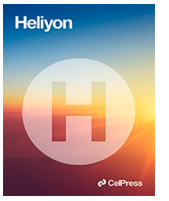
Heliyon 10 (2024) e33161

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Anemoside B4 attenuates necrotic enteritis of laying hens induced by *Clostridium perfringens* via inhibiting NF-κB and PI3K/Akt/mTOR signalling pathways

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A R T I C L E I N F O

Keywords:
Anemoside B4
Clostridium perfringens
Necrotic enteritis
NF-κB
PI3K/Akt/mTOR
Tight junctions

A B S T R A C T

Poultry necrotic enteritis is an important enteric disease which might be controlled by antibiotics. However, with the excessive use of antibiotics, the phenomenon of drug resistance of *Clostridium perfringens* is becoming increasingly prominent. Anemoside B4 exhibits important anti-inflammatory, antioxidant and immunomodulatory effects. This study was performed to estimate the effect of Anemoside B4 on chicken necrotic enteritis induced by *C. perfringens in vivo* and *in vitro*. In the *in vivo* experiment we investigated the efficacy of Anemoside B4 on the growth curve, biofilm formation, haemolytic activity, virulence-related gene expression and NF-κB and PI3K/AKT/mTOR activation in Caco-2 cells induced by *C. perfringens*. The results showed that 12.5–50 μg/mL Anemoside B4 had no antibacterial activity but could inhibit biofilm formation, attenuate haemolytic activity and virulence-related gene expression of *C. perfringens* and weaken NF-κB and PI3K/Akt/mTOR activation triggered by *C. perfringens* in Caco-2 cells. In the *in vivo* experiment, 60 17-day-old healthy White Leghorns were randomly divided into six groups. The growing laying hens of the control group were fed a basic diet, and those of the five challenged groups were fed a basic diet (infection group), added 0.43 g/kg Anemoside B4 (0.43 g/kg Ane group), 0.86 g/kg Anemoside B4 (0.86 g/kg Ane group), 1.72 g/kg Anemoside B4 (1.72 g/kg Ane group) and 40 mg/kg lincomycin (lincomycin group), respectively. All challenged laying hens were infected with 1×10^9 CFU *C. perfringens* from day 17–20. Blood and intestinal samples were obtained, and the data demonstrated that Anemoside B4 improved the blood biochemical parameters, attenuated jejunum tissue injury, increased the spleen, thymus, bursa of fabricius index, and decreased lesion scores of the jejunum and the ileum. In the jejunum, Anemoside B4 and lincomycin downregulated the expression of *IL-1β*, *IL-6*, *IL-10*, *TNF-α* and *IFN-γ* at mRNA levels. Moreover, Anemoside B4 significantly enhanced both mRNA and protein levels of tight junctions ZO-1, Claudin-1 and MUC-2 in the jejunum. Anemoside B4 weakened p-P65, p-PI3K, *p*-Akt and *p*-mTOR protein expression in the jejunum infected by *C. perfringens*. Diets supplemented with

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https://doi.org/10.1016/j.heliyon.2024.e33161
Received 19 March 2024; Received in revised form 13 June 2024; Accepted 14 June 2024
Available online 15 June 2024

Anemoside B4 alleviated *C. perfringens*-induced necrotic enteritis in laying hens by inhibiting NF-κB and PI3K/Akt/mTOR signalling pathways and improving intestinal barrier functions.

1. Introduction

Clostridium perfringens (*C. perfringens*) is a bacterial species commonly found in the environment, including the intestines of humans and animals [1]. As a spore-forming bacterium, it can produce toxins, causing various diseases in both animals and humans [2]. In livestock, this species can cause diseases such as gas gangrene, and in sheep and goats, it is linked to enterotoxaemia [3]. It also causes necrotic enteritis in poultry, specifically chickens [4], where is damages the intestinal lining of the birds, contributing to serious economic losses in the poultry industry because of higher mortality rates and growth rate reductions [5].

C. perfringens triggers necrotic enteritis and related subclinical disease, causing substantial economic problems in the broiler industry [6]. However, the pathogenesis of causing avian necrotic enteritis is still unclear. Toxins are considered important factors involved in bacterial pathogenesis [7]. Based on a previous study, *C. perfringens* produces alpha toxin, which is a potent cytolytic toxin that damages host cells by disrupting cell membranes [8]. Alpha toxin can also cause tissue damage and inflammation, contributing to the pathogenesis of necrotic enteritis [9]. *C. perfringens* also secretes NetB toxin, which is associated to the development of avian necrotic enteritis in chickens [10]. Specifically, NetB toxin damages the intestinal epithelial cells, leading to inflammation and necrosis of the gut tissue [11]. *C. perfringens* can also produce collagenase and hyaluronidase, enhancing tissue degradation and facilitating the spread of this bacterium [12]. According to previous findings, *C. perfringens* possesses adhesins, allowing it to adhere to host tissues and initiate colonisation, thereby contributing to subsequent infection [6]. A previous study showed that *C. perfringens* is capable of forming biofilms, improving its survival and resistance to host immune responses and antimicrobial treatments [13]. These virulence-related factors contribute to the pathogenesis of necrotic enteritis caused by *C. perfringens*.

Necrotic enteritis elicited by *C. perfringens* could be controlled by antibiotics. However, against the background of antimicrobial resistance due to the excessive use of antibiotics, traditional Chinese medicine might be a better choice for controlling necrotic enteritis caused by this bacterial species [14]. Anemoside B4 (AneB), as a natural compound that belongs to a class of triterpenoid saponins, is derived from the plant species *Pulsatilla chinensis* [15] and has important biological activities, such as anti-inflammatory, antioxidant and immunomodulatory effects [16]. In a previous study, Anemoside B4 ameliorated dextran sulphate sodium-induced ulcerative colitis through inhibiting NLRP3 inflammasome [17]. In addition, Anemoside B4 provides protection against acute lung injury by attenuating inflammation responses through blocking NLRP3 inflammasome activation and TLR4 dimerisation [18] and modulating mTOR/p70S6K-mediated autophagy [19]. Anemoside B4 also inhibits cisplatin-induced nephrotoxicity in mice through NF-κB- and MAPK-mediated apoptosis pathways [20]. However, it is still unclear whether Anemoside B4 can attenuate necrotic enteritis of chickens induced by *C. perfringens*.

The aim of this study was to investigate the effect of Anemoside B4 on chicken necrotic enteritis induced by *C. perfringens*. In this context, Anemoside B4 can be considered a novel drug to control chicken necrotic enteritis induced by *C. perfringens*.

2. Materials and methods

2.1. Ethics statement

The animal studies were approved by the Animal Care and Use Committee of Wuhan Polytechnic University, Hubei Province, China (WPU202308002). All experimental animals were euthanised at the end of the experiment.

2.2. Bacterial and cell culture condition

Anemoside B4 was bought from Chengdu Purechem-Standard co., LTD (Sichuan, China).

Type G strain, *C. perfringens* DK2 producing NetB toxin, was isolated from a commercially produced chicken small intestine which showed typical characteristic of necrotic enteritis. *C.* perfringens was grown in TGY (tryptose glucose yeast extract) broth (BD, USA) or on *Brucella* agar (Hopebio, Qingdao, China) at 37 °C under anaerobic conditions.

The Caco-2 cells were cultured in MEM culture medium (Thermo Fisher Scientific, NY, USA) and supplemented with 20 % foetal bovine serum (FBS) (Thermo Fisher Scientific, USA) at 37 °C under 5 % CO_2.

2.3. Determination of the effect of Anemoside B4 on the growth curve of C. perfringens

The growth curve showing the effects of Anemoside B4 on *C. perfringens* was determined as previously described [21]. Briefly, C. *perfringens* (3×10^8 CFU/mL) and Anemoside B4 (0, 12.5, 25, and 50 μg/mL) were co-cultured in tubes under 37 °C. Following co-culturing for 0, 2, 4, 6, 8, 10, 12 and 24 h, the bacterial samples were collected, diluted with sterile phosphate-buffered saline (PBS) and plated onto TSA plates. After incubation for 24 h under 37 °C, the colony numbers were determined, and the kinetic curves were generated by plotting the log10 CFU/mL.

