Heliyon 10 (2024) e26114

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Clostridium perfringens α toxin damages the immune function, antioxidant capacity and intestinal health and induces PLCγ1/AMPK/mTOR pathway-mediated autophagy in broiler chickens

Tong Zhang [a,1], Xiaohui Wang [a,1], Wenli Li [a], Heliang Wang [b], Lei Yan [c], Lianwen Zhao [a], Xiaowen Zhang [a], Nianxue Wang [a], Wendong An [a], Tongyue Liu [a], Wenlei Fan [a], Beibei Zhang [a,*]

[a] College of Animal Science and Technology, Qingdao Agricultural University, Qingdao, 266109, China
[b] Qingdao Sino-science Gene Technology Co., Ltd, Qingdao, 266114, China
[c] Shandong New Hope Liuhe Group, Qingdao, 266000, China

A R T I C L E I N F O

Keywords:
Clostridium perfringens α toxin
Immune function
Antioxidant capacity
Intestinal health
Autophagy
Signaling pathway
Broiler chickens

A B S T R A C T

Clostridium perfringens α toxin is generated by all types of *C. perfringens* and is closely related to necrotic enteritis in poultry. This study was conducted to investigate the effects of α toxin on immune function, antioxidant capacity, intestinal health and the underlying mechanisms in broiler chickens. A total of 144 twenty-day-old broiler chickens were randomly assigned to four treatments. On d 21, the birds were intraperitoneally injected with PBS (control group) or α toxin at 0.025, 0.1 or 0.4 U/kg of body weight. Samples were collected at 3 h and 24 h post injection (p.i.). Results showed that α toxin challenge linearly decreased the average daily gain during the 3 days after infection and decreased plasma IgA and IgM levels 3 h p.i. Plasma diamine oxidase and D-lactate levels were linearly elevated by α toxin challenge at 3 h p.i. and 24 h p.i. Alpha toxin challenge linearly decreased plasma and jejunal mucosal catalase, glutathione peroxidase and total superoxide dismutase activities at 3 h p.i. and linearly decreased glutathione peroxidase and total superoxide dismutase activities at 24 h p.i. The ileal villus height to crypt depth ratio decreased linearly with increasing α toxin levels at 3 h p.i. and 24 h p.i. Alpha toxin challenge linearly elevated jejunal *IL-1β*, *IL-6*, *IL-8* and tumor necrosis factor α mRNA expression at 3 h p.i. Additionally, α toxin challenge linearly reduced the jejunal *claudin-1*, *claudin-3* and *zonula occludens* 1 mRNA expression at 3 h p.i. and the *claudin-3*, *occludin* and *zonula occludens* 1 mRNA expression at 24 h p.i. What's more, α toxin linearly increased the jejunal *PLCγ1*, *AMPKα1* and *ATG5* mRNA expression and linearly decreased the *mTOR* mRNA expression. In conclusion, *C. perfringens* α toxin challenge decreased body weight gain, impaired immune function, antioxidant capacity and intestinal health, and induced PLCγ1/AMPK/mTOR pathway-mediated autophagy. The recommended intraperitoneal injection dose for moderate injury was 0.1 U/kg of body weight and the recommended sampling time was 3 h p.i. in broiler chickens.

* Corresponding author. Qingdao Agricultural University, No. 700 Changcheng Road, Chengyang District, Qingdao 266109, China.
E-mail address: zhangbeibei@qau.edu.cn (B. Zhang).

[1] The authors contributed equally to the work.

https://doi.org/10.1016/j.heliyon.2024.e26114
Received 5 June 2023; Received in revised form 2 February 2024; Accepted 7 February 2024
Available online 14 February 2024
2405-8440/Â© 2024 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Clostridium perfringens is a gram-positive, anaerobic, spore-forming bacterium that is found in soil and the guts of most animal species and humans [1]. This bacterium is recognized as the causative pathogen of various diseases, including cellulitis, necrotic enteritis, enterotoxemia, and gas gangrene. Necrotic enteritis is an enteric disease in poultry, that is characterized by dampened growth performance, decreased feed efficiency, and depression in its chronic form, as well as anorexia, severe morbidity, and significant mortality in its acute form [2].

In recent years, the incidence of necrotic enteritis has been increasing, resulting in the loss of over 6 billion dollars annually in the global poultry industry due to the reduced use or removal of antibiotics [3]. Recently, *C. perfringens* has been updated for seven types (types A through G) depending on its major toxins production, such as α toxin, β toxin, ε toxin and ι toxin, enterotoxin and NetB [4,5]. Studies in the past few decades have shown that α toxin is an important virulence factor in the pathogenesis of avian necrotic enteritis [1,6,7].

The α toxin is a zinc-metalloenzyme produced by all types of *C. perfringens*. This toxin has phospholipase C and sphingomyelinase activities that can damage cell membranes [8] and cause hemolysis, necrosis, increased vascular permeability, and platelet aggregation [9]. Alpha toxin caused a mild to moderate infiltration of neutrophils in the lamina propria and submucosa of the ileum of lambs [10]. Also, Rehman et al. [11] revealed that α toxin can impair the intestinal mucosal barrier of laying hens by measuring the transmural tissue conductance. Alpha toxin can cause inflammatory responses, as evidenced by increased serum interleukin (IL)-1β, IL-6 and tumor necrosis factor α (TNF-α) release in mice given the α toxin challenge [12]. Guo et al. [13] also discovered that α toxin upregulated the mRNA expression of *IL-6*, *IL-8* and *TNF-α* in chicken primary intestinal epithelial cells. However, limited information is available about the effects of α toxin on immune function, antioxidant capacity and intestinal health in broiler chickens.

Autophagy is a process that all eukaryotes use to deal with stress and defense. It is regulated by several autophagy-related genes (ATG) and signaling pathways [14]. Phospholipases C (PLC) γ, which includes PLCγ1 and PLCγ2, can be activated by a number of extracellular factors to start a cascade of signal pathways that regulate cellular metabolism and inflammation [15,16]. PLCγ1 plays a crucial role in the pathogenesis induced by α toxin [17]. Recent studies have shown that PLC could modulate the AMP-activated protein kinase (AMPK) pathway in inflammation [18] and autophagy [19]. AMPK, a key regulator of cell metabolism, is triggered by the induction of cellular stress (such as an increase in Ca^{2+} concentration or the production of reactive oxygen species) [20]. When activated, it blocks the mammalian target of rapamycin (mTOR) while promoting autophagy [14]. However, whether PLCγ1 would modulate autophagy via regulating AMPK/mTOR pathway in α toxin pathological progression remains unclear.

Therefore, this research was designed to study the effects of α toxin on plasma immunoglobulin levels, antioxidant capacity, intestinal morphology, inflammatory cytokines and barrier function in broiler chickens and investigate the underlying mechanism.

2. Materials and methods

2.1. Experimental design and diet

All experimental procedures were approved by the Animal Care and Use Committee of Qingdao Agricultural University (approval number: 20210715099). A total of 200 one-day-old male Arbor Acres broilers were obtained from a local commercial hatchery. On d 20 (one day before injection), all birds were weighted individually and 144 birds with similar weights were chosen and randomly assigned into 4 groups with 4 replicates of 9 birds. There was no significance in the initial body weights among the groups. On d 21, the four groups were intraperitoneally injected with α toxin (phospholipase C from *C. perfringens*, Type I, Sigma Chemical Co., St Louis, MO) at doses of 0 (control group), 0.025, 0.1 and 0.4 U/kg of body weight, respectively. The α toxin was dissolved in PBS at 0, 25, 100 and 400 U/L, respectively, and each bird was injected at 1 mL/kg of body weight. All birds were fed a corn-soybean-based diet in mash form. The diet was formulated to meet or exceed the China feeding standard of chicken (NY/T 33–2004). The components of the diet are as follows: 55.69% corn, 36.74% soybean meal (46% crude protein), 3.4% soybean oil, 1.15% limestone, 2.1% calcium hydrophosphate, 0.3% NaCl, 0.18% D,L-met, 0.1% vitamin premix, 0.15% mineral premix, 0.18% choline chloride and 0.01% ethoxyquin. The nutrient levels (calculated values) of the diet consist of 3.01 Mcal/kg metabolizable energy, 21.53% crude protein, 1.01% calcium, 0.46% non-phytate phosphorus, 1.23% Lys and 0.52% Met. The birds were reared in wire-floored cages with continuous light. Feed and water were provided ad libitum.

2.2. Sample collection

Eight birds per group (two birds per replicate cage) with an average body weight were randomly selected at 3 h and 24 h post injection (p.i.). The birds were fasted overnight before slaughter. Blood samples was aseptically obtained via the wing vein. Heparin plasma was collected after centrifugation at 4000 rpm for 15 min at 4 °C, aliquoted and stored at −20 °C until analysis. After blood collection, the birds were killed by exsanguination. The middle jejunum segments were collected and immediately frozen in liquid nitrogen and stored at −80 °C for the determination of mRNA expression. Jejunal mucosa was scraped by a glass slide, put into a microtube, rapidly frozen with liquid nitrogen, and stored at −20 °C until the antioxidant index assay. The middle sections of the ileum were obtained and fixed in a 4% paraformaldehyde solution for intestinal morphology analysis.

2.3. Body weight determination

Body weights for each replicate cage were weighted at 21 and 24 days of age after an overnight fast. Average daily gains from d 21 to 24 were calculated.

