 94 g; Sodium selenite: 11.25 g; Potassium Iodide: 8.75 g; DL-Methionine: 300 g; Polysaccharide vitamin: 100 g; Sodium chloride: 2,000 g; Mountain flour: 13,000 g; Calcium hydrogen phosphate: 6,000 g.

Hematoxylin-Eosin Staining

Hematoxylin-eosin (**H&E**) staining and observation was carried out as in Lin et al. (2018). Briefly, the ilea were fixed for at least 24 h in 4% polyformaldehyde, embedded in paraffin wax, sectioned into approximately 5-μm-thick pieces, and stained with hematoxylin. The pathological changes in the ilea were evaluated under a light microscope (Olympus, Japan).

Immunofluorescence Staining

Immunofluorescence (**IF**) staining observation was carried out as in Huan et al. 2020. In brief, the sections were incubated with myeloperoxidase (**MPO**) (1:200, diluted) at 4°C overnight. After washing out excess antibody with phosphate-buffered saline (**PBS**), the sections were incubated with fluorescein (**FITC**)-conjugated goat anti-rabbit or anti-mouse immunoglobulin (**Ig**)G (1:1,000, diluted). The sections were washed with PBS and stained with 4′, 6-Diamidino-2-Phenylindole (**DAPI**). The sections were observed and photographed under a fluorescence microscope (Nikon Eclipse C1, Tokyo, Japan). Then, the fluorescence intensity values were measured with ImageJ software.

Determination of Oxidative Stress Indices

Determination of antioxidant indices was carried out as in Zhang et al. (2021). Measurements of the activity of superoxide dismutase (**T-SOD**) (NanJing JianCheng Bioengineering Institute, China), catalase (**CAT**) (NanJing JianCheng Bioengineering Institute), and glutathione peroxidase (**GSH-Px**; NanJing JianCheng Bioengineering Institute, China) and the content of malondialdehyde (**MDA**; NanJing JianCheng Bioengineering Institute) in the ileum were performed strictly according to the commercial kit manuals. All the indexes were measured by microplate method.

Real-Time Quantitative Polymerase Chain Reaction Analysis

Total RNA was isolated from ileum samples using TransZol Up Reagent (Vazyme, Nanjing, China) according to the manufacturer's instructions, and then a GeneQuant 1300 spectrophotometer was used to determine the RNA concentration. The total RNA extraction method followed that of Dai et al. (2019). The RNA was reverse transcribed into cDNA using HiScript III RT-PCR (Vazyme, Nanjing, China) according to the manufacturer's instructions. The primers, shown in Table 2, were designed by using NCBI. Then, real-time quantitative polymerase chain reaction (**RT-qPCR**) was performed using ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China) and carried out using the Real-time PC Detection System (Bio-Rad CFX384 Touch, Foster City, CA). The relative mRNA expression levels were calculated by the $2^{-\Delta\Delta CT}$ method, and GAPDH was used as an internal reference gene to normalize gene transcription.

Table 2. Sequence of target genes primer.

Gene names	Sequence of primer (5′-3′)
GAPDH	F: AGTCGGAGTCAACGGATTTGG
	R: AAGATAGTGATGGCGTGCCC
IL-1β	F: GGTCAACATCGCCACCTACA
	R: CATACGAGATGGAAACCAGCAA
IL-6	F: GCCAGAGCCAGGGAGAATATC
	R: CCCTCACGGTCTTCTCCATAAA
IL-8	F: GCAAGGTAGGACGCTGGTAA
	R: GCGTCAGCTTCACATCTTGA
TNF-α	F: CAGATGGGAAGGGAATGAAC
	R: CACACGACAGCCAAGTCAAC
HO-1	F: ACAACGCTGAAAGCATGTCC
	R: GGATGCTTCTTGCCAACGAC
NQO-1	F: CGCACCCTGAGAAAACCTCT
	R: ACTGCAGTGGGAACTGGAAG
SOD-1	F: CCAAAAGATGCAGATAGGCACG
	R: GCAGTGTGGTCCGGTAAGAG
CAT	F: AGCTTGCAAAATGGCTGACG
	R: ATAGCCAAAGGCACCTGCTC
ZO-1	F: GGCAGCTATCAGACCACTCT
	R: ACTTGTAGCACCATCTGCCT
ZO-2	F: CGGACTGTCATCTCGTTCAGGCAC
	R: GCTGGGAAGGAAGAGAACCT
ZO-3	F: CGGCAAGATCGCCAACATTA
	R: CCATGAGGGTCGTAGTCCTC
Claudin1	F: TACAGCCCTTGGCCAATACA
	R: CCAAGAAACAACCACCAGCA
Claudin2	F: GATACGTGTAGCAGCAGCAG
	R: AGCTGGGATTTCTGAGCAGT
Claudin3	F: AAGGTGTACGACTCCATGCT
	R: CGATGGTGATCTTGGCCTTG
Occludin	F: CCTCATCGTCATCCTGCTCT
	R: GGTCCCAGTAGATGTTGGCT
MUC2	F: CAGGATACGTGTGTGCCCAT
	R: GGACGCGTTGCAATCAAAGT
AvBD1	F: ATGCGGATCGTGTACCTGCTC
	R: CTGCTTGGGATGTCTGGCTCT
AvBD2	F: CTCTCTCCTCTTCCTGGCAC
	R: GAGGGGTCTTCTTGCTGCTG
AvBD4	F: ACGCTGATCTGCAGGACTAC
	R: GAGAACGGGAAAAGCCCACAG
Lyz	F: TCTGGGGAAAGTCTTTGGACG
	R: TTTTGCAACACACACCCAGTT
PI3K	F: TTAAACGCGAAGGCAACGA
	R: CAGTCTCCTCCTGCTGTCGAT
AKT	F: AAAGAGCGCATGAGTGGACG
	R: CGTGGTCCTCCTTGTAGTAG
NF-Kb	F: CCACCAACTACAACGGCCA
	R: CTGGTATGGGGAGTAGCCCT