2.4. Determination of the effect of Anemoside B4 on the biofilm formation of C. perfringens

The effect of Anemoside B4 on biofilm formation by *C. perfringens* was determined using a quantitative assay as described previously [22]. Briefly, the wells of a microplate treated with Anemoside B4 (12.5, 25 and 50 μg/mL) for 3 h at 37 °C, and biofilm formation by *C. perfringens* was determined, and the wells treated with PBS was set as the negative control. A 24-h treatment culture of *C. perfringens* was diluted with TSB to obtain an OD_{660} of 0.1. Subsequently, the samples (100 μL) were added to the treated wells in the 96-well microplate. After 24 h of incubation, the free-floating bacteria and spent media were discarded using a 13-g needle. After washing five times with PBS, the *C. perfringens* biofilms were stained with 0.05 % crystal violet (100 μL) for 1 h. Subsequently, the wells were washed five times with PBS to discard unbound crystal violet dye and dried for 1 h at 37 °C. After the addition of 100 μL 95 % (v/v) ethanol to each well, the plate was shaken for 15 min, and absorbance was read at 570 nm.

2.5. Determination of the effect of Anemoside B4 on the haemolytic activity of C. perfringens

The effect of Anemoside B4 on the haemolytic activity of *C. perfringens* was assessed as described elsewhere [23]. Briefly, the bacteria were grown overnight and resuspended with TSB to obtain an OD_{600} of 0.5. After treatment with Anemoside B4 for 6 h, the bacterial culture was centrifuged at 10,000 rpm for 15 min, and the supernatant was collected. Subsequently, sterile sheep red blood cells were added and cocultured at 37 °C for 30 min; PBS with and without Triton X-100 was used as the positive and negative control, respectively. The Anemoside B4 haemolysis rate was explored by determining absorption at 543 nm.

2.6. Determination of the effect of Anemoside B4 on the expression of virulence-related genes of C. perfringens

The RT-qPCR method was used to explore the effect of Anemoside B4 on the expression of virulence-related genes of *C. perfringens* [24]. Briefly, *C. perfringens* was grown in TGY broth with Anemoside B4 at concentrations of 12.5, 25 and 50 μg/mL to obtain anabsorptionof 0.8 of OD_{600} at 37 °C under anaerobic conditions. The *C. perfringens* RNA was extracted using TRIzol. After synthesising the cDNA by reverse transcriptase (TaKaRa, Dalian, China) from the purified RNA, cDNA amplification was carried out using the SYBR Green PCR Kit (TaKaRa, Beijing, China) in an ABI 7500 PCR System. The 16S rRNA gene was employed as the internal control. The

Table 1
Primer sequences for qRT-PCR analysis.

Gene		Nucleotide Sequence (5′-3′)	Tm (°C)
16S RNA	Forward	GGGGGTTTCAACACCTCC	56.5
	Reverse	GCAAGGGATGTCAAGTGT	52.4
netB	Forward	ACGGAAAAATGAAATGGCCTGAA	55.0
	Reverse	GCATTTATTCCAGCACCAGCA	56.0
pfoA	Forward	AGATGTACAAGCTGCTTTCA	51.0
	Reverse	TGTGCATCTCCTCCTAAAAC	51.7
plc	Forward	CCTGACACAGGGGAATCACAA	55.61
	Reverse	CTGCGCTATCAACGGCAGTA	54.2
virS	Forward	TCCTTCAATACAGGCTATGTGAT	53.36
	Reverse	TAAAGGACAAGTTAGAAATGGAAT	50.66
virR	Forward	CTAAAAGCACGAACTTCATAACC	52.63
	Reverse	ACCTTTGAGACAGGAGAGGATC	54.94
agrB	Forward	CATTAGAGGATTCTGAATGTGCT	52.73
	Reverse	TTATTCAGTATGGAACTTATGCTCT	52.52
agrD	Forward	TTTGGTTCCTCTGGTTGGTG	55.2
	Reverse	AAAACTTATTAACATTATTTGCTGC	47.1
IL-1β	Forward	CGAGGAGCAGGGACTTTGC	59.4
	Reverse	GAAGGTGACGGGCTCAAAAA	55.7
IL-6	Forward	CAGGACGAGATGTGCAAGAA	54.9
	Reverse	TAGCACAGAGACTCGACGTT	55.4
IL-10	Forward	CGCTGTCACCGCTTCTTCA	58.5
	Reverse	CGTCTCCTTGATCTGCTTGATG	55.7
TNF-α	Forward	CCCATCCCTGGTCCGTAAC	58.5
	Reverse	ATACGAAGTAAAGGCCGTCCC	57.0
IFN-γ	Forward	AAAGCCGCACACATCAAACACA	58.0
	Reverse	GCCATCAGGAAGGTTGTTTTC	54.3
ZO-1	Forward	GGAGTACGAGCAGTCAACATAC	55.2
	Reverse	GAGGCGCACGATCTTCATAA	55.6
Occludin-1	Forward	ACGGCAGCACCTACCTCAA	59.5
	Reverse	GGGCGAAGAAGCAGATGAG	56.4
Claudin-1	Forward	CATACTCCTGGGTCTGGTTGGT	59.0
	Reverse	GACAGCCATCCGCATCTTCT	57.8
MUC-2	Forward	TTCATGATGCCTGCTCTTGTG	55.5
	Reverse	CCTGAGCCTTGGTACATTCTTGT	57.0
GAPDH	Forward	GGTGAAAGTCGGAGTCAACGG	56.2
	Reverse	CGATGAAGGGATCATTGATGGC	58.7

primers used in this study are shown in Table 1.

2.7. Dosing schedule effect of Anemoside B4 on Caco-2 cell viability in vitro

The Caco-2 cell viability was examined using the cell counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Japan) [25]. Briefly, 1×10^6 Caco-2 cells were plated into 96-well plates (Costar, USA) and treated with 0, 6.25, 12.5, 25, 50 and 100 μg/mL Anemoside B4 for 24 h at 37 °C with 5 % CO_2. After the addition of 10 μL CCK-8 solution to each well and incubation for 1 h, the optical density at 450 nm was examined. The Caco-2 cell viability was determined using the following formula: cell viability (%) = (experimental well - blank well/control well - blank well) × 100 %. The data are presented as mean ± SD. The experiment was repeated at least three times.

2.8. Determination of the effect of Anemoside B4 on the activation of NF-κB and PI3K/Akt/mTOR signalling pathways in Caco-2 cells induced by C. perfringens

The efficacy of Anemoside B4 on NF-κB and PI3K/Akt/mTOR activation was measured using the Western blot method. Briefly, 1×10^6 Caco-2 cells were seeded into 24-well plates and treated with Anemoside B4 at concentrations of 12.5, 25 or 50 μg/mL for 2 h. Subsequently, 1×10^7 CFU/mL *C. perfringens* were added into the 24-well plate cells. After coculturing for 6 h, the Caco-2 cells were collected, and the NF-κB and PI3K/Akt/mTOR expressions were determined by Western blot.

2.9. Western blot

The protein expression levels were determined by the Western blot method. Briefly, total cell protein or tissue protein was isolated using RIPA Lysis Buffer (Beyotime Biotechnology, Shanghai, China). After measuring the protein concentration using a Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China), the total cell proteins were isolated by 12 % SDS-PAGE and transferred onto a PVDF (polyvinylidene difluoride) membrane. After blocking with 5 % skim milk for 90 min and washing three times with TBST, the membrane was incubated with primary antibody of Claudin-1 (ABclonal, Wuhan, China, 1:2000), MUC-2 (ABclonal, 1:2000), PI3K (ABclonal, 1:1000), p-PI3K (ABclonal, 1:1000), ZO-1 (proteintech, wuhan, China, 1:2000), Occludin-1 (proteintech, 1:2000), Akt (proteintech, 1:2000), *p*-Akt (proteintech, 1:2000), mTOR (proteintech, 1:5000), *p*-mTOR (proteintech, 1:2000), P65 (proteintech, 1:2000), p-P65 (proteintech, 1:2000), or GAPDH antibody (proteintech, 1:5000) for 12 h at 4 °C, respectively. After washing with TBST five times and culturing with corresponding HRP Goat Anti-Rabbit IgG (Claudin-1, MUC-2, PI3K, p-PI3K, ZO-1, Occludin-1, Akt, P65, p-P65, GAPDH) (Abbkine, Wuhan, China, 1:5000) or HRP Goat Anti-Mouse IgG (H + L) (*p*-Akt, mTOR, *p*-mTOR) (ABclonal, 1:5000) at 37 °C for 1 h, the membranes were visualised with the ECL Enhanced Kit (ABclonal). The protein expression levels were determined using ImageJ software on FluorChem™ FC2 AIC system (Alpha Innotech, USA).