2.4. Detection of plasma immunoglobulin and intestinal permeability-related indices

Plasma IgG, IgA, and IgM levels were detected followed by the protocols of the chicken ELISA kit (Shanghai Meilian Biological Engineering Co. Ltd., Shanghai, China) with a microplate reader (TECAN, Infinite M Nano, Männedorf, Switzerland). The blood diamine oxidase (DAO) and D-lactate levels are sensitive indicators of intestinal permeability, which were measured according to the manufacturer's procedure stated in the commercial chicken ELISA kit (Shanghai Meilian Biological Engineering Co. Ltd., Shanghai, China).

2.5. Antioxidant capacity analysis

The activities of catalase (CAT), glutathione peroxidase (GSH-Px) and total superoxide dismutase (T-SOD) and the level of malondialdehyde (MDA) in plasma and intestinal mucosa were determined using the kits of the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Intestinal mucosa was homogenized in ice-cold sterile physiological saline for the preparation of 10% mucosa homogenates. The homogenates were centrifuged at 3000 rpm for 10 min at 4 °C and then the supernatants were collected for further assays. The protein level of supernatants was quantified using a Bicinchoninic Acid Protein Assay Kit (Cwbio, Beijing, China). Finally, antioxidant indices in the intestinal mucosa were expressed as units per milligram of protein in the sample.

Table 1
Sequences of quantitative real-time PCR primers.

Gene name[a]	Accession number	Primer sequence[b] (5′ to 3′)	Product size
IL-1β	XM_015297469.1	F: ACTGGGCATCAAGGGCTA R: GGTAGAAGATGAAGCGGGTC	131
IL-6	XM_015281283.1	F: CGCCCAGAAATCCCTCCTC R: AGGCACTGAAACTCCTGGTC	152
IL-8	XM_015301388.1	F: ATGAACGGCAAGCTTGGAGCTG R: TCCAAGCACACCTCTCTTCCATCC	233
IL-10	NM_001004414.2	F: CGCTGTCACCGCTTCTTCA R: TCCCGTTCTCATCCATCTTCTC	88
IL-17	NM_204460.1	F: CTCCGATCCCTTATTCTCCTC R: AAGCGGTTGTGGTCCTCAT	292
IL-22	NM_001199614.1	F: GGTTGTCTTCTGCTGTTGTTGCTG R: GCCAAGGTGTAGGTGCGATTCC	156
TNF-α	XM_046927265.1	F:GAGCGTTGACTTGGCTGTC R:AAGCAACAACCAGCTATGCAC	64
Claudin-1	NM_001013611.2	F: CATACTCCTGGGTCTGGTTGGT R: GACAGCCATCCGCATCTTCT	100
Claudin-2	NM_001277622.1	F: TTTGATGGAGGGCTGAGGA R: CAAGGACCGAGTGGCAGTG	289
Claudin-3	NM_204202.2	F:GCTCTGCCGTTACCAGCTACG R:CTGCACACAGCTCTCCTGGCAAC	343
Occludin	NM_205128.1	F: ACGGCAGCACCTACCTCAA R: GGGCGAAGAAGCAGATGAG	123
ZO-1	XM_015278975.1	F: CTTCAGGTGTTTCTCTTCCTCCTC R: CTGTGGTTTCATGGCTGGATC	131
PLCγ1	XM_015296747.4	F: GAGCCTGGTGGACTTGA R: GCGTCTCCTCGTTGATG	86
AMPKα1	NM_001039603.2	F: TGGCATTTGGGGATACGGAG R: GATTCTTCCGTCGAACACG	130
mTOR	XM_040689168.2	F: GGAGCAGCAAGAAGAGTC R: AAGGATGGTGAGGAGGAAT	92
Beclin1	XM_015299595.4	F: CACCAGCCAGAATGATGT R:ACCAGTAACCTTCAGTCTTC	110
ATG5	XM_046938875.1	F: TTATCAGGCAACAACAGAGA R: GATAGCACTAGGACATACATCT	115
GAPDH	NM_204305.1	F: TGCTGCCCAGAACATCATCC R: ACGGCAGGTCAGGTCAACAA	142

[a] *TNF-α*, tumor necrosis factor alpha; *ZO-1*, zonula occludens 1; *PLCγ1*, Phospholipases Cγ1; *AMPKα1*, AMP-activated protein kinase α1; *mTOR*, mammalian target of rapamycin; *Beclin1*, programmed cell death 1; *ATG5*, autophagy-related gene 5; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

[b] F, forward; R, reverse.

2.6. Intestinal morphology analysis

The intestinal tissues were dehydrated stepwise with ethanol, embedded in paraffin, and sectioned (5 μm). The tissue slices were stained with hematoxylin and eosin and sealed with gum. Images were captured at 100 × magnifications under a light microscope (DM2000 LED, Leica, Wetzlar, Germany) and analyzed by a microscopic photography analysis system (ZEN, Zeiss, Jena, Germany). Villus height was determined from the tip of the villus to the villus-crypt junction. Crypt depth was defined as the depth of the invagination between adjacent villi, and the villus height to crypt depth ratio (VCR) was calculated. For each section, ten pairs of villus and crypts were observed, and the average was calculated as the final value.

2.7. Real-time quantitative PCR

Trizol reagent (Invitrogen Biotechnology Inc., Carlsbad, CA) was used to extract total RNA from jejunum tissues. The purity and concentration of the total RNA were determined with a nanophotometer (Nano Photometer NP80, Implen, München, Germany). Reverse transcription into cDNA was performed using a PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology Inc., Tokyo, Japan). TB Green® Premix Ex Taq™ (Takara Biotechnology Inc., Tokyo, Japan) was used in CFX96 Real-Time PCR Detection Systems (Bio-rad, Hercules, CA) to perform quantitative real-time PCR on intestinal genes. The reaction volume of the 25 μL mixture contained 12.5 μL of TB Green Premix Ex Taq (Tli RnaseH Plus) (2X), 0.5 μL of each forward and reverse primer (10 μmol/L), 2 μL of cDNA and 9.5 μL of sterile nuclease-free water. The PCR condition was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The specificity of the PCR products was assessed using a melting curve. The primer sequences for the target and reference genes are listed in Table 1. The relative mRNA expression levels of target genes relative to those of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.8. Statistical analysis

A one-way analysis of variance was conducted to analyze the data using SPSS software version 25.0 (SPSS Inc., Chicago, IL). Duncan multiple comparison was performed to compare the differences among group means. The impact of α toxin levels was investigated using linear and quadratic polynomials. Graphs were designed with the use of GraphPad Prism 8 (GraphPad Software, San Diego, CA). Data are shown as means with SEM. All statements of significance were based on $P < 0.05$.

3. Results

3.1. Average daily gain

As shown in Fig. 1, α toxin challenge linearly decreased the average daily gain of broiler chickens during days 21–24 ($P < 0.01$). Compared with the control group, the 0.1 and 0.4 U/kg groups significantly decreased the average daily gain ($P < 0.01$), but the 0.025 U/kg group did not affect the average daily gain.

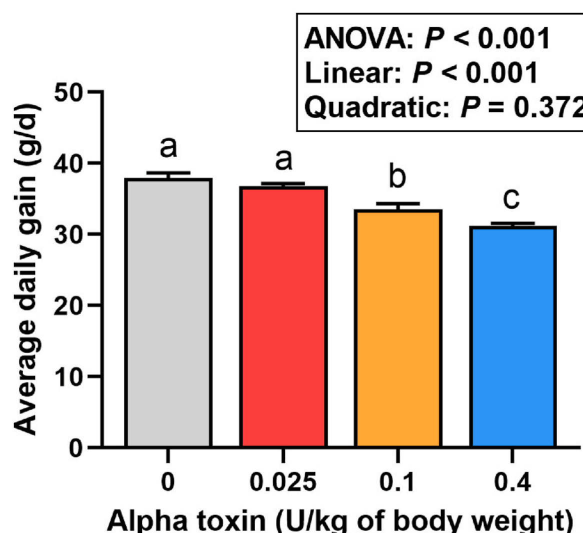

Fig. 1. The average daily gain of broiler chickens during 3 days after α toxin injection (d 21 to 24). The four groups were injected with either PBS or α toxin at doses of 0.025, 0.1, and 0.4 U/kg of body weight, respectively. Values are means with their SEM represented by vertical bars. Mean values with different letters were significantly different ($P < 0.05$).

3.2. Plasma immunoglobulin levels and intestinal permeability-related indices

At 3 h p.i., α toxin challenge linearly decreased plasma IgA levels ($P < 0.01$) (Table 2). Compared with the control group, the 0.1 and 0.4 U/kg groups significantly decreased plasma IgA levels ($P < 0.05$), but the 0.025 U/kg group did not affect plasma IgA levels ($P > 0.05$) at 3 h p.i. Relative to the control group, the 0.025 and 0.4 U/kg groups significantly decreased the plasma IgM level ($P < 0.05$) at 3 h p.i. Plasma DAO ($P < 0.01$) and D-lactate levels ($P < 0.01$) were linearly increased with the elevation of α toxin level at 3 h p. i. The plasma levels of DAO in the 0.1 and 0.4 U/kg groups were significantly increased relative to those in the control and 0.025 U/kg groups ($P < 0.05$) at 3 h p.i. Compared with the control group, the 0.4 U/kg group significantly increased the plasma D-lactate levels ($P < 0.05$), while no significance was observed among the control, 0.025 and 0.1 U/kg groups at 3 h p.i. Plasma DAO level was increased in a linear ($P < 0.01$) and quadratic ($P = 0.01$) manner as α toxin level increased at 24 h p. i. Increasing levels of α toxin elevated plasma D-lactate concentration in a linear manner at 24 h p. i. ($P < 0.01$). Relative to the control and 0.025 U/kg groups, the 0.1 and 0.4 U/kg groups significantly increased the plasma DAO and D-lactate levels ($P < 0.01$) at 24 h p.i.

