

Dietary Ellagic Acid Ameliorated *Clostridium Perfringens*-Induced Subclinical Necrotizing Enteritis In Broilers Via Regulating Inflammation Signaling Pathways And Cecal Microbiota to Inhibit Intestinal Barrier Damage

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Abstract

Background: Subclinical necrotizing enteritis (SNE) is a common intestinal disease caused by *Clostridium perfringens* in broilers, which cause chronic intestinal damage, affect the digestion and absorption of nutrients, and reduce production performance. Ellagic acid (EA) has been reported to have antioxidant and anti-inflammatory properties in many aspects. This study was conducted to evaluate the effect and mechanism of EA in relieving subclinical necrotizing enteritis in broilers induced by *C. perfringens*.

Results: *C. perfringens* challenge decreased body weight (BW), average daily gain (ADG); jejunal villi height/crypt depth (V/C); the activity of catalase (CAT), and the mRNA expression of *zonula occludens 1* (ZO-1) in jejunum mucosa of broilers. While it increased feed conversion ratios (FCR); jejunal crypt depth (CD); activities of myeloperoxidase (MPO) and diamine oxidase (DAO), as well as concentrations of interleukin 6 (IL-6), C-reactive protein (CRP), and procalcitonin (PCT) in serum; activities of inducible nitric oxide synthase (iNOs) and lysozyme (LZM), the concentration of malondialdehyde (MDA), and the mRNA expression of *claudin-2*, *TNF- α* , *IL-1 β* , *TLR4*, *TLR2*, *NF- κ B*, *JAK3*, *STAT6* and *iNOs* in jejunum mucosa of broilers. Dietary EA supplement relieved these adverse effects, and heightened jejunal villi height (VH); the concentration of D-xylose in plasma; activities of superoxide dismutase (SOD), and the mRNA expression of *occludin* in jejunum mucosa of broilers. The alpha diversity of cecal microbiota exhibited dietary EA supplement increased observed species and Shannon index. *C. perfringens* challenge increased the relative abundance of *Firmicutes* and decreased the relative abundance of *Desulfobacterota*. Similarly, EA increased relative abundance of *Firmicutes*. LEfSe analysis showed that *C. perfringens* challenge harmed the cecal microbiota of broiler chickens, dietary EA supplementation led to a small beneficial effect, while the simultaneous effect of them seems to stimulate the immune function of broilers and made broilers possess a better cecal microbiota.

Conclusions: Dietary EA ameliorated *C. perfringens*-induced SNE in broilers via regulating jejunal inflammation signaling pathways *TLR/NF- κ B* and *JAK3/STAT6*, relieving jejunal oxidative stress, and balancing cecal microbiota to inhibit intestinal barrier damage, prevent systemic inflammatory response, and improve nutrient absorption capacity, finally protect and enhance growth performance of broilers.

Introduction

Necrotizing enteritis (NE) is a common inflammatory disease of small intestine caused by *Clostridium perfringens*. NE poses an important threat for various animals, including chickens, pigs, sheep, and goats. *C. perfringens* is a spore-forming, anaerobic, gram-positive bacterium and an opportunistic pathogen found in the environment and the intestinal microbiota of humans and animals [1]. *C. perfringens* strains vary significantly in toxin production and has been divided into types A-G based on the presence of encoding genes for alpha (CPA), beta (CPB), epsilon (ETX), iota (ITX), NetB, and CPE toxins [2]. In poultry, NE is caused mainly by type A strains, which lead to economic losses of \$6 billion annually in the global poultry industry [3].

NE usually occurs in broiler at 2- to 6-week-old, including acute clinical NE and subclinical NE (SNE)[4]. Acute clinical NE is characterized by diarrhea, bloody feces, intestinal ulcer erosion, peracute course, and high mortality [5]. Whereas the flock suffering from SNE presents no overt clinical signs and low mortality [4], in most cases, even only an overall reduction in broiler performance is observed [6]. As a result, SNE is difficult to diagnose and control timely, leading to more widespread infections and greater economic losses than acute

clinical NE [6]. In 2015, a study reported that in drug-free broiler flocks of eight commercial farms in Canada, 27.45% of the flocks suffered from acute clinical NE, and 49.02% of the flocks suffered from SNE [1]. In addition, previous studies [7, 8] have demonstrated that SNE usually results in pathological changes of intestinal structure, damages of intestinal barrier function, activation of intestinal inflammatory pathways, disorders of intestinal microflora, poor digestion and absorption, and depressing growth performance of broilers. Therefore, modulation in intestinal health may be a potential strategy to prevent and treat SNE in broiler.