2.10. Laying hen infection models

A total of 60 1-day-old healthy White Leghorns were bought from Beijing Boehringer Ingelheim Vital Biotechnology Co., Ltd. (Beijing, China). The laying hens were randomly divided into six groups, namely the control group (Control), the *C. perfringens* infection group (Infection), the Anemoside B4 treatment group (0.43, 0.86, 1.72 g/kg AneB) and the lincomycin group (lincomycin) as the positive control group, each with 10 laying hens. All laying hens were freely drink water and feed food. The necrotic enteritis infection model was established as previously described, with some minor modifications [26]. Briefly, laying hens from all groups, except the control group, were challenged intragastrically with 1×10^9 CFU *C. perfringens* in 1 mL of sterile PBS from day 17–20 once daily, and the control group only received 1 mL of sterile PBS. After the challenge, the growing laying hens from the infection and the control groups were fed the basic diet, whereas those from the 0.43, 0.86 and 1.72 g/kg AneB groups were fed the basic diet with 0.43, 0.86 and 1.72 g/kg Anemoside B4, respectively. The growing laying hens from the lincomycin group were fed the basic diet supplemented with 40 mg/kg lincomycin. During the observation period, clinical signs and survival rate were recorded. All laying hens were euthanised on day 24.

2.11. Determination of the effect of Anemoside B4 on blood biochemical parameters

At 15 h after the last challenge with *C. perfringens*, blood samples were collected for the determination of blood biochemical parameters.

2.12. Determination of the effect of Anemoside B4 on the immune organ index, intestinal injury, inflammatory cytokines, and tight junction expression, NF-κB and PI3K/Akt/mTOR signalling activation

After the laying hens were euthanised, the immune organ index (spleen, thymus, bursa of fabricius) was evaluated as the immune organ weight divided by the body weight and multiplied by 100 % [27]. The lesion scores for the jejunum and ileum were estimated by using the lesion score system described elsewhere [4]. Tissue samples from the jejunum, ileum and spleen were collected for histopathological analyses. The mRNA expression levels of the inflammatory cytokines *IL-1β*, *IL-6*, *IL-10*, *TNF-α* and *IFN-γ* in the jejunum were determined using the RT-qPCR method. The tight junction expression levels in the jejunum were determined via RT-qPCR and

Western blot. The NF-κB and PI3K/Akt/mTOR activation in the jejunum induced by *C. perfringens* was measured by Western blot as described above. The primers used in this study are displayed in Table 1.

2.13. Statistical analyses

The data were analysed using the GraphPad Prism 8 software and presented as mean ± SD. Differences were analysed using analysis of variance (ANOVA). Lesion score analysis was carried out using the Mann–Whitney test. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate statistical significance.

3. Results

3.1. Effects of Anemoside B4 on antibacterial activity, biofilm formation, haemolytic activity and cell viability of C. perfringens

To determine the antibacterial activity of Anemoside B4 against *C. perfringens*, the growth curve was determined. Bacterial growth was not inhibited when *C. perfringens* was treated with 12.5–50 μg/mL Anemoside B4 (Fig. 1A). As shown in Fig. 1B, the biofilm formation ability of *C. perfringens* subjected to 12.5–50 μg/mL Anemoside B4 was significantly decreased, compared to that of *C. perfringens* not treated with Anemoside B4, suggesting that Anemoside B4 significantly inhibits the biofilm formation of this species ($P < 0.01$) (Fig. 1B). Haemolytic activity determination of *C. perfringens* demonstrated that when the bacterium was treated with Anemoside B4 at concentrations of 25–50 μg/mL, haemolytic activity was reduced by 45.1 % compared to that without Anemoside B4, indicating that Anemoside B4 can attenuate *C. perfringens* haemolytic toxin activity or production ($P < 0.01$) (Fig. 1C). As shown in Fig. 1D, Anemoside B4, at concentrations from 6.25 to 100 μg/mL, is not toxic to Caco-2 cells (Fig. 1D).

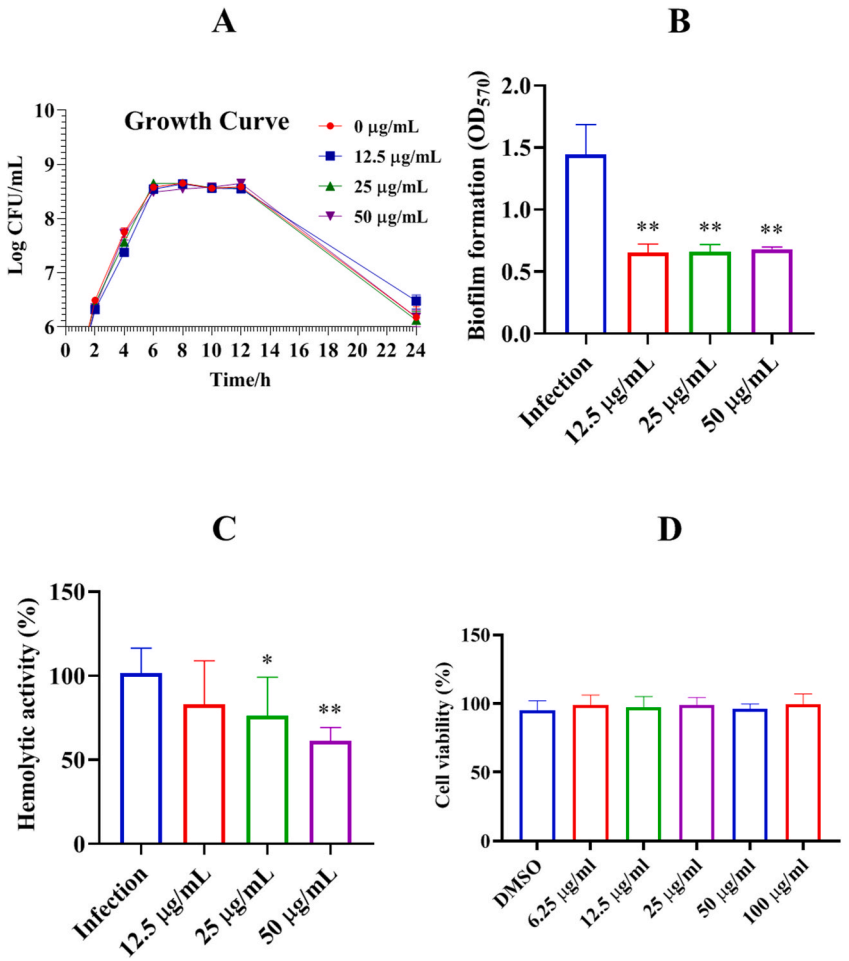

Fig. 1. Determination of the effects of Anemoside B4 on antibacterial activity (A), biofilm formation (B), haemolytic activity (C) and cell viability (D) of *C. perfringens*. *C. perfringens* and 12.5–50 μg/mL Anemoside B4 were co-cultured and the antibacterial activity, biofilm formation, and haemolytic activity were determined. Caco-2 cells were treated with 0, 6.25, 12.5, 25, 50 and 100 μg/mL Anemoside B4 for 24 h, the cell viability were measured. $^{*}P < 0.05$; $^{**}P < 0.01$*.

We also explored the effect of the multiplicity of infection (MOI) of *C. perfringens* in the Caco-2 cells infection model. When the MOI was 1:1, the cell morphology was changed (Supplemental Fig. 1B). When the MOI was 5:1 and 10:1, the cell morphology becomes significantly round, and a large number of cells die (Supplemental Figs. 1C and 1D), thus we chose MOI at 1:1 in this study.