3.3. Plasma and intestinal mucosal antioxidant status

As presented in Table 3, plasma CAT ($P < 0.01$), GSH-Px ($P < 0.01$) and T-SOD ($P < 0.01$) activities were decreased in a linear manner with an increased α toxin level at 3 h p.i. Among the four groups, the 0.4 U/kg groups had the lowest plasma CAT, GSH-Px and T-SOD activities at 3 h p.i. The activities of GSH-Px ($P < 0.01$) and T-SOD ($P < 0.01$) in the plasma were linearly decreased by the increase of the α toxin level at 24 h p.i. Compared with the control group, the other three groups significantly decreased plasma GSH-Px and T-SOD activities ($P < 0.01$) at 24 h p.i. There was no difference in MDA levels among all groups at 3 and 24 h p.i. ($P > 0.05$).

The jejunal CAT ($P < 0.01$), GSH-Px ($P < 0.01$) and T-SOD ($P < 0.01$) activities were linearly decreased as the α toxin level increased at 3 h p.i. (Table 4). The jejunal activities of GSH-Px and T-SOD in the 0.025, 0.1 and 0.4 U/kg groups were significantly decreased relative to those in the control group ($P < 0.01$) at 3 h p.i. There were linear decreases in jejunal GSH-Px ($P < 0.05$) and T-SOD ($P < 0.01$) activities with the increase of α toxin level at 24 h p.i. The jejunal MDA level was elevated with increasing levels of α toxin in a linear manner at 24 h p.i. ($P < 0.01$). Compared to the control birds, only the birds in the 0.4 U/kg group significantly decreased the GSH-Px and T-SOD activities ($P < 0.05$) and increased the MDA level ($P < 0.05$) at 24 h p.i.

3.4. Intestinal morphology

At 3 h p.i., α toxin challenge linearly increased the crypt depth ($P < 0.01$) and linearly decreased the VCR ($P < 0.01$) of the ileum (Table 5). Relative to the control and 0.025 U/kg groups, the 0.1 and 0.4 U/kg groups significantly increased the crypt depth ($P < 0.01$) and decreased the VCR ($P < 0.01$) at 3 h p.i. At 24 h p.i., α toxin challenge linearly decreased the villus height ($P < 0.01$) and linearly decreased the VCR ($P < 0.01$) of the ileum. Compared to the control birds, only the birds in the 0.4 U/kg group significantly decreased the ileal villus height and VCR ($P < 0.05$) at 24 h p.i.

3.5. Relative mRNA expression of inflammatory genes

At 3 h p.i., the relative mRNA expression of *IL-1β* ($P < 0.01$), *IL-6* ($P < 0.01$), *IL-8* ($P < 0.01$) and *TNF-α* ($P < 0.01$) in the jejunum was linearly up-regulated in response to the increase of α toxin level (Fig. 2 A-C and G). The mRNA expression of *IL-1β*, *IL-6* and *TNF-α* in the 0.025, 0.1, and 0.4 U/kg groups was significantly higher than that in the control group ($P < 0.05$) at 3 h p.i. Relative to the control birds, only the 0.4 U/kg group significantly upregulated the mRNA expression of *IL-8* ($P < 0.05$) at 3 h p.i. There was no significant effect on *IL-10*, *IL-17* and *IL*-22 mRNA expression among all groups at 3 h p.i. ($P > 0.05$) (Fig. 2D–F). There was no

Table 2
Effects of α toxin on plasma immunoglobulin levels and intestinal permeability-related indices of broiler chickens.

Items[1]	Alpha toxin (U/kg)				SEM	P-value		
	0	0.025	0.1	0.4		ANOVA	Linear	Quadratic
3 h								
IgA (μg/mL)	1.48[a]	1.34[ab]	1.32[bc]	1.19[c]	0.030	0.002	<0.001	0.901
IgM (μg/mL)	2.94[a]	2.50[b]	2.66[ab]	2.55[b]	0.063	0.041	0.057	0.151
IgG (μg/mL)	63.52	62.67	60.26	61.15	1.269	0.818	0.461	0.750
DAO (pg/mL)	86.98[b]	92.31[b]	112.03[a]	114.63[a]	2.581	<0.001	<0.001	0.623
D-Lactate (μg/L)	334.30[b]	336.96[b]	372.42[b]	449.01[a]	13.660	0.006	0.001	0.117
24 h								
IgA (μg/mL)	1.30	1.29	1.26	1.23	0.021	0.676	0.230	0.877
IgM (μg/mL)	2.90	2.56	2.54	2.65	0.066	0.175	0.186	0.090
IgG (μg/mL)	69.22	64.88	66.76	71.37	1.342	0.359	0.492	0.108
DAO (pg/mL)	82.49[c]	82.62[c]	88.58[b]	98.67[a]	1.569	<0.001	<0.001	0.010
D-Lactate (μg/L)	289.63[c]	290.12[c]	339.06[b]	384.49[a]	9.612	<0.001	<0.001	0.087

[1]DAO, diamine oxidase.
[a-c]Means within the same column with different superscripts differ significantly ($P < 0.05$).

Table 3
Effects of α toxin on the plasma antioxidant capacity of broiler chickens.

Items[1]	Alpha toxin (U/kg)				SEM	*P*-value		
	0	0.025	0.1	0.4		ANOVA	Linear	Quadratic
3 h								
CAT (U/mL)	1.42[a]	1.44[a]	0.90[b]	0.98[b]	0.063	<0.001	<0.001	0.705
GSH-Px (U/mL)	725.30[a]	573.01[b]	424.58[c]	296.39[d]	29.927	<0.001	<0.001	0.479
T-SOD (U/mL)	188.94[a]	177.89[a]	163.82[ab]	141.04[b]	5.270	0.049	<0.001	0.510
MDA (nmol/mL)	2.60	1.98	1.96	1.98	0.101	0.055	0.032	0.096
24 h								
CAT (U/mL)	0.729	0.649	0.604	0.429	0.063	0.400	0.104	0.706
GSH-Px (U/mL)	765.94[a]	569.64[b]	373.33[c]	235.73[d]	48.944	<0.001	<0.001	0.543
T-SOD (U/mL)	235.06[a]	206.44[b]	197.06[b]	167.83[c]	5.604	<0.001	<0.001	0.964
MDA (nmol/mL)	2.30	1.94	1.92	1.80	0.107	0.395	0.121	0.577

[1]CAT, catalase; GSH-Px, glutathione peroxidase; T-SOD, total superoxide dismutase; MDA, malondialdehyde.
[a-d]Mean values in the same row with no common superscript were significantly different ($P < 0.05$).

Table 4
Effects of α toxin on the jejunal mucosal antioxidant capacity of broiler chickens.

Items[1]	Alpha toxin (U/kg)				SEM	*P*-value		
	0	0.025	0.1	0.4		ANOVA	Linear	Quadratic
3 h								
CAT (U/mgprot)	23.09[a]	19.97[ab]	18.95[ab]	16.24[b]	0.786	0.014	0.002	0.880
GSH-Px (U/mgprot)	22.09[a]	19.62[b]	16.09[c]	11.07[d]	0.839	<0.001	<0.001	0.131
T-SOD (U/mgprot)	108.92[a]	95.39[b]	90.38[b]	80.92[c]	2.267	<0.001	<0.001	0.431
MDA (nmol/mgprot)	41.73	42.49	43.92	47.17	1.109	0.297	0.074	0.578
24 h								
CAT (U/mgprot)	41.33	39.36	33.09	30.37	1.754	0.076	0.012	0.906
GSH-Px (U/mgprot)	429.58[a]	427.16[a]	417.97[a]	341.37[b]	13.622	0.045	0.020	0.142
T-SOD (U/mgprot)	339.61[a]	344.44[a]	295.83[ab]	279.54[b]	9.955	0.001	<0.001	0.089
MDA (nmol/mgprot)	57.65[b]	78.92[a]	78.28[a]	84.29[a]	2.903	0.037	0.008	0.550

[1]CAT, catalase; GSH-Px, glutathione peroxidase; T-SOD, total superoxide dismutase; MDA, malondialdehyde.
[a-d]Mean values in the same row with no common superscript were significantly different ($P < 0.05$).

Table 5
Effects of α toxin on the ileal morphology of broiler chickens.

Items[1]	Alpha toxin (U/kg)				SEM	*P*-value		
	0	0.025	0.1	0.4		ANOVA	Linear	Quadratic
3 h								
Villus height (μm)	780.46	731.47	690.61	680.02	15.049	0.060	0.010	0.474
Crypt depth (μm)	130.52[b]	135.32[b]	168.88[a]	178.65[a]	6.362	0.005	0.001	0.801
VCR	6.03[a]	5.64[a]	4.12[b]	3.90[b]	0.227	<0.001	<0.001	0.779
24 h								
Villus height (μm)	725.14[a]	647.47[a]	613.03[ab]	467.82[b]	31.561	0.023	0.004	0.528
Crypt depth (μm)	152.51	150.69	135.25	115.84	5.435	0.055	0.011	0.374
VCR	5.02[a]	4.57[ab]	4.41[ab]	3.92[b]	0.140	0.044	0.006	0.920

[1]VCR, villus height to crypt depth ratio.
[a,b]Mean values in the same row with no common superscript were significantly different ($P < 0.05$).

significant effect on *IL-1β, IL-6, IL-8, IL-10, IL-17* and *IL*-22 mRNA expression among all groups at 24 h p.i. ($P > 0.05$) (Fig. 3A–F). At 24 h p.i., the relative mRNA expression of *TNF-α* was increased in a linear manner by the increase in α toxin level ($P < 0.01$) (Fig. 3 G). Compared to the control group, the 0.1 and 0.4 U/kg groups significantly upregulated *TNF-α* mRNA expression ($P < 0.05$), while the 0.025 U/kg group did not affect its expression ($P > 0.05$) at 24 h p.i.