Western Blotting

Western blotting was carried out as in Zhuang et al. (2019). The protein levels of GAPDH, p-PI3K, PI3K, p-AKT, AKT, p-NF-κB, NF-κB, zonula occludens (**ZO**)-1, Claudin1, Occludin, SOD1, and heme Oxygenase-1 (**HO-1**) were determined by Western blot analysis. The primary antibodies used in this study were p-PI3K (1:1,000; BioGot, China), PI3K (1:1,000; Proteintech, Wuhan, China), p-AKT (1:1,000; Bimake, China), AKT1 (1:1,000; Bimake), p-NF-κB (1:1,000; Bimake), NF-κB (1:500; BioGot), ZO-1 (1:1,000; Abclonal, China), Claudin1 (1:500; Wanleibio, China), Occludin (1:500; Wanleibio, China), SOD-1 (1:2,000;

Proteintech, Wuhan, China), HO-1 (1:500; Wanleibio). The anti-rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Protein bands were imaged by Image Lab Software (Bio-Rad) and analyzed by ImageJ software. The GAPDH (1:5,000; Proteintech, Wuhan, China) band was used as a control to perform standardized quantitative analysis on Western blots.

Statistical Analysis

The statistical analysis was performed using IBM SPSS Statistics 26 and Microsoft Excel 2019 software. The data are expressed as the mean ± standard deviation (**SD**). Date among all treatments were analyzed by One-way analysis of variance (**ANOVA**) followed by Dunnett multiple comparison if the data were Gaussian distribution and had equal variance, or analyzed by Kruskal-Wallis followed by Dunn's multiple comparisons if the date were not normally distributed. The Gaussian distribution of data was analyzed by D'Agostino-Pearson omnibus normality test and Kolmogorov-Smirnov test was tested for multiple comparisons. Finally, data graphs were generated using Prism 8.0 software. Differences were considered significant at $P < 0.05$ and are indicated as follows: *ns* $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

RESULTS

Effect of Dietary BA Supplementation on Diarrhea Rate and Mortality

After injecting the *E. coli* fluid, the number of diarrhea events and the mortality rate of the chicks were recorded. The results showed that when BA was added to the diet 15 d prior to *E. coli* infection, the BAI10 group and BAI20 group demonstrated a reduction in the diarrhea rate after injection of *E. coli* from 73.3 to 33.3 and 20%, respectively, in a dose-dependent manner (Table 3). Additionally, the mortality rate was decreased from 53.3 to 6.7% in the BA prevention groups (BAI10 and BAI20 groups) after injection of *E. coli* (Table 3). Overall, the results indicate that dietary BA supplementation could decrease the incidence of diarrhea and the mortality rate.

Table 3. Diarrheic rate and mortality.

Group	Diarrheic rate (%)	Dead rate (%)
Control	0.0	0.0
E. coli	73.3	53.3
BAI10	33.3	6.7
BAI20	20.0	6.7

Histopathological Analysis of Ileum Tissues

HE staining of chick ileum tissues showed that the intestinal villi were intact and clear in the control group and that columnar epithelial cells were tightly connected. However, compared with that in the control group, the intestinal villi in the *E. coli* group were atrophied, and the crypts were hyperplastic. In particular, inflammatory cell infiltration and swelling and bleeding of the intestinal villi were observed in the *E. coli* group (arrowheads in Figure 1B). Nevertheless, compared with that in the *E. coli* group, the numbers of lesions in the BAI10 and BAI20 groups were reduced, and goblet cells were expanded to resist invasion (Figures 1C−1D).

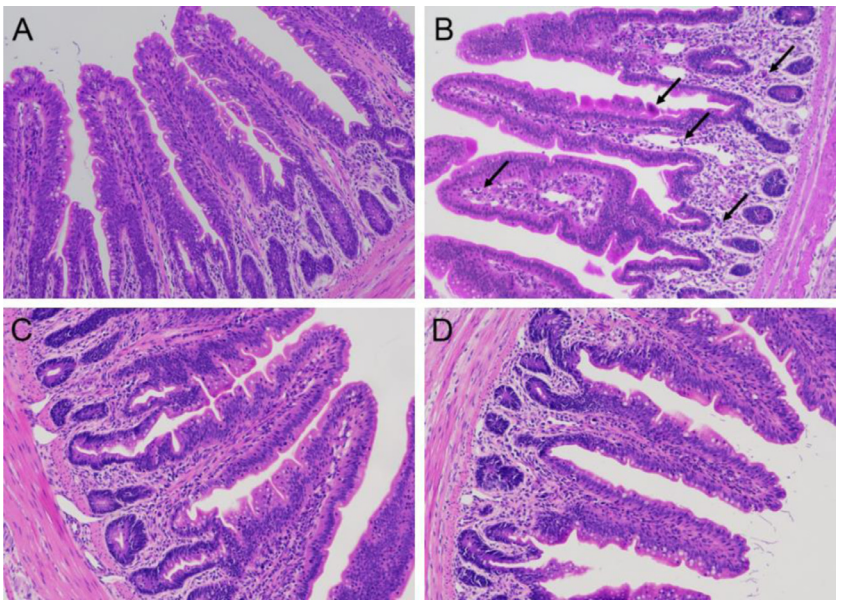

Figure 1. Histopathology with hematoxylin and eosin staining (200 ×) of ileal sections from the control group (A), *E. coli* group (B), BAI10 (10 mg/kg baicalin + *E. coli*) group (C), and BAI20 (20 mg/kg baicalin + *E. coli*) group (D). In the images, the arrowheads indicate the location of the lesions (B).