3.2. Anemoside B4 inhibits virulence-related gene expressions of C. perfringens

To determine the effect of Anemoside B4 on the expression of virulence-related genes in *C. perfringens*, the bacterium was treated with different concentration of Anemoside B4. Based on the results, 12.5–50 μg/mL Anemoside B4 significantly inhibited the expression of the genes *plc*, *pfoA*, *netB*, *agrB*, *virS* and *virR* at mRNA level ($P < 0.01$) (Fig. 2), and 25–50 μg/mL Anemoside B4 attenuated *agrD* gene mRNA expression ($P < 0.01$) (Fig. 2).

3.3. Anemoside B4 impedes the NF-κB and PI3K/Akt/mTOR signalling pathways in Caco-2 cells infected by C. perfringens

To determine the effects of Anemoside B4 on the NF-κB and PI3K/Akt/mTOR signalling pathways, 1×10^7 CFU *C. perfringens* and 1×10^6 Caco-2 cells were cocultured for 2 h. The results demonstrated that p-PI3K, *p*-AKT, *p*-mTOR were upregulated when the Caco-2 cells were infected by *C. perfringens*; however, 50 μg/mL Anemoside B4 attenuated p-PI3K, *p*-AKT, *p*-mTOR expression compared to the infection group ($P < 0.05$) (Fig. 3A, B, C, D). Also *C. perfringens* induced p-P65 protein expression compared to the control group, whereas 50 μg/mL Anemoside B4 downregulated the p-P65 protein expression compared to that observed in the infection group ($P < 0.05$) (Fig. 3E and F).

3.4. Anemoside B4 improves the blood biochemical parameters of laying hens infected by C. perfringens

As shown in Table 2, the expression levels of glutamic oxaloacetic transaminase (AST), totalbilirubin (T-Bil), direct bilirubin (D-Bil), and glucose (GLU) were downregulated, and those of high-density lipoprotein-cholesterol (HDL-C) and bloodurea nitrogen (BUN) were upregulated in the infection group compared to the control group (Table 2). Concentrations of 0.43–1.72 g/kg Anemoside B4 and lincomycin could improve the high-density lipoprotein-cholesterol and bloodurea nitrogen concentrations compared to the infection

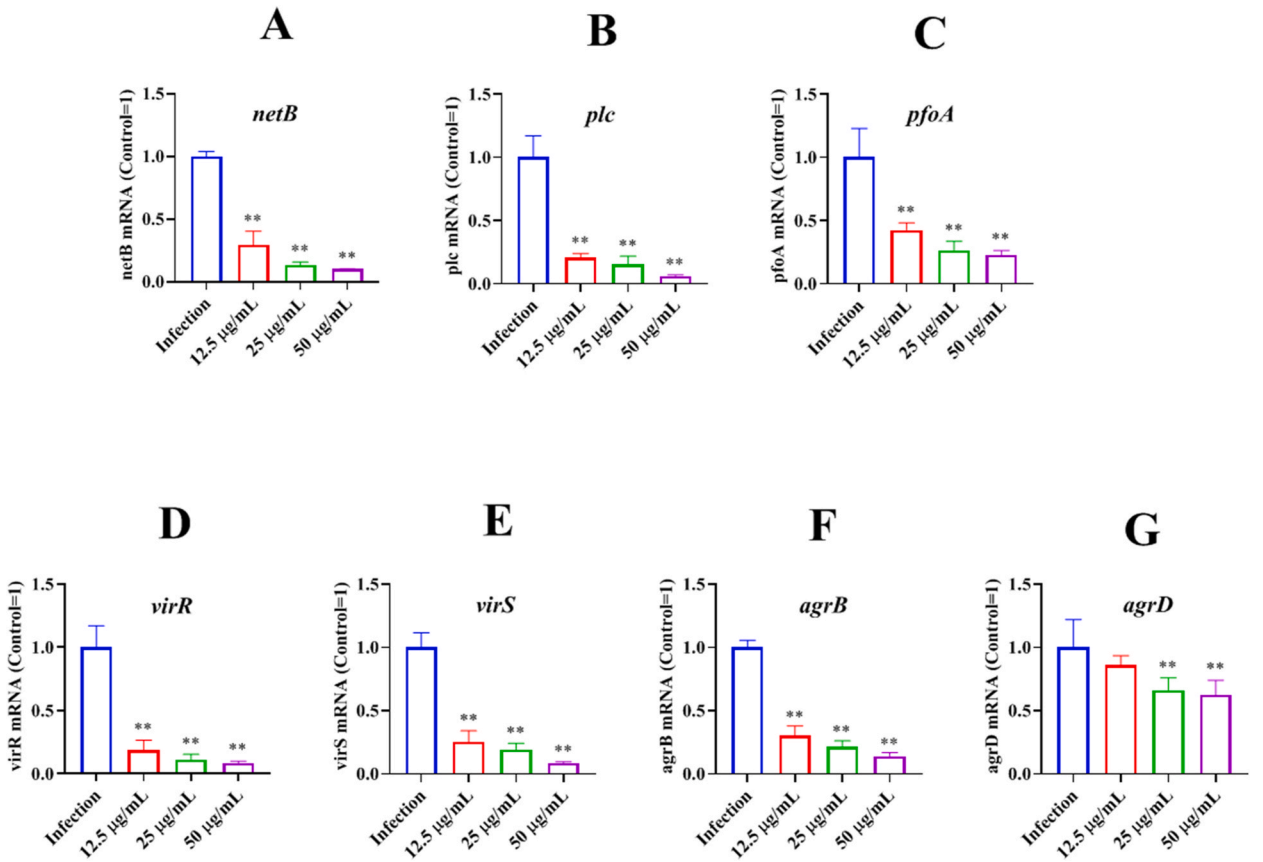

Fig. 2. Measurement of the effect of Anemoside B4 on virulence-related gene expressions of *C. perfringens*. The RNA of *C. perfringens* was extracted and *netB* (A), *plc* (B), *pfoA* (C), *virS (D)*, *virR* (E), *agrB* (F), *and agrD* (G) expression at mRNA levels were determined by RT-qPCR. $^{**}P < 0.01$.