3.6. *Relative mRNA expression of tight junction molecules*

There were linear decreases in jejunal relative mRNA expression of *claudin-1* ($P < 0.01$), *claudin-3* ($P < 0.01$) and *zonula occludens 1* (*ZO-1*) ($P < 0.05$) with the increase of α toxin level at 3 h p.i. (Fig. 4 A, C and E). The *claudin*-1 mRNA expression in the control and 0.025 U/kg groups was significantly higher than that in the 0.1 and 0.4 U/kg groups ($P < 0.05$) at 3 h p.i. Compared with the control and 0.025 U/kg groups, only the 0.4 U/kg group remarkably decreased the *claudin*-3 mRNA expression ($P < 0.05$) at 3 h p.i. The *ZO*-1 mRNA expression in the control group was the highest and no significance was observed among the other three groups ($P > 0.05$) at 3 h

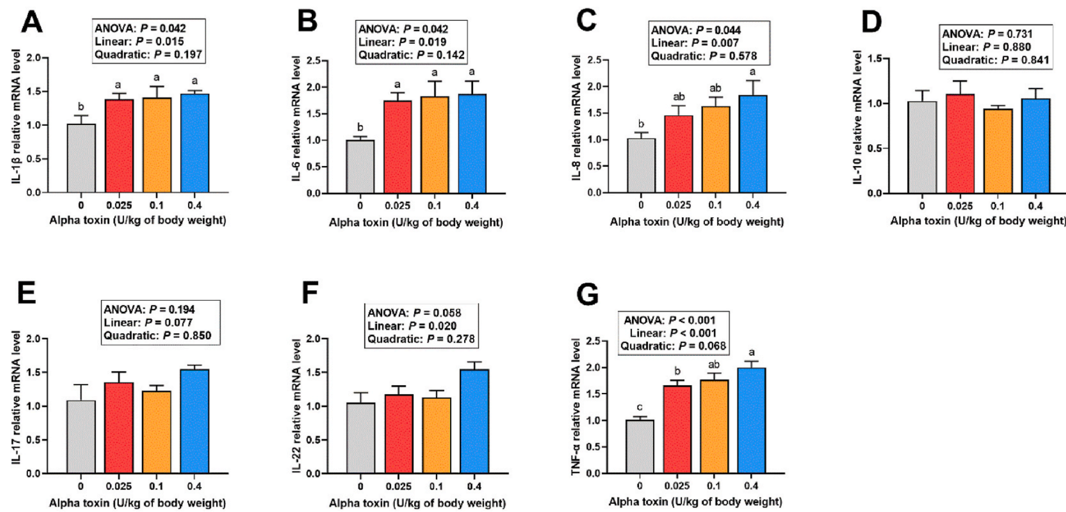

Fig. 2. Effects of α toxin on the jejunal relative mRNA expression of inflammatory genes of broiler chickens at 3 h. The four groups were injected with either PBS or α toxin at doses of 0.025, 0.1, and 0.4 U/kg of body weight, respectively. TNF-α, tumor necrosis factor alpha. Values are means with their SEM represented by vertical bars. Mean values with different letters were significantly different ($P < 0.05$).

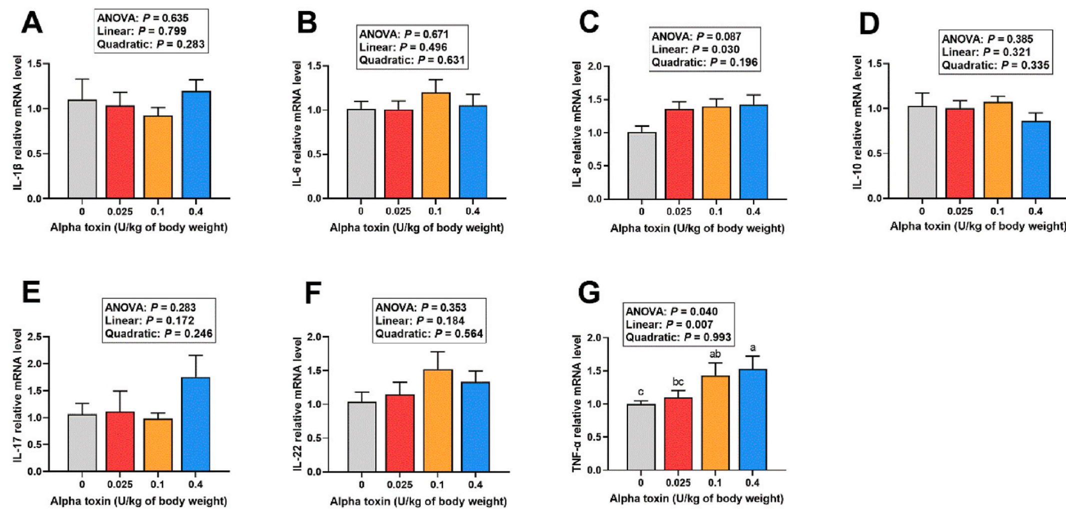

Fig. 3. Effects of α toxin on the jejunal relative mRNA expression of inflammatory genes of broiler chickens at 24 h. The four groups were injected with either PBS or α toxin at doses of 0.025, 0.1, and 0.4 U/kg of body weight, respectively. TNF-α, tumor necrosis factor alpha. Values are means with their SEM represented by vertical bars. Mean values with different letters were significantly different ($P < 0.05$).

p.i. At 24 h p.i., α toxin challenge linearly decreased the relative mRNA expression of *claudin-3* ($P < 0.01$), *occludin* ($P < 0.01$) and *ZO-1* ($P < 0.01$) in the jejunum (Fig. 5C–E). The *claudin*-3 mRNA expression in the 0.4 U/kg group was the lowest and no significance was observed among the other three groups ($P > 0.05$) at 24 h p.i. Compared with the control group, all of the α toxin-challenged groups remarkably decreased the *occludin and ZO*-1 mRNA expression ($P < 0.05$) at 24 h p.i. No significant difference was found among all groups in the jejunal relative mRNA expression of *claudin-2* and *occludin* at 3 h p.i (Fig. 4 B and D). and *claudin-1* and *claudin-2* at 24 h p.i. (Fig. 5A and B) ($P > 0.05$).

3.7. Relative mRNA expression of PLCγ1/AMPK/mTOR pathway

At 3 h p.i., α toxin challenge linearly increased the mRNA expression of *PLCγ1* ($P < 0.01$) and linearly decreased the mRNA expression of *mTOR* ($P < 0.01$) (Fig. 6 A and C). The mRNA expression of *PLCγ1* in the control and 0.025 groups was significantly higher than that in the 0.1 and 0.4 groups ($P < 0.05$) at 3 h p.i. Relative to the control birds, the 0.1 and 0.4 U/kg groups significantly upregulated the mRNA expression of *mTOR* ($P < 0.05$) at 3 h p.i. The relative mRNA expression of *AMPKα1* was increased in a linear ($P < 0.05$) and quadratic ($P < 0.05$) manner with the increased α toxin level at 3 h p.i. (Fig. 6 B). The *AMPKα1* mRNA expression in the 0.4

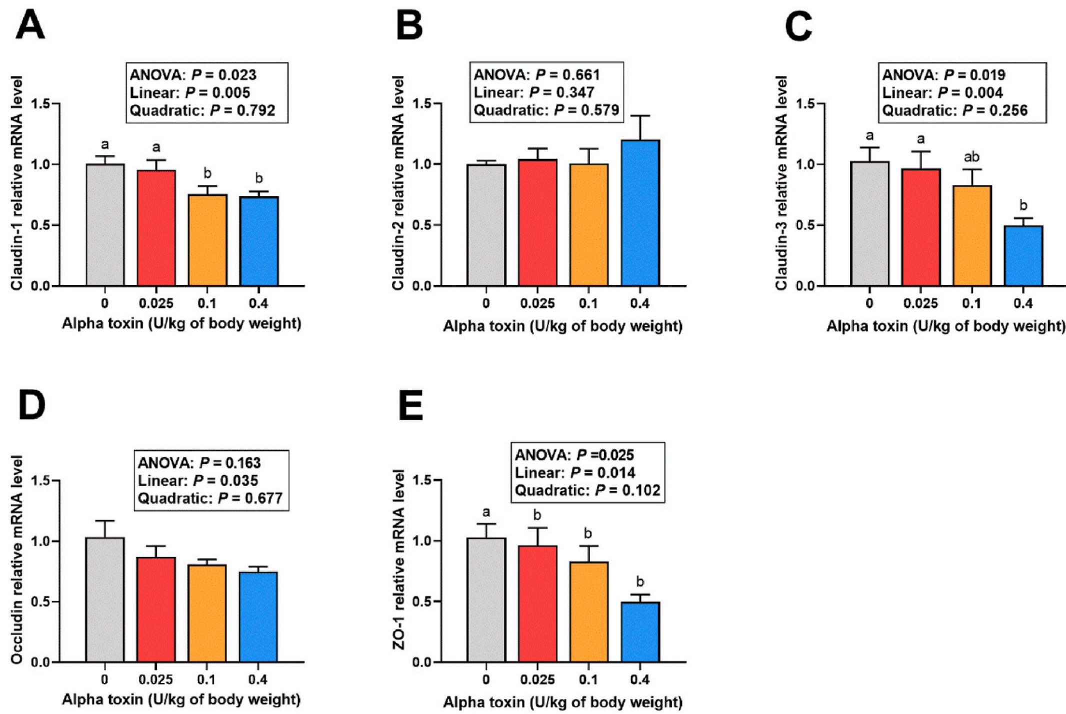

Fig. 4. Effects of α toxin on the jejunal relative mRNA expression of tight junction proteins of broiler chickens at 3 h. The four groups were injected with either PBS or α toxin at doses of 0.025, 0.1, and 0.4 U/kg of body weight, respectively. ZO-1, zonula occludens 1. Values are means with their SEM represented by vertical bars. Mean values with different letters were significantly different ($P < 0.05$).