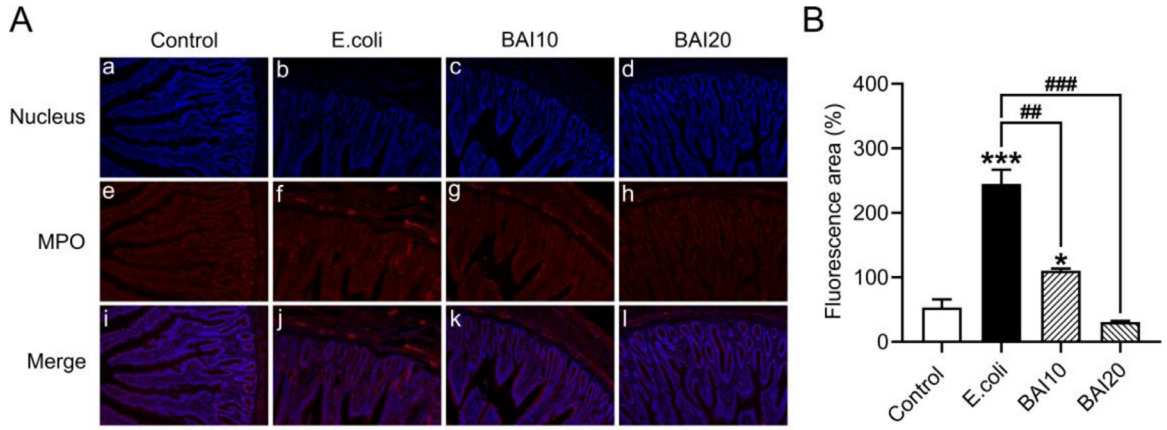

Figure 2. Immunofluorescence images (200 ×) of ileal sections from the control group, *E. coli* group, BAI10 group, and BAI20 group. (A) In the images, nuclear staining is shown in blue (a, b, c and d), MPO staining is shown in red (e, f, g and h), and the colocalization signals are shown in merged images (i, j, k and l). (B) Quantitative analysis of MPO staining. Data are presented as the mean ± SD of at least three independent experiments. "*" indicates a significant difference compared with the control group ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). "#" indicates a significant difference between the corresponding groups ($\#P < 0.05$, $\#\#P < 0.01$ and $\#\#\#P < 0.001$). Below is the same.

IF Staining

IF staining of chick ileum tissue showed that the MPO content in the *E. coli* group was dramatically ($P < 0.001$) improved compared with that in the control group, indicating an increased inflammatory response and elevated oxidative stress levels in vivo. However, in the BAI10 and BAI20 groups, the expression of MPO was noticeably ($P < 0.01$ or $P < 0.001$) decreased compared with that in the *E. coli* group, and the expression levels were basically the same as that in the control group (Figures 2A−2B).

BA Increase Mucosal Tight-Junction Proteins

The results in Figures 3A−3B show that in the BAI10 and BAI20 groups, in contrast to the *E. coli* group and the control group, the mRNA levels of ZO-1, ZO-2, ZO-3, Claudin2, Occludin, and mucin (**MUC**)2 were significantly ($P < 0.05$ or $P < 0.01$) decreased, and the mRNA levels of Claudin1 and Claudin3 were decreased, but not significantly ($P > 0.05$). However, there was a remarkable ($P < 0.05$ or $P < 0.001$) increase in ZO-1, ZO-2, ZO-3, Claudin1, Claudin2, Claudin3, Occludin, and MUC2 mRNAs in the BAI10 and BAI20 groups compared with the *E. coli* group. Similarly, as shown in Figures 3C−3D, the mRNA levels of avian β-defensin (**AvBD**)1, AvBD2, AvBD4, and lysozyme (**Lyz**) were significantly ($P < 0.05$ or $P < 0.001$) elevated in the BAI10 and BAI20 groups compared with the *E. coli* group. Consistently, the protein levels of ZO-1, Claudin1, and Occludin were markedly ($P < 0.05$ or $P < 0.01$) decreased compared with those in the *E. coli* group and the control group. The protein levels of ZO-1, Claudin1, and Occludin were significantly ($P < 0.05$ or $P < 0.01$) increased in the BAI10 and BAI20 groups compared with the *E. coli* group (Figures 3E−3F).

Effects of E. coli and BA on Oxidative Stress Response

As shown in Figures 4A−4D, the activities of CAT, GSH-Px, and T-SOD were considerably depressed in the *E. coli* group ($P < 0.05$ or $P < 0.01$), whereas the activity of MDA was increased but not significantly ($P > 0.05$) compared with the control group. However, in the BAI10 and BAI20 groups, there was a significant ($P < 0.05$ or $P < 0.01$, or $P < 0.001$) increase in the activities of CAT and T-SOD and a decrease ($P > 0.05$) in the content of MDA compared with that in the *E. coli* group.

The RT-qPCR results verified that the mRNA levels of HO-1 and NQO-1 were increased in the *E. coli* group compared with the control group; however, the levels of HO-1 and NQO-1 were downregulated in the BAI10 and BAI20 groups compared with the *E. coli* group (Figures 4E−4F). In contrast, compared to those in the *E. coli* and the control groups, the mRNA levels of SOD-1 and CAT were markedly ($P < 0.05$ or $P < 0.01$) decreased, but the mRNA levels of SOD-1 and CAT were markedly ($P < 0.01$ or $P < 0.001$) increased, in the BAI10 and BAI20 groups (Figures 4E−4F). Consistently, compared with the control group, the protein level of HO-1 was upregulated ($P < 0.05$), notably in the *E. coli* group (Figures 4G−4H). In addition, compared to that in the *E. coli* and control groups, the protein expression of SOD-1 in the BAI10 and BAI20 groups was noticeably ($P < 0.01$) downregulated (Figures 4G−4H). However, the BAI10 and BAI20 groups showed robust decreases ($P < 0.05$ or $P < 0.001$) in the protein level of HO-1 and increases ($P < 0.05$ or $P < 0.01$) in the protein level of SOD-1 compared with the *E. coli* group (Figures 4G−4H).