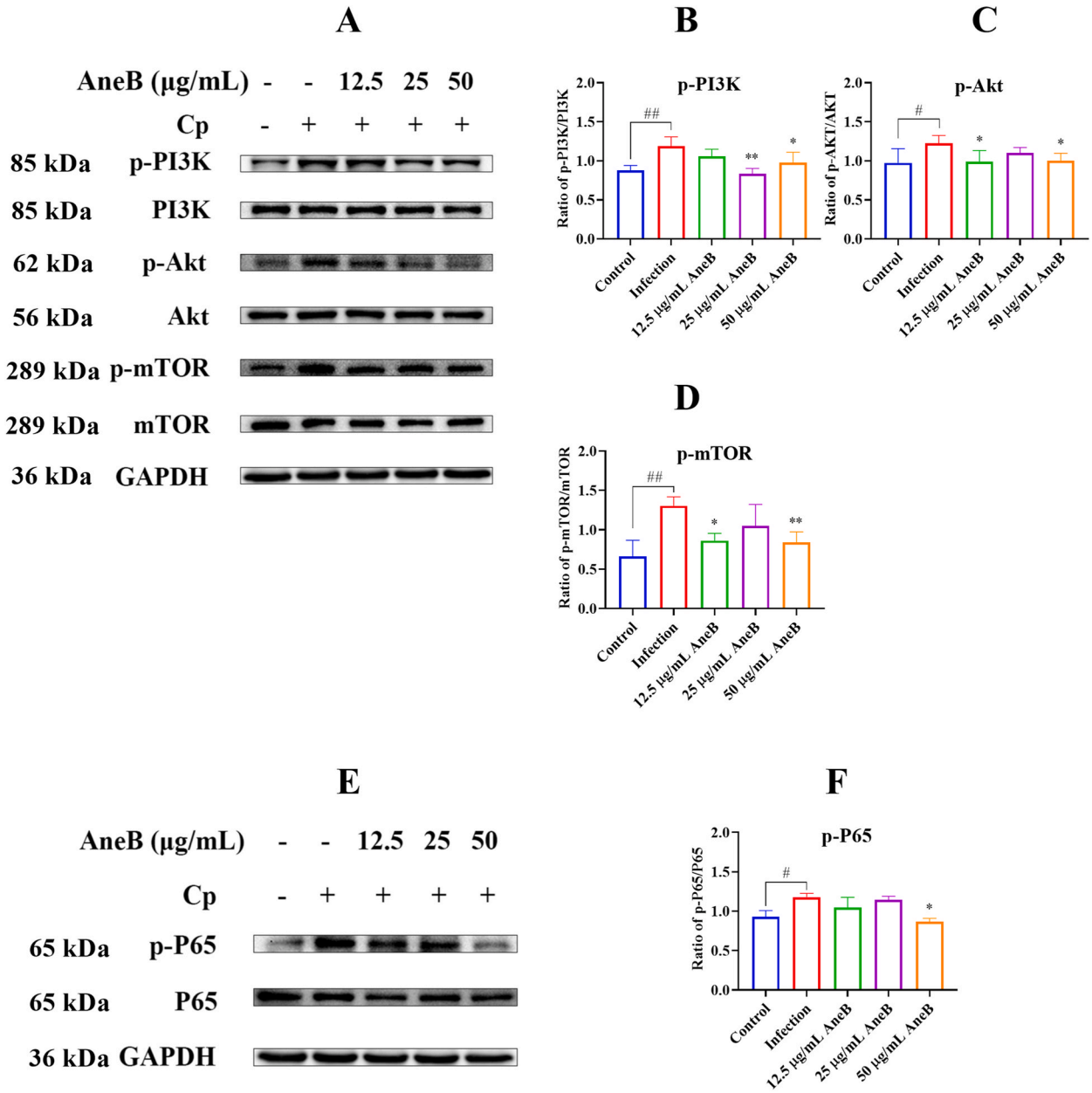

Fig. 3. The effect of Anemoside B4 on PI3K/Akt/mTOR (A–D) and NF-κB (E, F) pathways activation in Caco-2 cells infected by *C. perfringens*. The Caco-2 cells were pretreated with Anemoside B4 and cocultured with *C. perfringens*. The p-PI3K, *p*-Akt, *p*-mTOR, P65, and p-P65 protein expressions were determined by Western blotting. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus controls; $^{*}P < 0.05$; $^{**}P < 0.01$.

group (Table 2). In addition, the glucose and direct bilirubin levels were increased by the addition of 0.86–1.72 g/kg Anemoside B4 compared to the infection group (Table 2). A concentration of 0.86 g/kg Anemoside B4 and lincomycin promoted glutamic oxaloacetic transaminase production in the blood compared to the infection group (Table 2).

3.5. Anemoside B4 increases immune organ index, decreases necrotic lesion scores, and attenuates histopathological damage of laying hens infected by C. perfringens

After the laying hens were challenged by *C. Perfringens*, the immune organ index was determined. In the infection group, the spleen, thymus and bursa of fabricius index values were significantly decreased compared to those of the control group ($P < 0.01$) (Fig. 4A). At 0.86–1.72 g/kg Anemoside B4 and lincomycin improved the spleen index value compared to that of the infection group ($P < 0.05$) (Fig. 4A). However, 0.43 and 1.72 g/kg Anemoside B4 could not increase the thymus index value (Fig. 4A). Furthermore, 0.86 g/kg

Table 2
Detection of the blood biochemical parameters.

Item	Control	Infection	0.43 g/kg AneB	0.86 g/kg AneB	1.72 g/kg AneB	Linconmycin	SEM	*P* value				
	(A)	(B)	(C)	(D)	(E)	(F)		BvsA	CvsB	DvsB	EvsB	FvsB
T-Bil(μmol/L)	12.24	8.28	9.58	10.84	10.42	11.15	0.41	0.010	0.336	0.073	0.126	0.048
TP(g/L)	29.85	30.63	30.24	30.17	30.80	30.32	0.29	0.459	0.711	0.658	0.868	0.764
ALB (g/L)	12.62	13.37	13.04	13.23	13.31	13.10	0.14	0.086	0.421	0.735	0.872	0.517
AST (U/L)	251	199	217	232	216	234	5.05	0.001	0.198	0.023	0.206	0.019
TC(mmol/L)	3.49	3.86	3.80	3.55	3.79	3.73	0.09	0.118	0.769	0.188	0.736	0.549
TG(mmol/L)	1.27	0.98	1.07	0.95	0.89	1.03	0.12	0.173	0.661	0.883	0.650	0.832
GLU(mmol/L)	16.9	14.7	15.5	16.2	15.9	15.7	0.21	<0.001	0.089	0.005	0.019	0.045
IP(mmol/L)	1.94	2.02	2.02	2.07	2.05	2.05	0.11	0.360	0.969	0.563	0.728	0.671
HDL-C (mmol/L)	2.05	2.57	2.07	1.85	2.02	2.03	0.12	0.017	0.021	0.002	0.013	0.014
LDL-C (mmol/L)	0.85	0.99	1.01	1.00	0.98	1.01	0.12	0.115	0.821	0.970	0.850	0.880
BUN(mmol/L)	0.6	0.8	0.6	0.6	0.6	0.5	0.00	0.017	0.003	0.003	0.007	0.001
γ-GT(U/L)	24	24	23	23	23	23	0.25	1.000	0.493	0.493	0.493	0.310
CK(U/L)	1563	1470	1431	1369	1401	1250	41.09	0.520	0.783	0.483	0.631	0.141
D-Bil(μmol/L)	3.19	2.44	2.62	2.71	2.74	2.86	0.08	<0.001	0.104	0.020	0.011	0.001

total bilirubin: T-Bil; total protein: TP; albumin:ALB; glutamic oxaloacetic transaminase: AST; total cholesterol: TC; triglycerides: TG; glucose: GLU; inorganic phosphate: IP; high-density lipoprotein-cholesterol: HDL-C; low-density lipoprotein-cholesterol: LDL-C; blood urea nitrogen: BUN; γ-glutamyl transpeptidase: γ-GT; creatine kinase: CK; direct bilirubin: D-Bil.

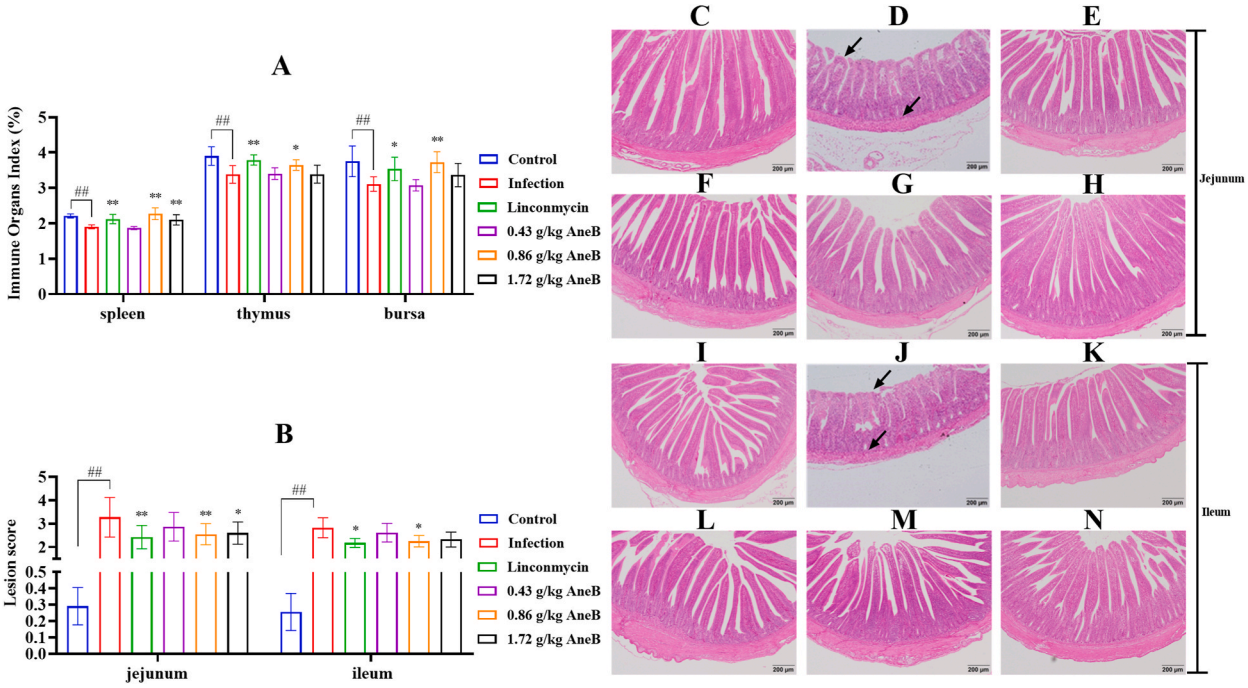

Fig. 4. Determination of the effect of Anemoside B4 on immune organ index, necrotic lesion scores, and histopathological damage of laying hens infected by *C. perfringens*. After the laying hens were challenged by *C. perfringens*, the immune organ index (A), necrotic lesion scores (B) and histopathological damage (C–N) were evaluated. Arrows represents shortening of intestinal villi and inflammation cell infiltration. C and I: the control group; D and J: the infection group; E and K: the lincomycin group; F and L: 0.43 g/kg Anemoside B4 group; G and M: 0.86 g/kg Anemoside B4 group; H and N: 1.72 g/kg Anemoside B4 group. $^{\#\#}P < 0.01$ versus controls; $^{*}P < 0.05$; $^{**}P < 0.01$.