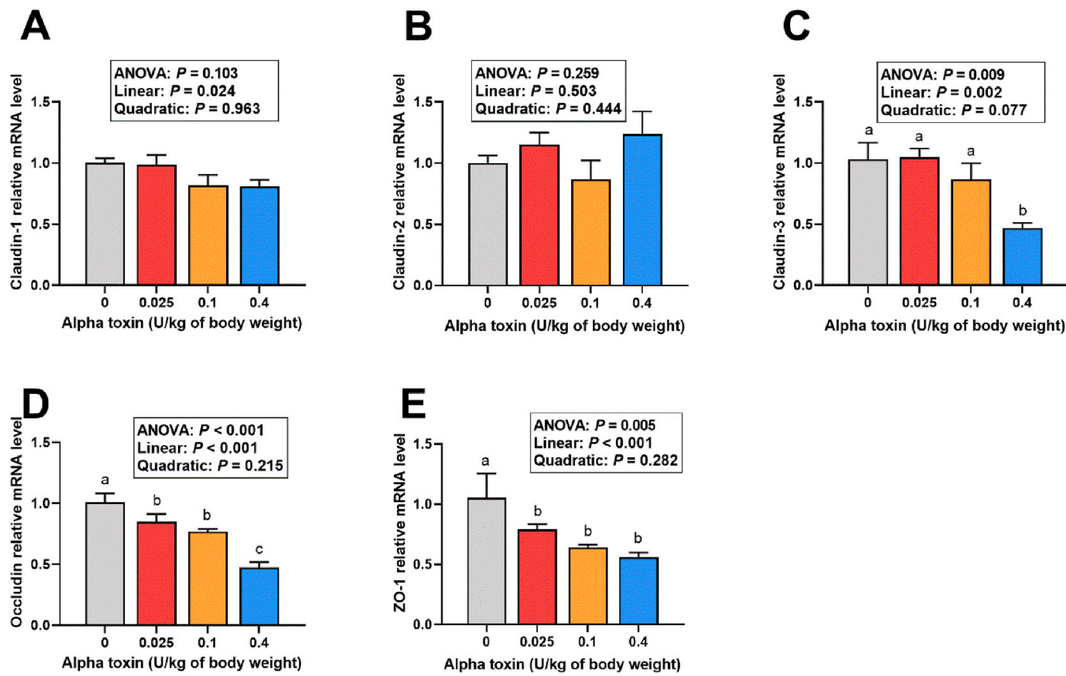

Fig. 5. Effects of α toxin on the jejunal relative mRNA expression of tight junction proteins of broiler chickens at 24 h. The four groups were injected with either PBS or α toxin at doses of 0.025, 0.1, and 0.4 U/kg of body weight, respectively. ZO-1, zonula occludens 1. Values are means with their SEM represented by vertical bars. Mean values with different letters were significantly different ($P < 0.05$).

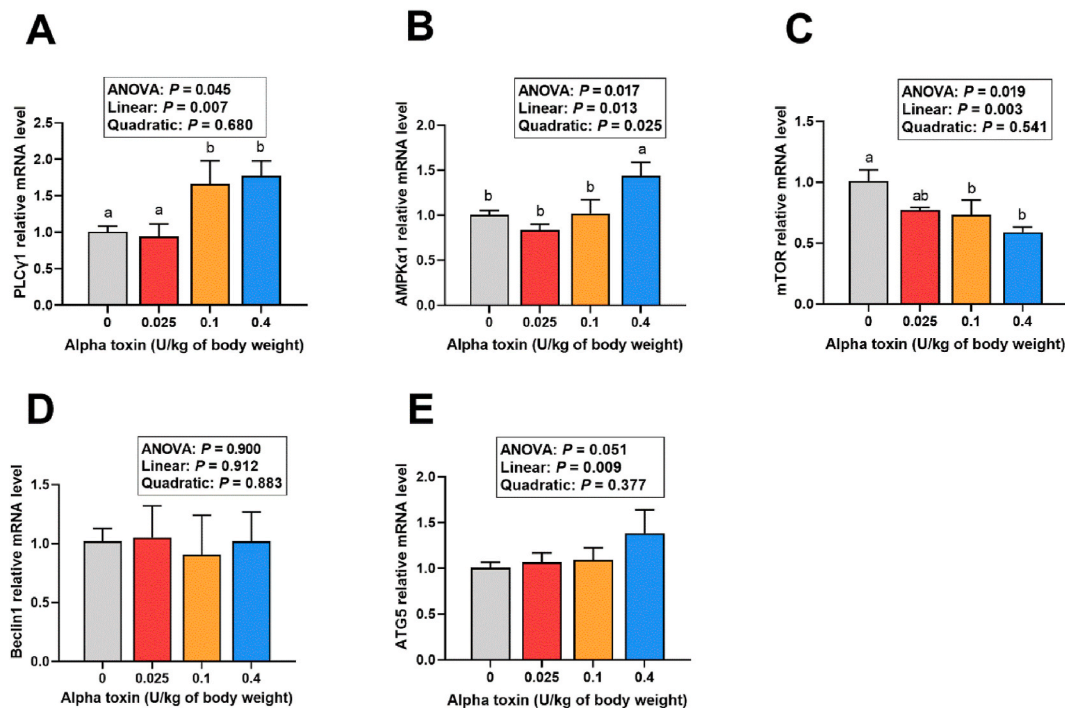

Fig. 6. Effects of α toxin on the jejunal relative mRNA expression of autophagy-related signaling pathway of broiler chickens at 3 h. The four groups were injected with either PBS or α toxin at doses of 0.025, 0.1, and 0.4 U/kg of body weight, respectively. PLCγ1, Phospholipases Cγ1; AMPKα1, AMP-activated protein kinase α1; mTOR, mammalian target of rapamycin; Beclin1, programmed cell death 1; ATG5, autophagy-related gene 5. Values are means with their SEM represented by vertical bars. Mean values with different letters were significantly different ($P < 0.05$).

U/kg group was the highest and no significance was observed among the other three groups ($P > 0.05$) at 3 h p.i. At 24 h p.i., α toxin challenge linearly increased the relative mRNA expression of *AMPKα1* ($P < 0.01$) and *ATG5* ($P < 0.01$) in the jejunum (Fig. 7 B and E). Relative to the control group, the 0.1 and 0.4 U/kg groups significantly upregulated the mRNA expression of *AMPKα1* ($P < 0.05$) at 24 h p.i. Only the 0.4 U/kg group significantly increased the *ATG5* mRNA expression when compared to the control group ($P < 0.05$) at 24 h p.i. No significant difference was found among all groups in the jejunal relative mRNA expression of *Beclin1* and *ATG5* at 3 h p.i (Fig. 6D and E). and PLC*γ1, mTOR* and *Beclin1* at 24 h p.i. (Fig. 7 A, C and D) ($P > 0.05$).

4. Discussion

The incidence of necrotic enteritis caused by *C. perfringens* infection in commercial poultry is growing at an alarming rate. It has been reported that the *C. perfringens* alpha toxin was involved in the pathogenesis of chicken necrotic enteritis with diarrhea and macroscopic lesions in the small intestine [9]. In this study, broiler chickens were challenged with α toxin to investigate the oxidative damage, inflammatory reaction and intestine injury evoked by this toxin. Our results showed that α toxin reduced the average daily gain during the 3 days after infection. This detrimental effect may be associated with oxidative damage, intestinal inflammatory responses, damaged intestinal morphology and barrier function.

Plasma immunoglobulins, especially IgA, IgM and IgG, are key indicators reflecting the non-specific immune status of animals and play crucial roles in defense against foreign antigens [21,22]. In the present study, α toxin challenge significantly reduced plasma IgA and IgM levels at 3 h p.i., possibly associated with the increased pro-inflammatory cytokines. Patients whose peripheral blood mononuclear cells exhibited higher production of TNF-α and IL-1β after stimulation with LPS had lower serum IgA levels at diagnosis [23]. Additionally, Mishra et al. [24] reported that lower doses of IgA triggered the release of pro-inflammatory cytokines like IL-1β and IL-6 from peripheral blood mononuclear cells, while higher doses of IgA led to a reduction in pro-inflammatory cytokine production. However, the release of the anti-inflammatory cytokines IL-10 and TGF-β1 was not significantly changed. Consistent with our results, Gharib-Naseri et al. [25] also found decreased expression of IgA and IgM in chickens challenged with *C. perfringens* compared to unchallenged birds. Furthermore, it has been reported that antibody titers against *C. perfringens* α toxin were higher in healthy chickens than those in necrotic enteritis-afflicted chickens [26].