Effect of BA on Inflammatory Factors

RT-qPCR analysis confirmed that the relative mRNA levels of proinflammatory factors (IL-1β, IL-6, IL-8, and

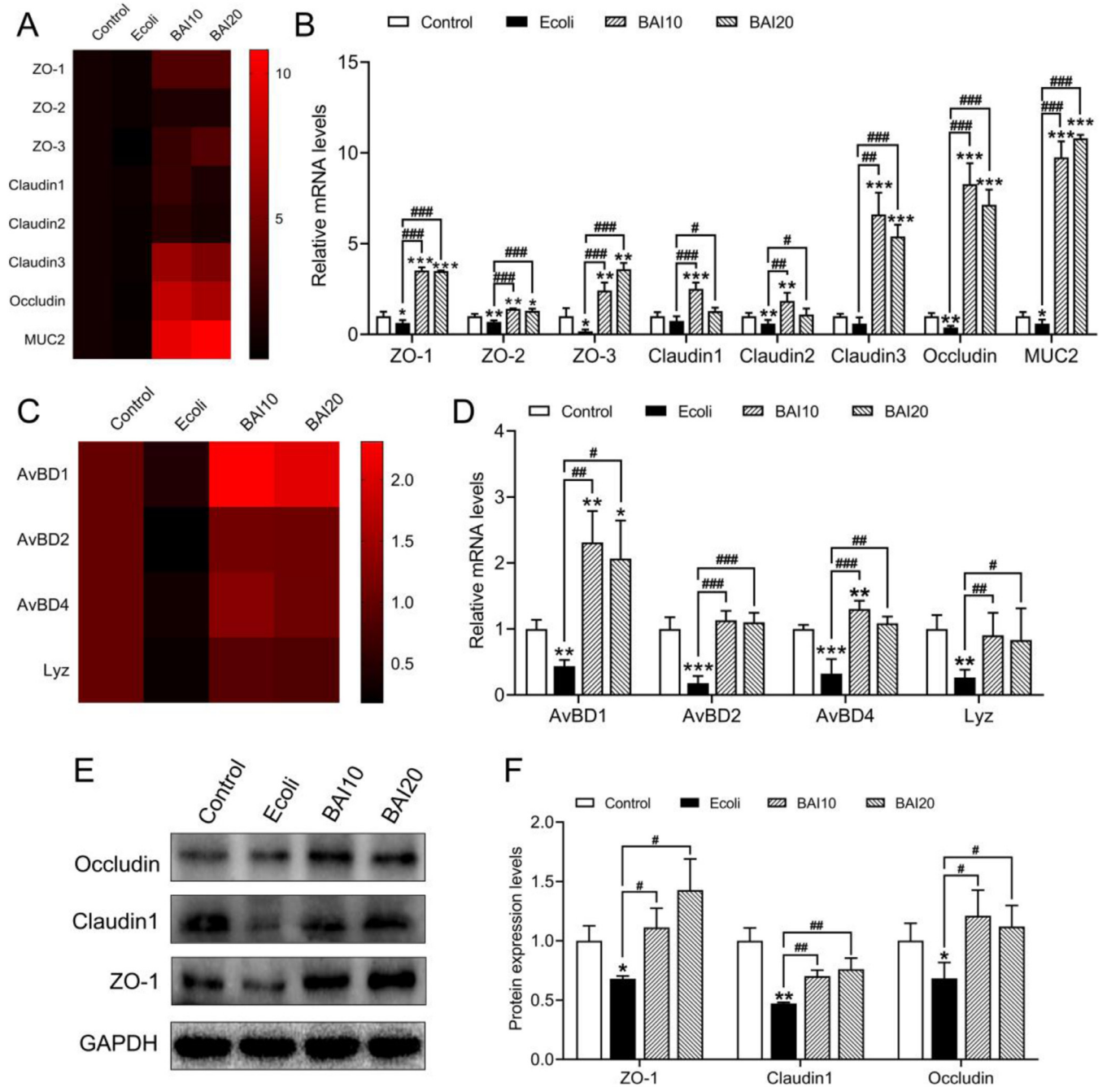

Figure 3. Baicalin ameliorated APEC-induced impairment of the intestinal barrier. (A) The heat map shows the mRNA levels of the intestinal barrier genes. (B) Effects of the control, *E. coli*, BAI10, and BAI20 groups on the mRNA levels of intestinal barrier genes. (C, D) Effects of the control, *E. coli*, BAI10, and BAI20 groups on the mRNA levels of AvBD1, AvBD2, AvBD4 and Lyz. (E, F) Effects of the control, *E. coli*, BAI10 and BAI20 treatments on the protein levels of ZO-1, Claudin1, and Occludin.

TNF-α) were markedly ($P < 0.05$ or $P < 0.001$) increased in the *E. coli* group compared with the control group (Figures 5A−5B). The BAI10 and BAI20 groups exhibited a significant ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) reduction in the levels of the above proinflammatory factors compared with the *E. coli* group (Figures 5A−5B). The relative mRNA expression levels of anti-inflammatory factors (IL-4, IL-10, and TGF-β) were markedly ($P < 0.01$ or $P < 0.001$) decreased in the *E. coli* group compared with the control group. However, compared with the *E. coli* group, the BAI10 and BAI20 groups exhibited a noticeable ($P < 0.05$ or $P < 0.001$) increase in the expression of the above anti-inflammatory factors (Figures 5C−5D).