Anemoside B4 and lincomycin also increased the bursa of fabricius index value compared to the infection group ($P < 0.05$) (Fig. 4A).

In the control group, we detected no obvious necrotic lesions in the jejunum and ileum of laying hens (Fig. 4B). When the laying hens were challenged by *C. perfringens*, the necrotic lesion scores were increased both in the jejunum and the ileum ($P < 0.01$); when these hens were fed a diet supplemented with 0.86 g/kg Anemoside B4, or lincomycin, the lesion scores were significantly decreased in the jejunum and the ileum compared to those in the infection group ($P < 0.05$) (Fig. 4B).

Via haematoxylin-eosin staining, we also determined histopathological damage in the jejunum and ileum, induced by *C. perfringens*. In the infection group of jejunum and ileum, we found serious pathological damage such as shortening of intestinal villi and inflammation cell infiltration (Fig. 4D–J), while there was displayed minor histopathological damage in the lincomycin group and 0.43–1.72 g/kg Anemoside B4 groups (Fig. 4E–H, K-N).

3.6. Anemoside B4 attenuates inflammatory cytokine expression in the jejunum of laying hens infected by C. perfringens

After the laying hens were infected with *C. perfringens*, the expression of *IL-1β*, *IL-6*, *IL-10*, *TNF-α* and *IFN-γ* at mRNA levels in the jejunum in the infection group was significantly increased compared to that in the control group ($P < 0.01$) (Fig. 5). When the laying hens were fed with lincomycin, the *IL-1β*, *IL-6*, *TNF-α* and *IFN-γ* mRNA expression levels were significantly decreased compared to those in the infection group ($P < 0.05$) (Fig. 5). Anemoside B4, at concentrations of 0.43, 0.86 and 1.72 g/kg, could inhibit *IL-1β*, *IL-6*, *TNF-α* and *IFN-γ* expression compared to the infection group ($P < 0.01$) (Fig. 5).

3.7. Anemoside B4 improves the tight junction expressions in the jejunum of laying hens infected by C. perfringens

The tight junctions ZO-1, Claudin-1, Occludin-1 and MUC-2 were determined in the jejunum after the laying hens were infected by

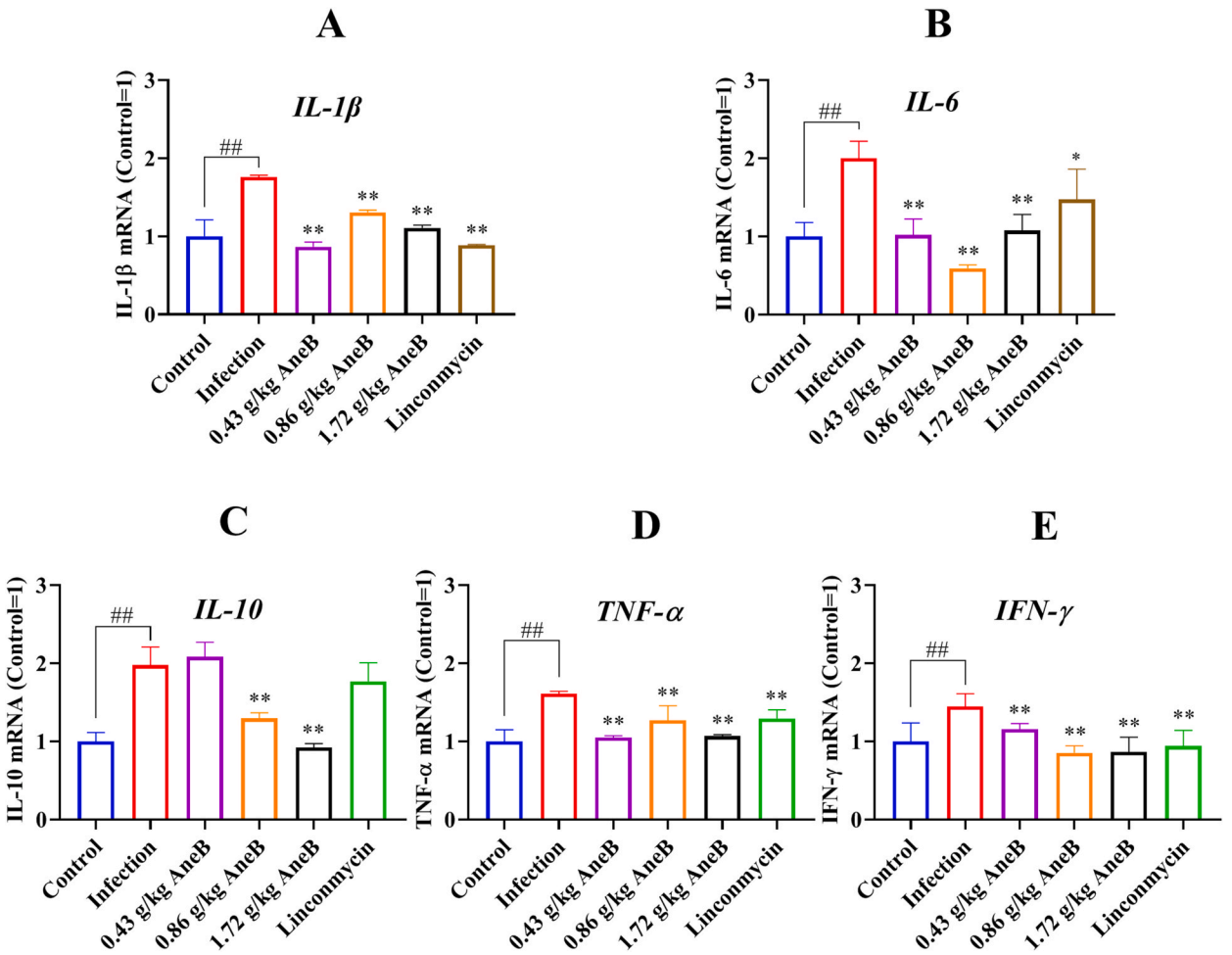

Fig. 5. Detection of the Anemoside B4 on inflammatory cytokine expression in the jejunum of laying hens infected by *C. perfringens*. The jejunum RNA was obtained and the *IL-1β* (A), *IL-6* (B), *IL-10* (C), *TNF-α* (D) and *IFN-γ* (E) mRNA expression levels were explored by RT-qPCR. $^{\#\#}P < 0.01$ versus controls; $^{*}P < 0.05$; $^{**}P < 0.01$.