Oxidative stress usually occurs when there is an imbalance between oxidation and antioxidation in the body, which leads to cell and tissue damage [27]. The principal antioxidant enzymes include CAT, GSH-Px and SOD. The SOD is capable of converting the superoxide radicals to H_2O_2, and the excess H_2O_2 can be removed by CAT and members of the peroxidase family, including GSH-Px [28]. MDA is a crucial biomarker of oxidative damage when peroxides break down into aldehydes (such as MDA) in the final stage of the peroxidation process [28]. In this study, chicken plasma and mucosal CAT, GSH-Px and SOD activities were decreased and mucosal

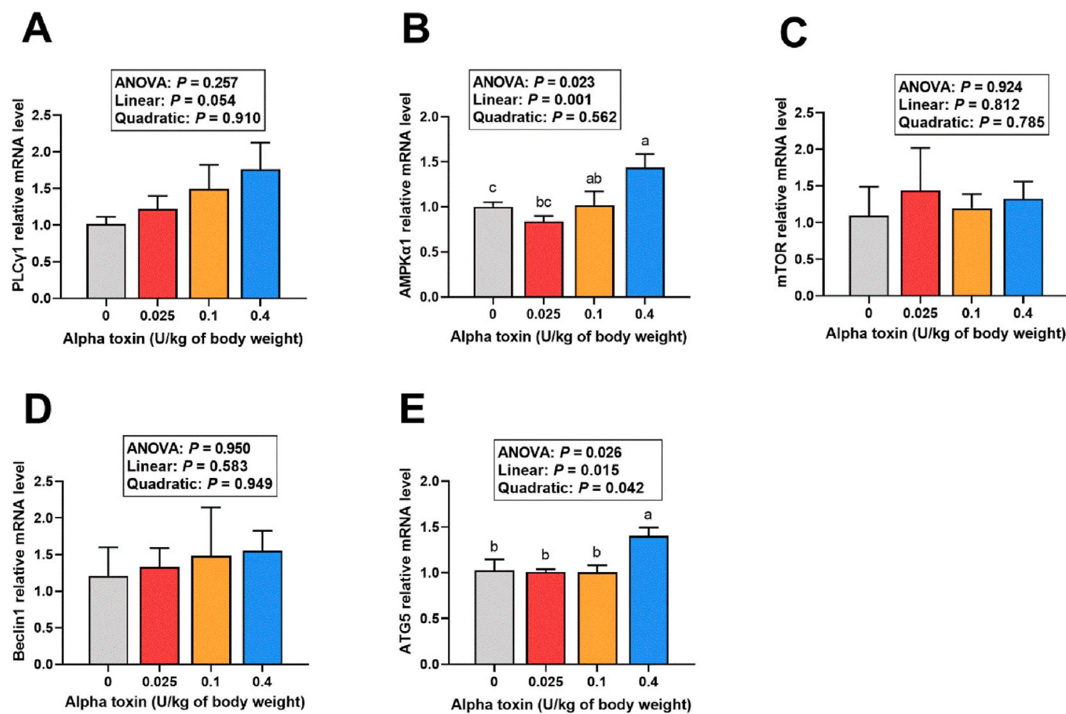

Fig. 7. Effects of α toxin on the jejunal relative mRNA expression of autophagy-related signaling pathway of broiler chickens at 24 h. The four groups were injected with either PBS or α toxin at doses of 0.025, 0.1, and 0.4 U/kg of body weight, respectively. PLCγ1, Phospholipases Cγ1; AMPKα1, AMP-activated protein kinase α1; mTOR, mammalian target of rapamycin; Beclin1, programmed cell death 1; ATG5, autophagy-related gene 5. Values are means with their SEM represented by vertical bars. Mean values with different letters were significantly different ($P < 0.05$).

MDA content was elevated with the increased α toxin level in a linear manner, which clearly demonstrated that α toxin challenge induced oxidative stress. The results were also consistent with the findings of Monturiol-Gross et al. [29], who found that α toxin caused oxidative stress by stimulating the generation of reactive oxide species in Chinese hamster fibroblast cells.

Intestinal barrier function is an important factor in the productivity and livability of broilers. When intestinal barrier damage occurs, intestinal permeability will rise. DAO is an especially active intracellular enzyme in intestinal mucosa, and when intestinal mucosa is impaired, the DAO is leaked from villus tips into blood circulation [30]. D-lactate is a metabolic end product of intestinal microflora and would pass into the bloodstream through the damaged intestinal barrier [31]. These two indices are considered sensitive biomarkers to evaluate intestinal permeability. The present results showed that α toxin challenge linearly increased plasma DAO and D-lactate levels at 3 and 24 h p.i., suggesting that α toxin could impair the intestinal barrier integrity of broiler chickens. The decreased intestinal integrity was further supported by the damaged intestinal morphology and the decreased gene expression of the intestinal tight junction in this study. Similarly, Rehman et al. [11] found that *C. perfringens* α toxin can impair the intestinal mucosal barrier of laying hens by measuring the transmural tissue conductance with Ussing Chamgbers.

The intestinal morphology reveals the host's ability to absorb nutrients and defend itself against external antigens. Shorter villus height indicates decreased nutrition absorptive area and poor absorptive capacity [32]. A deeper crypt indicates faster cellular turnover to renew the villus in response to epithelial cell injury, inflammation and sloughing [33]. Greater VCR is usually related to improved intestinal function and integrity. In the current research, α toxin challenge linearly increased crypt depth and linearly decreased villus height and VCR in the ileum of broiler chickens. The results indicated that α toxin administration caused intestine structure damage, which has also been reported by Morris et al. [34] in calves, as evidenced by large amounts of exfoliated epithelial cells in the lumen of alpha toxin-treated intestinal loops.

The balance between pro- and anti-inflammatory cytokines is essential for intestinal immune homeostasis [35]. *IL-1β, IL-6, IL-8* and *TNF-α* are major pro-inflammatory cytokines associated with cytokine storms [36]. IL-10 is a crucial anti-inflammatory cytokine to prevent the generation of pro-inflammatory mediators [37]. IL-17 and IL-22 are cytokines involved in tissue signaling, supporting the protection and regeneration of barrier organs like the skin, lungs, and gastrointestinal tract [38]. In this study, the preponderance of proinflammatory cytokines, such as *IL-1β, IL-6, IL-8* and *TNF-α*, triggered by α toxin, indicated the occurrence of inflammation. Previous research has revealed that TNF-α plays a significant role in α toxin-induced mortality and its production is dependent on the activation of extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signal transduction via tyrosine kinase A (TrkA) [12]. Production of IL-8 was triggered by α toxin through the activation of two separate pathways, the ERK1/2/nuclear factor kappa-B (NF-κB) and p38 MAPK pathways [39]. Consistent with our results, Guo et al. [13] reported that 1000 U/L of α toxin upregulated *IL-6*, *IL-8* and *TNF-α* mRNA expression compared with the control group in an *in vitro* study of chicken primary intestinal

epithelial cells. Also, it has been discovered that the α toxin induces serum IL-1β, IL-6 and TNF-α release in mice [12].

Tight junctions seal the paracellular space and act as a fence to prevent macromolecular translocation [40,41]. The main components of tight junction proteins are claudins, occludin and zonula occludens. The disrupted tight junctions could lead to increased mucosal permeability and pathogen invasion [41]. In previous studies, claudin-1 and claudin-3 were considered pore-sealing claudins, and claudin-2 was a pore-forming claudin [42]. Occludin was important in maintaining barrier function as its expression was negatively correlated with the translocation of fluorescein isothiocyanate transfer from the gut to the blood [43]. Compared with ZO-2 and ZO-3, ZO-1 plays a major role in forming tight junctions in epithelial cells [44]. However, few studies are available regarding tight junction protein expression in α toxin-challenged birds. In the current study, α toxin administration linearly downregulated the jejunal mRNA expression of *claudin-1*, *claudin-3*, *occludin* and *ZO-1*, suggesting that α toxin was detrimental to the intestinal mucosal barrier. Hence, our results indicated that the toxin's destruction of intestinal morphology and barrier function may contribute to the compromised body weight gain of broiler chickens.

Alpha toxin has been reported to alter cell membrane dynamics via specifically activated endogenous PLCγ1 [17]. The inhibition of PLCγ1 could decrease the release of IL-8, which is a chemokine causing acute inflammation by recruiting and activating neutrophils [17]. Autophagy is intimately connected with inflammatory reactions and their relationship is extremely complicated [45]. ATG5 binds to microtubule-associated protein light chain 3B-II (LC3B-II) and initiates the formation of autophagosomes in the early autophagy stage [19]. The AMPK/mTOR pathway plays a crucial role in regulating autophagy [14]. In our study, α toxin increased the *AMPKα1* mRNA expression and decreased the *mTOR* mRNA expression. AMPK is associated with energy and nutrients sensing and activates autophagy by blocking mTOR [46,47]. mTOR has been proven to negatively regulate autophagy, and its inactivation is necessary to initiate autophagy [19,45]. In this study, the activation of the AMPK/mTOR signaling pathway by α toxin may be due to the stimulation of Ca^{2+} release. Alpha toxin can activate endogenous PLC with the formation of diacylalycerol and inositol trisphosphate; the latter increases intrcytoplasmic Ca^{2+} [48,49]. A recent study has demonstrated that an increase in Ca^{2+} mediated by the PLC pathway triggers autophagy through the calmodulin-dependent protein kinase kinase β (CaMKKβ)/AMPK/mTOR signaling pathway [19]. Collectively, α toxin may activate autophagy through the PLCγ1/AMPK/mTOR signaling pathway in broiler chickens.