BA Increases p-PI3K, p-AKT and p-NF-κB mRNA, and Protein Levels

As shown in Figures 6A−6B, the RT-qPCR results revealed that the mRNA levels of AKT and NF-κB in the *E. coli* group were dramatically ($P < 0.05$ or $P < 0.01$) improved compared with those in the control group. However, the mRNA levels of PI3K were

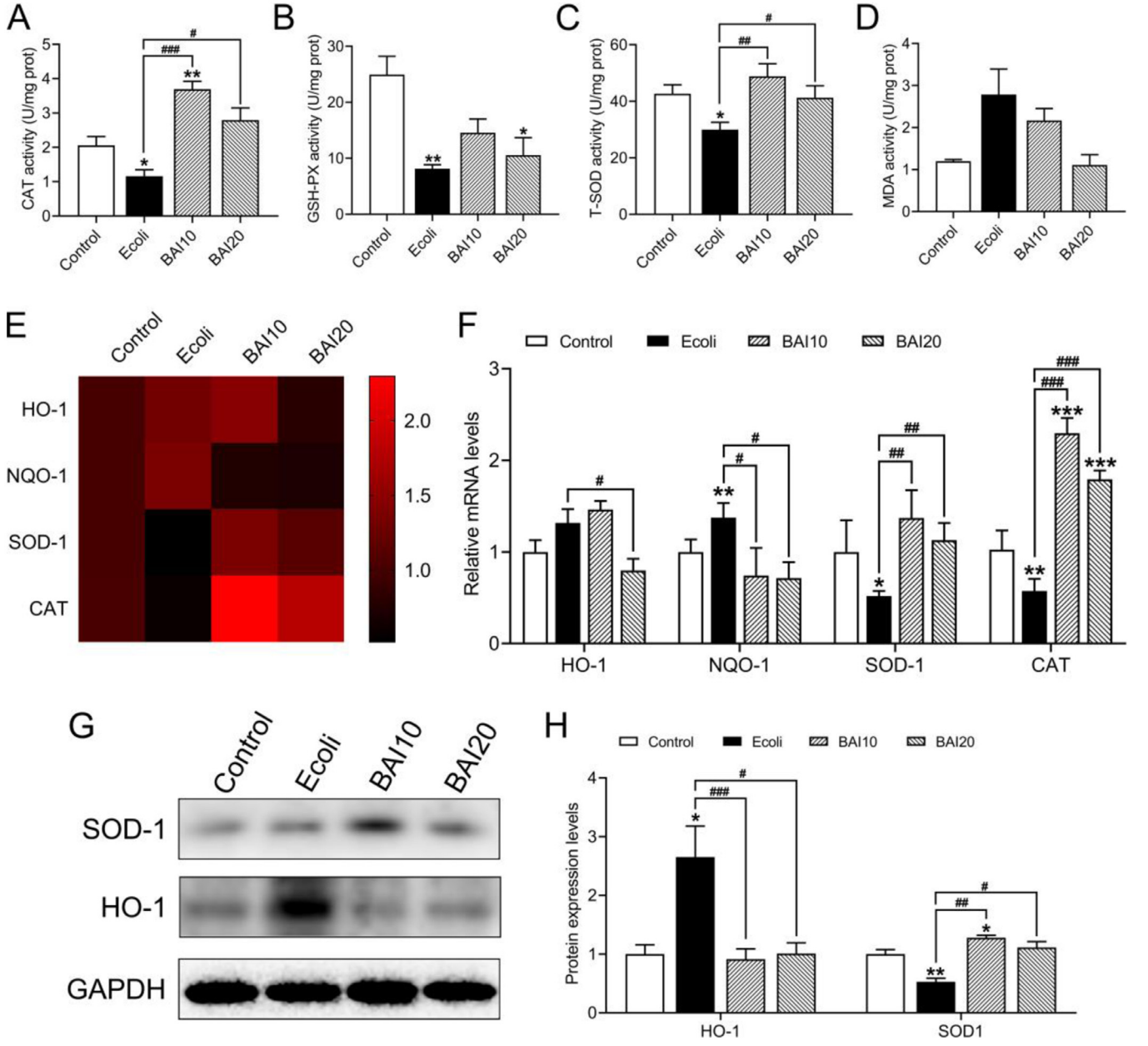

Figure 4. Baicalin ameliorated APEC-induced oxidative stress. (A) CAT activity; (B) GSH-Px activity; (C) T-SOD activity; (D) MDA activity; (E) heat map showing the mRNA levels of antioxidant genes; (F) effects of the control, *E. coli*, BAI10 and BAI20 groups on the mRNA levels of antioxidant genes; (G, H) effects of the control, *E. coli*, BAI10 and BAI20 groups on the protein levels of HO-1 and SOD-1.

increased but were not significantly different ($P > 0.05$) from those in the control group. However, the mRNA levels of PI3K, AKT, and NF-κB in the BAI10 and BAI20 groups were decreased dramatically ($P < 0.05$ or $P < 0.01$) compared with those in the *E. coli* group. Further confirming the levels of PI3K/AKT/NF-κB signaling pathway-related proteins, there were notable ($P < 0.01$) increases in the protein levels of p-PI3K, p-AKT, and p-NF-κB in the *E. coli* group compared with the control group (Figures 6C–6G). Additionally, the protein levels of p-PI3K/PI3K, p-AKT/AKT, and p-NF-κB/NF-κB were increased ($P < 0.01$ or $P < 0.001$) in the *E. coli* group compared with the control group (Figure 6F). However, the BAI10 and BAI20 groups exhibited a marked ($P < 0.05$) decrease in the protein levels of p-PI3K/PI3K, p-AKT/AKT, and p-NF-κB/NF-κB compared with the *E. coli* group (Figure 6F).