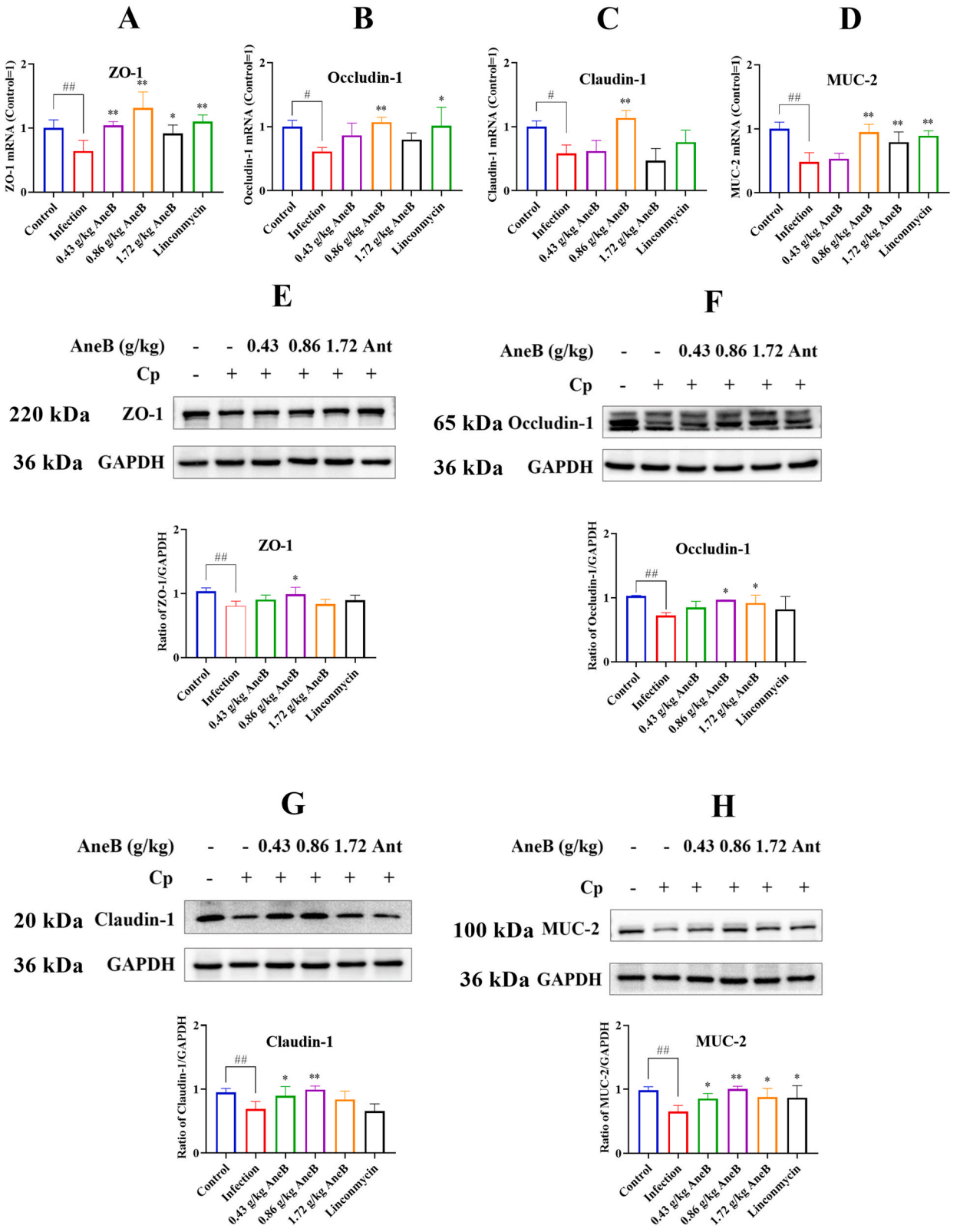

Fig. 6. Determination of Anemoside B4 on tight junction expressions in the jejunum of laying hens challenged by *C. perfringens*. ZO-1, Occludin-1, Claudin-1, and MUC-2 expression at mRNA and protein levels were measured by RT-qPCR (A–D) and Western blotting (E–H), respectively. Ant: lincomycin; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus controls; $^{*}P < 0.05$; $^{**}P < 0.01$.

C. perfringens. Based on the results, ZO-1, Claudin-1, Occludin-1 and MUC-2 expression at mRNA level was significantly downregulated in the infection group compared to the control group ($P < 0.05$) (Fig. 6). In the lincomycin group, ZO-1, Occludin-1 and MUC-2 were upregulated compared to the infection group ($P < 0.05$) (Fig. 6). At concentrations of 0.86 and 1.72 g/kg, Anemoside B4 enhanced ZO-1and MUC-2 expression compared to the infection group ($P < 0.05$) (Fig. 6).

We also determined the protein expression levels in the jejunum by Western blot. The expression levels of ZO-1, Claudin-1, Occludin-1 and MUC-2 were decreased compared to those of the control group ($P < 0.01$) (Fig. 6). Lincomycin promoted MUC-2 protein expression compared to the infection group ($P < 0.05$) (Fig. 6). At concentrations of 0.43 and 0.86 g/kg, Anemoside B4 upregulated Claudin-1 and MUC-2 protein expression compared to the infection group ($P < 0.05$) (Fig. 6).

3.8. Anemoside B4 inhibits PI3K/Akt/mTOR and NF-κB signalling pathways activation in the jejunum of laying hens infected by C. perfringens

In the PI3K/Akt/mTOR pathway, *C. perfringens* induced the expression of p-PI3K, *p*-Akt and *p*-mTOR compared to the control group ($P < 0.05$) (Fig. 7A, B, C, D). In the lincomycin group, the p-PI3K, *p*-AKT and *p*-mTOR expression levels were decreased compared to those in the infection group ($P < 0.05$) (Fig. 7A, B, C, D). Anemoside B4 at concentrations of 1.72 g/kg, 0.43–0.86 g/kg and 0.86–1.72 g/kg attenuated p-PI3K, *p*-AKT and *p*-mTOR protein expression, respectively, compared to the infection group ($P < 0.05$) (Fig. 7A, B, C, D).

The p-P65 expression level was increased in the infection group compared to the control group ($P < 0.05$) (Fig. 7E and F). Lincomycin and Anemoside B4 at concentrations of 0.43–1.72 g/kg, decreased p-P65 protein expression compared to the infection group ($P < 0.05$) (Fig. 7E and F).

4. Discussion

In the present study, we explored the effects of Anemoside B4 supplementation in laying hens challenged by *C. perfringens* on histopathological damage, inflammatory cytokine expression and NF-κB and PI3K/Akt/mTOR signalling pathways. Anemoside B4 supplementation mitigated histopathological damage and attenuated inflammatory cytokine secretion. These results are of paramount importance to reveal the anti-inflammatory effect of Anemoside B4.

Regarding the macroscopic and microscopic findings, the severity of the lesions detected was consistent with previously published findings for the jejunum and ileum when a similar infection model was adopted [28,29]. In the present study, we demonstrate that Anemoside B4 relieved tissue lesions, especially in the jejunum. Notably, Anemoside B4 could attenuate the various macroscopic lesions detected. We therefore speculate that Anemoside B4 reduces the inflammation responses reduction, mediated by *IL-1β*, *IL-6*, *IL-10*, *TNF-α* and *IFN-γ* attenuation, and decreases inflammation cell infiltration, but the specific mechanism needs to be further studied. The downregulation of inflammation cell infiltration in the jejunum by Anemoside B4 has not been reported previously, and it is essential to investigate the effects of Anemoside B4 supplementation on laying hens with necrotic enteritis.

Based on previous studies, quorum-sensing (QS) regulates bacterial virulence-related gene expression through regulating bacterial responses to local cell density [5]. Two important QS systems, (accessory gene-regulator) Agr (Agr A, Agr B, Agr C, Agr D), and VirR/Virs of *C. perfringens*, have been identified and are involved in virulence-related gene expressions [24,30]. The secretion of toxins by *C. perfringens* is mainly controlled by the (accessory gene-regulator) Agr QS system [31]. However, the production of netB toxin, triggering necrotic enteritis, depends on the Agr QS system and VirR [32]. The virulence-related genes *plc* and *pfoA* are also controlled by the QS system [33]. Considering the important roles of virulence-related genes in the pathogenesis of necrotising enteritis, the effect of Anemoside B4 on virulence-related gene expression of *C. perfringens* was investigated. According to our results, Anemoside B4 inhibited the expression of the genes *netB*, *plc*, *pfoA*, *agrB*, *agrD*, *virS* and *virR*, which might also explain the alleviation of the tissue damage induced by *C. perfringens*.