In conclusion, the present data revealed that *C. perfringens* α toxin challenge dampened body weight gain, decreased plasma immunoglobulin levels, induced oxidative damage and intestinal inflammation, impaired intestinal morphology and barrier function, and induced PLCγ1/AMPK/mTOR pathway-mediated autophagy in broiler chickens. The recommended intraperitoneal injection dose for moderate injury was 0.1 U/kg of body weight and recommended sampling time was 3 h p.i. in broiler chickens. This research provides a better understanding of the role of α toxin in pathogenesis of necrotic enteritis and offers information with respect to building an experimental model of α toxin challenge for future nutrition intervention against necrotic enteritis.

Ethics statement

This study was reviewed and approved by the Animal Care and Use Committee of Qingdao Agricultural University, with the approval number: 20210715099.

Data availability statement

The data that has been used is confidential.

CRediT authorship contribution statement

Tong Zhang: Writing – original draft, Methodology, Data curation. **Xiaohui Wang:** Methodology, Formal analysis, Data curation. **Wenli Li:** Supervision, Project administration, Conceptualization. **Heliang Wang:** Resources, Methodology, Data curation. **Lei Yan:** Resources, Methodology, Investigation. **Lianwen Zhao:** Formal analysis, Data curation. **Xiaowen Zhang:** Methodology, Data curation. **Nianxue Wang:** Software, Methodology. **Wendong An:** Formal analysis, Data curation. **Tongyue Liu:** Methodology, Investigation. **Wenlei Fan:** Validation, Software. **Beibei Zhang:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Beibei Zhang reports financial support was provided by the National Natural Science Foundation of China (32102580). Beibei Zhang reports financial support was provided by the Natural Science Foundation of Shandong Province (ZR2020QC181). Beibei Zhang reports financial support was provided by the Doctoral Science Research Starup Funding of Qingdao Agricultural University (663–1120008). Beibei Zhang reports financial support was provided by the Open Project of State Key Laboratory of Animal Nutrition. Beibei Zhang reports financial support was provided by the National College Student Innovation and Entrepreneurship Training Program Project (202210435024). Wenlei Fan reports financial support was provided by the Natural Science Foundation of Shandong Province (ZR2020QC182). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (32102580), the Natural Science Foundation of Shandong Province (ZR2020QC181), the Doctoral Science Research Starup Funding of Qingdao Agricultural University (663/1120008), the Open Project of State Key Laboratory of Animal Nutrition, the National College Student Innovation and Entrepreneurship Training Program Project (202210435024), Natural Science Foundation of Shandong Province (ZR2020QC182), and Higher Education Department of the Ministry of Education, China.

References

[1] K.K. Cooper, J.G. Songer, Necrotic enteritis in chickens: a paradigm of enteric infection by Clostridium perfringens type A, Anaerobe 15 (2009) 55–60, https://doi.org/10.1016/j.anaerobe.2009.01.006.
[2] G. Chalmers, H.L. Bruce, D.L. Toole, D.A. Barnum, P. Boerlin, Necrotic enteritis potential in a model system using Clostridium perfringens isolated from field outbreaks, Avian Dis. 51 (2007) 834–839, https://doi.org/10.1637/7959-022807-regr.1.
[3] B. Wade, A. Keyburn, The true cost of necrotic enteritis, Worlds Poult. Sci. 31 (2015) 16–17.
[4] R. Kiu, L.J. Hall, An update on the human and animal enteric pathogen Clostridium perfringens, Emerg. Microb. Infect. 7 (2018) 141, https://doi.org/10.1038/s41426-018-0144-8.
[5] I. Mehdizadeh Gohari, A.N. M, J. Li, A. Shrestha, F. Uzal, A.M. B, Pathogenicity and virulence of Clostridium perfringens, Virulence 12 (2021) 723–753, https://doi.org/10.1080/21505594.2021.1886777.
[6] F. Al-Sheikhly, R.B. Truscott, The pathology of necrotic enteritis of chickens following infusion of crude toxins of Clostridium perfringens into the duodenum, Avian Dis. 21 (1977) 241–255.
[7] C.F. Coursodon, H.T. Trinh, M. Mallozzi, G. Vedantam, R.D. Glock, J.G. Songer, Clostridium perfringens alpha toxin is produced in the intestines of broiler chicks inoculated with an alpha toxin mutant, Anaerobe 16 (2010) 614–617, https://doi.org/10.1016/j.anaerobe.2010.09.006.
[8] J. Sakurai, M. Nagahama, M. Oda, Clostridium perfringens alpha-toxin: characterization and mode of action, J. Biochem. 136 (2004) 569–574, https://doi.org/10.1093/jb/mvh161.
[9] H. Rehman, W.A. Awad, I. Lindner, M. Hess, J. Zentek, Clostridium perfringens alpha toxin affects electrophysiological properties of isolated jejunal mucosa of laying hens, Poultry Sci. 85 (2006) 1298–1302, https://doi.org/10.1093/ps/85.7.1298.
[10] M.E. Fernández Miyakawa, F.A. Uzal, Morphologic and physiologic changes induced by Clostridium perfringens type A alpha toxin in the intestine of sheep, Am. J. Vet. Res. 66 (2005) 251–255, https://doi.org/10.2460/ajvr.2005.66.251.
[11] H. Rehman, A. Ijaz, A. Specht, D. Dill, P. Hellweg, K. Manner, J. Zentek, In vitro effects of alpha toxin from Clostridium perfringens on the electrophysiological parameters of jejunal tissues from laying hens preincubated with inulin and N-acetyl-L-cysteine, Poultry Sci. 88 (2009) 199–204, https://doi.org/10.3382/ps.2008-00054.
[12] M. Oda, A. Kihara, H. Yoshioka, Y. Saito, N. Watanabe, K. Uoo, M. Higashihara, M. Nagahama, N. Koide, T. Yokochi, J. Sakurai, Effect of erythromycin on biological activities induced by clostridium perfringens alpha-toxin, J. Pharmacol. Exp. Therapeut. 327 (2008) 934–940, https://doi.org/10.1124/jpet.108.143677.
[13] S. Guo, C. Li, D. Liu, Y. Guo, Inflammatory responses to a Clostridium perfringens type A strain and α-toxin in primary intestinal epithelial cells of chicken embryos, Avian Pathol. 44 (2015) 81–91, https://doi.org/10.1080/03079457.2015.1005573.
[14] X. Chen, X. Zhou, X. Cheng, L. Lin, Q. Wang, R. Zhan, Q. Wu, S. Liu, Protective effect of ferulic Acid on lipopolysaccharide-induced BV2 microglia inflammation via AMPK/mTOR signaling pathway, Molecules 28 (2023) 3482, https://doi.org/10.3390/molecules28083482.
[15] X. Chen, Y. Wang, N. Qu, B. Zhang, C. Xia, PLCγ1 inhibition-driven autophagy of IL-1β-treated chondrocyte confers cartilage protection against osteoarthritis, involving AMPK, Erk and Akt, J. Cell Mol. Med. 25 (2021) 1531–1545, https://doi.org/10.1111/jcmm.16245.
[16] S.A. Alqarni, A. Bineid, S.F. Ahmad, N.O. Al-Harbi, F. Alqahtani, K.E. Ibrahim, N. Ali, A. Nadeem, Blockade of tyrosine kinase, LCK leads to reduction in airway inflammation through regulation of pulmonary Th2/treg balance and oxidative stress in cockroach extract-induced mouse model of allergic asthma, Metabolites 12 (2022) 793, https://doi.org/10.3390/metabo12090793.
[17] T. Takagishi, M. Oda, M. Kabura, M. Kurosawa, K. Tominaga, S. Urano, Y. Ueda, K. Kobayashi, T. Kobayashi, J. Sakurai, Y. Terao, M. Nagahama, Clostridium perfringens alpha-toxin induces Gm1a clustering and trka phosphorylation in the host cell membrane, PLoS One 10 (2015) e0120497, https://doi.org/10.1371/journal.pone.0120497.
[18] L. Hou, F. Jiang, B. Huang, W. Zheng, Y. Jiang, G. Cai, D. Liu, C.Y. Hu, C. Wang, Dihydromyricetin ameliorates inflammation-induced insulin resistance via phospholipase C-CaMKK-AMPK signal pathway, Oxid. Med. Cell. Longev. 2021 (2021) 8542809, https://doi.org/10.1155/2021/8542809.
[19] T. Cui, X. Wang, J. Hu, T. Lin, Z. Hu, H. Guo, G. Huang, G. Hu, C. Zhang, Molybdenum and cadmium co-exposure induces CaMKKβ/AMPK/mTOR pathway mediated-autophagy by subcellular calcium redistribution in duck renal tubular epithelial cells, J. Inorg. Biochem. 236 (2022) 111974, https://doi.org/10.1016/j.jinorgbio.2022.111974.
[20] J. Finley, Oocyte activation and latent HIV-1 reactivation: AMPK as a common mechanism of action linking the beginnings of life and the potential eradication of HIV-1, Med. Hypotheses 93 (2016) 34–47, https://doi.org/10.1016/j.mehy.2016.05.012.
[21] L.N. Long, B.J. Kang, Q. Jiang, J.S. Chen, Effects of dietary Lycium barbarum polysaccharides on growth performance, digestive enzyme activities, antioxidant status, and immunity of broiler chickens, Poultry Sci. 99 (2020) 744–751, https://doi.org/10.1016/j.psj.2019.10.043.
[22] H.W. Schroeder Jr., L. Cavacini, Structure and function of immunoglobulins, J. Allergy Clin. Immunol. 125 (2010) S41–S52, https://doi.org/10.1016/j.jaci.2009.09.046.
[23] M.E. González-Serrano, I. Estrada-García, D. Mogica-Martínez, A. González-Garay, G. López-Herrera, L. Berrón-Ruiz, S.E. Espinosa-Padilla, M.A. Yamazaki-Nakashimada, A. Vargas-Hernández, L. Santos-Argumedo, S.A. Estrada-Parra, F.J. Espinosa-Rosales, Increased pro-inflammatory cytokine production after lipopolysaccharide stimulation in patients with X-linked agammaglobulinemia, J. Clin. Immunol. 32 (2012) 967–974, https://doi.org/10.1007/s10875-012-9706-z.
[24] K.P. Mishra, A.P. Yadav, Shweta, S. Chanda, D. Majumdar, L. Ganju, Serum levels of immunoglobulins (IgG, IgA, IgM) in Antarctic summer expeditioners and their relationship with seasickness, Cell. Immunol. 271 (2011) 29–35, https://doi.org/10.1016/j.cellimm.2011.05.01.
[25] K. Gharib-Naseri, J.C. de Paula Dorigam, K. Doranalli, S. Kheravii, R.A. Swick, M. Choct, S.B. Wu, Modulations of genes related to gut integrity, apoptosis, and immunity underlie the beneficial effects of Bacillus amyloliquefaciens CECT 5940 in broilers fed diets with different protein levels in a necrotic enteritis challenge model, J. Anim. Sci. Biotechnol. 11 (2020) 104, https://doi.org/10.1186/s40104-020-00508-4.
[26] K.W. Lee, H.S. Lillehoj, M.S. Park, S.I. Jang, G.D. Ritter, Y.H. Hong, W. Jeong, H.Y. Jeoung, D.J. An, E.P. Lillehoj, Clostridium perfringens alpha-toxin and NetB toxin antibodies and their possible role in protection against necrotic enteritis and gangrenous dermatitis in broiler chickens, Avian Dis. 56 (2012) 230–233, https://doi.org/10.1637/9847-070711-ResNote.1.
[27] S. Liu, J. Tan, Y. Hu, X. Jia, M.H. Kogut, J. Yuan, H. Zhang, Dietary l-arginine supplementation influences growth performance and B-cell secretion of immunoglobulin in broiler chickens, J. Anim. Physiol. Anim. Nutr. 103 (2019) 1125–1134, https://doi.org/10.1111/jpn.13110.
[28] R. Kohen, A. Nyska, Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification, J. Toxicol. Pathol. 30 (2002) 620–650, https://doi.org/10.1080/01926230290166724.