DISCUSSION

Avian colibacillosis is a complex disease in poultry that is caused by APEC and frequently has serious effects on mortality, diarrhea rates, and egg production losses in birds and causes extreme economic losses in global poultry production (Korf et al., 2020). Because of the imbalance in the nutrition levels of chicks and the instability of their intestinal flora, chicks are more likely to be affected by external pathogens at the early stage of growth, especially *E. coli* (Wang et al., 2021).

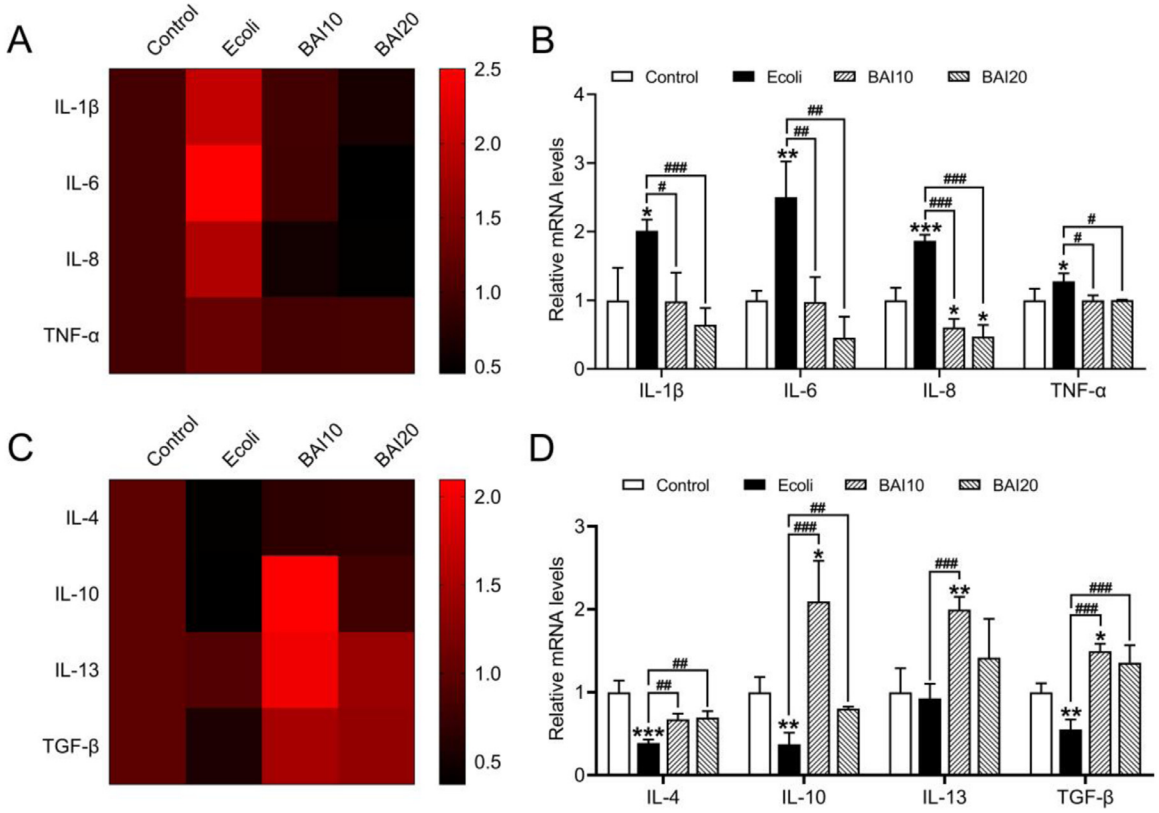

Figure 5. Effect of baicalin on inflammatory factors mRNA levels. (A) Heat map shows the mRNA levels of pro-inflammatory (IL-1β, IL-6, IL-8, and TNF-α) genes. (B) Effects of Control, E. coli, BAI10, and BAI20 groups on the mRNA levels of proinflammatory (IL-1β, IL-6, IL-8, and TNF-α) genes. (C) Heat map shows the mRNA levels of anti-inflammatory (IL-4, IL-10, IL-13, and TGF-β) genes. (D) Effects of Control, E. coli, BAI10, and BAI20 groups on the mRNA levels of anti-inflammatory (IL-4, IL-10, IL-13, and TGF-β) genes.

BA is a polyphenolic compound isolated from a variety of traditional Chinese medicines that has been reported to possess multiple biological activities, such as antioxidant, anti−inflammatory, and anticancer activities (Dinda et al., 2017; Ren et al., 2017). In the present study, we verified that BA can effectively alleviate intestinal damage and intestinal inflammation induced by APEC by improving the antioxidant capacity of the body and upregulating the expression of intestinal barrier proteins.

Severe watery diarrhea and dehydration are the main symptoms of APEC-induced colibacillosis, which causes high mortality in chicks (Peng et al., 2019). Recent studies have indicated that the intestinal tract is considered to be one of the primary target organs affected by APEC-induced colibacillosis (Kathayat et al., 2021). Moreover, chicks infected by APEC frequently show intestinal morphological damage, destruction of intestinal barrier integrity and so on. (Kathayat et al., 2021). Therefore, the intestinal barrier, which can resist the invasion of external pathogens, is particularly important. In the present study, BA significantly reduced the diarrhea rate and mortality of chicks and improved intestinal villus atrophy, bleeding, inflammatory cell infiltration, and other conditions caused by APEC infection. MPO is a marker of intestinal damage that can regulate the inflammatory response of the organism, leading to the formation of oxides and tissue cell damage (Alvarenga et al., 2016). In addition, the amount of MPO can be used to represent the degree of intestinal injury and the degree of inflammatory response. Interestingly, in the present study, BA significantly reduced the amount of MPO in the ileum after APEC injection.