Innate immunity is crucial for host defense against pathogenic infections [34], and the NF-κB pathway plays important roles in anti-bacterial immunity [35]. According to a previous study, the NF-κB pathway participates in inflammatory responses and barrier disruption in the trachea of chicks following *Mycoplasma gallisepticum* infection [36]. In another study, Jingfang granule alleviated *Pseudomonas aeruginosa*-induced acute lung inflammation through suppression of the NF-κB pathway [37]. Xinfeng capsule can alleviate NF-κB-p65-induced immuno-inflammation in ankylosing spondylitis [38]. Bazedoxifene attenuated intestinal injury in sepsis by suppressing the NF-κB signaling pathway [39]. Thus, NF-κB is considered an important potential target in the treatment of inflammation responses [40]. In this study, *C. perfringens* induced NF-κB pathway activation, and Anemoside B4 inhibited NF-κB pathway activation, which might have provided some potential targets to prevent *C. perfringens*-induced inflammation damage.

In a previous study, liensinine attenuated excessive apoptosis of intestinal cells caused by LPS by inhibiting the PI3K/Akt/mTOR pathway [41]. Inulin can alleviate perfluorooctanoic acid-induced intestinal injury in mice by modulating the PI3K/Akt/mTOR signalling pathway [42], whereas dihydromyricetin can alleviate inflammatory bowel disease-associated intestinal fibrosis by inducing autophagy through the PI3K/Akt/mTOR signalling pathway [43]. Similar, rhein has an anti-inflammatory function in ulcerative colitis via inhibiting the PI3K/Akt/mTOR signalling pathway [44]. Based on the important role of the PI3K/Akt/mTOR pathway in intestinal inflammation, we explored whether *C. perfringens* can activate the PI3K/Akt/mTOR pathway. Based on our results, the PI3K/Akt/mTOR pathway was activated in the jejunum infected by *C. perfringens*, and Anemoside B4 weakened PI3K/Akt/mTOR pathway activation triggered by *C. perfringens*. Our results provide important insights into necrotic enteritis treatment and suggest Anemoside B4 as a potential therapeutic candidate to treat intestinal injury and reduce inflammation responses in necrotic enteritis.

Tight junctions are cell-cell junction structures which are critical for building the epithelial barrier and keeping epithelial polarity

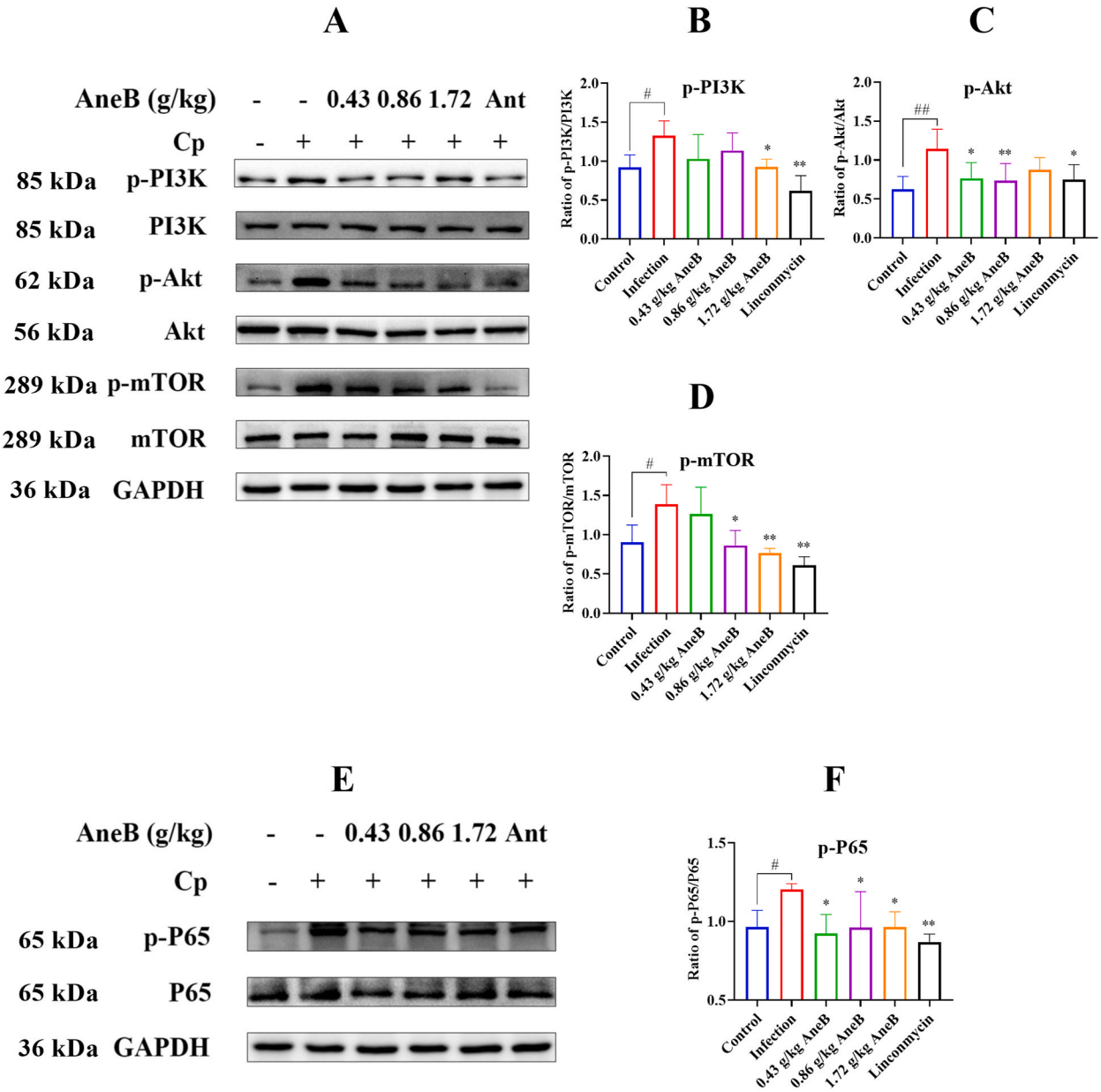

Fig. 7. Detection of Anemoside B4 on PI3K/Akt/mTOR (A–D) and NF-κB (E, F) pathways activation in the jejunum of laying hens infected by *C. perfringens*. The p-PI3K, *p*-Akt, *p*-mTOR and p-P65 protein expressions were explored by Western blotting. Ant:lincomycin; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus controls; *P < 0.05; **$P < 0.01$.

[45], with important roles in intestinal barrier function [46]. According to a previous study, intestinal inflammation is closely related to intestinal epithelial barrier disruption, contributing to the progression of intestinal diseases [47]. Pathogenic *Escherichia coli* damages the intestinal barrier leading to intestinal diseases in humans and livestock [48], and barrier dysfunction is associated to Crohn's disease and ulcerative colitis [49]. Thus, we investigated the tight junction expressions in the jejunum of necrotising enteritis induced by *C. perfringens* and found that ZO-1, Claudin-1, Occludin-1 and MUC-2 were decreased and Anemoside B4 improved the intestinal barriers functions, explaining the antiinflammation effect of Anemoside B4.

5. Conclusion

Overall, Anemoside B4 supplementation induced an antiinflammation response in laying hens in a necrotic enteritis infection model. The main results supporting this conclusion were inflammation cytokine reduction, tissue damage alleviation and NF-κB and PI3K/Akt/mTOR pathway inhibition. We therefore suggest Anemoside B4 as a novel drug to control chicken necrotic enteritis elicited

by *C. perfringens*.

Funding statement

This work was supported by the Key Research and Development Plan of Hubei Province, China (2023BBB069).

Data availability statement

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

Ethical statement

The animal studies and euthanasia procedure were approved by the Animal Care and Use Committee of Wuhan Polytechnic University, Hubei Province, China (WPU202308002) and the study complies with all regulations.

CRediT authorship contribution statement

Xinyue Tian: Investigation, Formal analysis. **Jingyang Li:** Formal analysis. **Siyu Liu:** Formal analysis. **Qiaoli Dong:** Data curation. **Yunjian Fu:** Formal analysis. **Ronghui Luo:** Formal analysis. **Yamin Sun:** Formal analysis. **Ling Guo:** Formal analysis. **Qirong Lu:** Methodology. **Chun Ye:** Formal analysis. **Jin Liu:** Formal analysis. **Shulin Fu:** Writing – review & editing, Writing – original draft, Formal analysis. **Yinsheng Qiu:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33161.

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