[29] L. Monturiol-Gross, M. Flores-Diaz, C. Araya-Castillo, M.J. Pineda-Padilla, G.C. Clark, R.W. Titball, A. Alape-Giron, Reactive oxygen species and the MEK/ERK pathway are involved in the toxicity of clostridium perfringens alpha-toxin, a prototype bacterial phospholipase C, J. Infect. Dis. 206 (2012) 1218–1226, https://doi.org/10.1093/infdis/jis496.
[30] Y. Honzawa, H. Nakase, M. Matsuura, T. Chiba, Clinical significance of serum diamine oxidase activity in inflammatory bowel disease: importance of evaluation of small intestinal permeability, Inflamm. Bowel Dis. 17 (2011) E23–E25, https://doi.org/10.1002/ibd.21588.
[31] M.D. Levitt, D.G. Levitt, Quantitative evaluation of D-lactate pathophysiology: new insights into the mechanisms involved and the many areas in need of further investigation, Clin. Exp. Gastroenterol. 13 (2020) 321–337, https://doi.org/10.2147/ceg.s260600.
[32] W.F. Caspary, Physiology and pathophysiology of intestinal absorption, Am. J. Clin. Nutr. 55 (1992) 299s–308s, https://doi.org/10.1093/ajcn/55.1.299s.
[33] D. Paiva, C. Walk, A. McElroy, Dietary calcium, phosphorus, and phytase effects on bird performance, intestinal morphology, mineral digestibility, and bone ash during a natural necrotic enteritis episode, Poultry Sci. 93 (2014) 2752–2762, https://doi.org/10.3382/ps.2014-04148.
[34] W.E. Morris, M.V. Dunleavy, J. Diodati, G. Berra, M.E. Fernandez-Miyakawa, Effects of Clostridium perfringens alpha and epsilon toxins in the bovine gut, Anaerobe 18 (2012) 143–147, https://doi.org/10.1016/j.anaerobe.2011.12.003.
[35] Y. Yue, Y. Guo, Y. Yang, Effects of dietary L-tryptophan supplementation on intestinal response to chronic unpredictable stress in broilers, Amino Acids 49 (2017) 1227–1236, https://doi.org/10.1007/s00726-017-2424-3.
[36] J.G. Wilson, L.J. Simpson, A.M. Ferreira, A. Rustagi, J. Roque, A. Asuni, T. Ranganath, P.M. Grant, A. Subramanian, Y. Rosenberg-Hasson, H.T. Maecker, S. P. Holmes, J.E. Levitt, C.A. Blish, A.J. Rogers, Cytokine profile in plasma of severe COVID-19 does not differ from ARDS and sepsis, JCI Insight 5 (2020) e140289, https://doi.org/10.1172/jci.insight.140289.
[37] A.J. Smith, S.E. Humphries, Cytokine and cytokine receptor gene polymorphisms and their functionality, Cytokine Growth Factor Rev. 20 (2009) 43–59, https://doi.org/10.1016/j.cytogfr.2008.11.006.
[38] K. Eyerich, V. Dimartino, A. Cavani, IL-17 and IL-22 in immunity: driving protection and pathology, Eur. J. Immunol. 47 (2017) 607–614, https://doi.org/10.1002/eji.201646723.
[39] M. Oda, R. Shiihara, Y. Ohmae, M. Kabura, T. Takagishi, K. Kobayashi, M. Nagahama, M. Inoue, T. Abe, K. Setsu, J. Sakurai, Clostridium perfringens alpha-toxin induces the release of IL-8 through a dual pathway via TrkA in A549 cells, Biochim. Biophys. Acta 1822 (2012) 1581–1589, https://doi.org/10.1016/j.bbadis.2012.06.007.
[40] S.T. Ballard, J.H. Hunter, A.E. Taylor, Regulation of tight-junction permeability during nutrient absorption across the intestinal epithelium, Annu. Rev. Nutr. 15 (1995) 35–55, https://doi.org/10.1146/annurev.nu.15.070195.000343.
[41] J.R. Turner, Intestinal mucosal barrier function in health and disease, Nat. Rev. Immunol. 9 (2009) 799–809, https://doi.org/10.1038/nri2653.
[42] W.A. Awad, C. Hess, M. Hess, Enteric pathogens and their toxin-induced disruption of the intestinal barrier through alteration of tight junctions in chickens, Toxins 9 (2017) 60, https://doi.org/10.3390/toxins9020060.
[43] P.D. Cani, S. Possemiers, T. Van de Wiele, Y. Guiot, A. Everard, O. Rottier, L. Geurts, D. Naslain, A. Neyrinck, D.M. Lambert, G.G. Muccioli, N.M. Delzenne, Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability, Gut 58 (2009) 1091–1103, https://doi.org/10.1136/gut.2008.165886.
[44] S. Tsukita, M. Furuse, M. Itoh, Multifunctional strands in tight junctions, Nat. Rev. Mol. Cell Biol. 2 (2001) 285–293, https://doi.org/10.1038/35067088.
[45] T. Liu, S. Han, Q. Dai, J. Zheng, C. Liu, S. Li, J. Li, IL-17A-Mediated excessive autophagy aggravated neuronal ischemic injuries via src-PP2B-mTOR pathway, Front. Immunol. 10 (2019) 2952, https://doi.org/10.3389/fimmu.2019.02952.
[46] L. Li, L. Li, X. Zhou, Y. Yu, Z. Li, D. Zuo, Y. Wu, Silver nanoparticles induce protective autophagy via Ca(2+)/CaMKKβ/AMPK/mTOR pathway in SH-SY5Y cells and rat brains, Nanotoxicology 13 (2019) 369–391, https://doi.org/10.1080/17435390.2018.1550226.
[47] F.S. Liu, C. Jiang, Z. Li, X.B. Wang, J. Li, B. Wang, G.H. Lv, F.B. Liu, Ca(2+) regulates autophagy through CaMKKβ/AMPK/mTOR signaling pathway in mechanical spinal cord injury: an in vitro study, Neurochem. Res. 48 (2023) 447–457, https://doi.org/10.1007/s11064-022-03768-w.
[48] M. Oda, S. Ikari, T. Matsuno, Y. Morimune, M. Nagahama, J. Sakurai, Signal transduction mechanism involved in Clostridium perfringens alpha-toxin-induced superoxide anion generation in rabbit neutrophils, Infect. Immun. 74 (2006) 2876–2886, https://doi.org/10.1128/iai.74.5.2876-2886.2006.
[49] M.A. Navarro, B.A. McClane, F.A. Uzal, Mechanisms of action and cell death associated with Clostridium perfringens toxins, Toxins 10 (2018) 212, https://doi.org/10.3390/toxins10050212.