The intestinal epithelial barrier is an important barrier for resistance against pathogenic microorganisms and the maintenance of organism health, and a healthy intestinal barrier is also important in reducing the inflammatory response in vivo (Wang et al., 2020). The intestinal barrier primarily consists of physical (intestinal mechanical barrier and mucus layer), chemical, and immunological barriers (antimicrobial peptides and Lyz; Suzuki, 2020). Claudins, ZOs, and Occludin are key backbone proteins, peripheral membrane proteins and transmembrane proteins of tight junctions, respectively, and together, they maintain the integrity of tight junctions (**TJs**) and the intestinal mechanical barrier (Shen et al., 2020; Yuan et al., 2021). Wu et al. (2020) revealed that the protein levels of Claudin-1, Occludin, and ZO-1 were markedly decreased in patients with predominant rotavirus damp heat diarrhea. Mucoprotein 2 (MUC2), the major protective mucin in the mucus layers, is a large, gel-forming glycoprotein secreted by intestinal goblet cells. Antimicrobial peptides (AvBD1, AvBD2, and AvBD4) and Lyz are important components of the intestinal chemical barrier, which can protect the intestinal mucosa from damage (Fusco et al.,

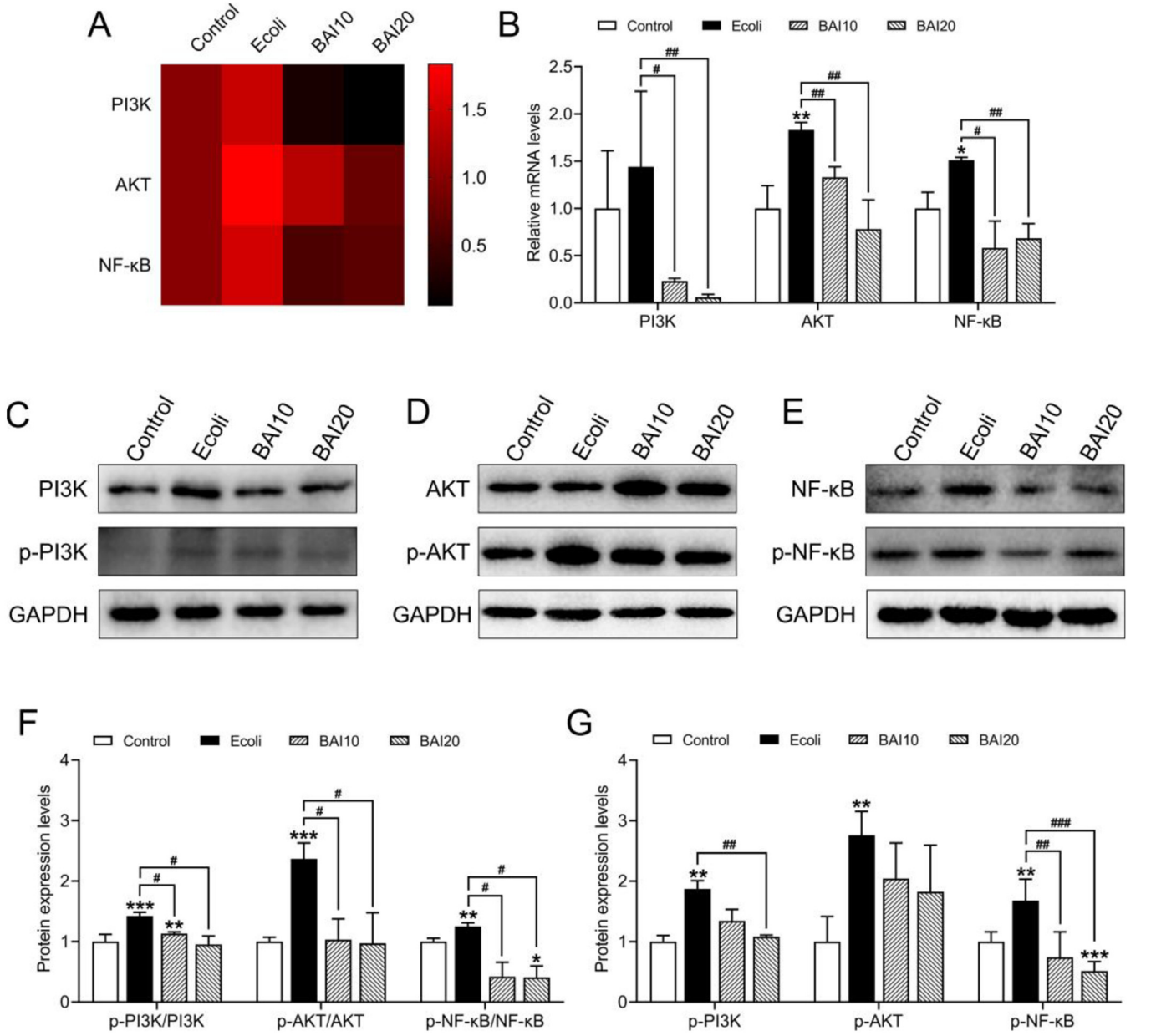

Figure 6. The effects of Baicalin on the relative mRNA and protein levels of phosphorylated PI3K/ AKT/ NF-κB. (A) Heat map shows the mRNA levels of PI3K, AKT, and NF-κB; (B) Effects of Control, E. coli, BAI10, and BAI20 groups on the mRNA levels of PI3K, AKT, and NF-κB; (C-G) Effects of Control, E. coli, BAI10 and BAI20 groups on protein levels of PI3K, p-PI3K, AKT, p-AKT, NF-κB, p-NF-κB, p-PI3K/PI3K, p-AKT/AKT, and p-NF-κB/ NF-κB.

2021). In addition, multiple studies have found that BA can strengthen the intestinal barrier and maintain intestinal health by affecting multiple functions of the body, including alleviating the inflammatory response and enhancing antioxidant capacity (Liu et al., 2020; Rizzo et al., 2021). Notably, the results of the present study showed that after APEC infection, the mRNA and/or protein levels of TJs, MUC2, AvBDs, and Lyz were markedly downregulated, indicating that the intestinal barrier had been destroyed; these results are consistent with Wu et al. (2020). However, BA treatment significantly upregulated the mRNA and/or protein levels of the above genes. These results are consistent with the intestinal morphological alterations in the group of chicks pretreated with BA. According to our results, APEC can affect intestinal health and damage the intestinal barrier, and BA can significantly improve these conditions.

Intestinal injury is manifested not only by damage to the intestinal barrier but also by weakening the antioxidant capacity of the body. Antioxidant enzymes (CAT, GSH-Px, and T-SOD) play key roles in protecting cells from oxidative stress by eliminating free radicals produced in metabolic reactions and/or activated by immunostimulants (Reyes-Becerril et al., 2019). In addition, as a marker of lipid peroxidation, the MDA content can reveal the extent of lipid peroxidation, which indirectly reflects the formation of free radicals and in turn the degree of organism damage (Duan et al., 2019). The present study showed that the activities of CAT, GSH-

Px, and T-SOD were significantly decreased and that the content of MDA was increased in APEC-treated chicks, which indicated that the antioxidative activities of APEC-treated chicks were damaged. As expected, dietary supplementation with BA can enhance the antioxidant defense system by enhancing the activities of CAT, GSH-Px, and T-SOD and decreasing the content of MDA. As oxidative stress occurs, HO-1, NOQ1, and other cytoprotective enzymes activated by Nrf2 can reduce ROS levels and ultimately maintain redox homeostasis to protect organisms against various stresses. The present study further showed that BA antagonizes APEC-induced intestinal injury in chicks by suppressing the transcription levels of HO-1 and NOQ1, downregulating the mRNA and protein levels of HO-1, and downregulating the transcription and protein levels of SOD-1. Taken together, these findings show that APEC could cause severe oxidative stress with resultant intestinal injury and that severe oxidative stress could lead to enterocyte damage, resulting in further intestinal inflammation.

Inflammation is the body's defense response to noxious stimuli, and APEC stimulation produces large numbers of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β. TNF-α is the foremost inflammatory cytokine that activates the NF-κB pathway and upregulates the expression of other inflammatory cytokines, thereby stimulating and aggravating the inflammatory response (Li et al., 2019; Yao et al., 2019). In addition, He et al. (2011) found that APEC increased the serum levels of TNF-α and IL-1, leading to inflammation. In line with previous studies, this study showed that APEC infection in chicks induced the secretion of inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8. Additionally, IL-4, IL-10, IL-13, and TGF-β are primarily anti-inflammatory cytokines and are responsible for restraining the production of proinflammatory cytokines. David et al., Dmitry et al. and other authors showed that IL-4, IL-10, and TGF-β could facilitate tissue repair and improve survival in animals challenged with noxious stress (Szczepankiewicz et al., 2018; Chistyakov et al., 2020; Xue et al., 2020). In the present study, BA significantly decreased the APEC-induced expression of inflammatory factors (TNFα, IL-1β, and IL-6) in ileal tissue and alleviated the damaging effects of APEC on the ileum. BA also promoted the secretion of anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) in chicks infected by APEC.

The PI3K/AKT signaling pathway has significant effects on both inflammation and cancer development, and the activation of NF-κB may result in the release of different types of inflammatory factors and ultimately induce intestinal inflammation (Borges et al., 2016; Rahmani et al., 2020). Qian et al. (2018) reported that the activation of the PI3K/AKT pathway could increase MMP production through multiple downstream targets, of which NF-κB has been identified as a pivotal regulator. In addition, upon the stimulation of cytokine receptors and other receptors, the membrane protein PI3K can directly or indirectly induce AKT phosphorylation and then activate NF-κB (Fu et al., 2016). Moreover, studies have shown that the PI3K/AKT signaling pathway mediates the anti-inflammatory response by inhibiting the NF-κB/ cyclooxygenase-2 (**COX-2**)/TNF-α/IL-1β inflammatory signaling pathway in experimental ischemic stroke (Tu et al., 2015). Therefore, we hypothesized that inhibition of the PI3K/AKT pathway could reduce the inflammatory response by inhibiting the activation of NF-κB. The results from the present study showed that the mRNA and protein levels of PI3K, AKT, and NF-κB were markedly increased, proving that the PI3K/AKT/NF-κB signaling pathway was activated by AEPC. However, the mRNA and protein levels of p-PI3K, p-AKT, and p-NF-κB were significantly decreased by BA treatment. It was further verified that BA can inhibit inflammation by inhibiting the activation of the PI3K/AKT/NF-κB signaling pathway.

Collectively, our findings showed that BA attenuated pathological changes in the ileum, oxidative stress and the inflammatory response in APEC-infected chicks. The mechanism may be attributed to the suppression of PI3K/AKT-mediated NF-κB signaling pathways. All the above results strongly suggest that BA is a viable therapeutic agent in the treatment of inflammatory diseases induced by bacteria, especially *E. coli*.

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DISCLOSURES

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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