With banning of antibiotics, various strategies have been used against SNE in broiler; apart from organic acids [9], polysaccharides [10], vaccines [11], prebiotics [8], and probiotics [7], plant extracts have been demonstrated to be effective for its protection on broiler health [12]. Ellagic acid (EA) is a chromene-dione derivative (2,3,7,8-tetrahydroxy-chromeno [5,4,3-cde]chromene-5,10-dione; C₁₄H₆O₈) extracted from various fruits, nuts, vegetables, and herbs [13]. EA possesses numerous pharmacological activities, including antioxidant activities [14], anti-inflammatory activities [15, 16], anti-cancer activities [13], and anti-metabolic syndrome activities [17]. In rats, EA exerted anti-inflammatory and antioxidant functions against streptozotocin-induced diabetic nephropathy via reduced the activation of NF- κ B and increased the nuclear translocation of Nrf2 to up-regulate GSH, γ -GCL, and SOD activities [18]. Meanwhile, the alleviating effect of EA on inflammatory mediators has also been widely reported in mice or rats, including TNF- α , IL-1 β , IL-6, IL-8, and iNOS, through TLRs, NF- κ B, and STAT signaling pathways [15, 16, 19]. In addition, EA can alter intestinal microbiota composition and be transformed to urolithins, possessing the potential protection against oxidative stresses and inflammatory diseases in gastrointestinal [20]. Ellagitannins (ETs), which can be hydrolyzed to EA in digestive tract, show a prebiotic effect on promoting the growth of *Lactobacillus* and *Bifidobacterium* [21]. In human, pomegranate ETs can increase the counts of *Akkermansia mucinifila* [22], improving the host metabolic functions and immune responses. However, no study has investigated the protective influences and mechanisms of EA against intestinal diseases (especially SNE) in poultry to date. Therefore, this study was undertaken to explore the preventing effects and mechanisms of EA on growth performance, immune response, intestinal barrier function, antioxidant capacity, and intestinal microflora of broilers suffered with SNE induced by *C. perfringens*.

Materials And Methods

Experimental animals and treatments

A 2 \times 2 factorial randomised complete block design was used to investigate the effects of dietary EA level (0 or 500 mg/kg), *C. perfringens* challenge (challenged or unchallenged), and their interactions on broiler. A total of 240 1-day-old male Arbor Acres broilers with an average weight at 40.4 g (SD 1.57) were purchased from Beijing Arbor Acres Poultry Breeding Co., Ltd (Beijing, China). Upon arrival, birds were weighed and randomly assigned to four groups. Each group had six replicates with ten birds per replicate. Each replicate was reared in a cage (1.0m \times 1.0m \times 0.6m, length \times width \times height) with a raised wire-netted floor. The treatments of groups were as follows: (1) control group (Control, basal diet); (2) *C. perfringens* challenge group (CP, basal diet + *C. perfringens* challenge); (3) ellagic acid and *C. perfringens* challenge group (EAXCP, basal diet extra 500mg/kg ellagic acid + *C. perfringens* challenge); (4) ellagic acid group (EA, basal diet extra 500mg/kg ellagic acid). Ellagic acid (99%, extracted from pomegranate peel) was purchased from Shaanxi Pioneer Biotech Co., Ltd (Shaanxi, China).

Corn–soybean meal basal diets were formulated according to the nutrient requirements for broilers as recommended by the National Research Council (NRC, 1994) [23]. The composition and nutrient levels of the basal diets are presented in Table S1. All diets were crumbled, and neither antibiotics nor antibacterial supplements were added. To avoid cross-contamination, the unchallenged birds and *C. perfringens*-challenged birds were reared in two separate parts in a light and climate controlled room at a 23-hours light/1-hour dark cycle, and provided with feed and water ad libitum. Room temperature was maintained at 33°C during the first 5 days and then gradually decreased by 5°C weekly until 23±1°C. In addition, birds were vaccinated against Newcastle disease virus and infectious bronchitis virus vaccines on day 7 and against bursa disease virus via drinking water on day 12 and 26 according to the routine immunization program.

Clostridium perfringens Challenge

Avian *C. perfringens* type A field strain (CVCC2030) was obtained from State Key Laboratory of Animal Nutrition (Beijing, China). *C. perfringens* culture and challenge was performed on the basis of the previous reports [24–26], with some modifications. Briefly, *C. perfringens* was anaerobically cultured in cooked meat medium with dried meat particles (CM605, CM607; Beijing Land Bridge Technology Co., Ltd) for 24 h at 37°C, then aseptically transferred into thioglycolate broth (70157, Millipore) and incubated anaerobically for 18 h at 37°C. Birds from CP and EAXCP groups were challenged with 1.0 mL of actively growing culture of *C. perfringens* at $2\sim3 \times 10^8$ CFU/mL by oral gavage each day from day 16 to 20, while those from Control and EA groups received an equal volume of thioglycolate broth.

Growth performance

On day 21 and 42, the birds were feed-deprived for 8 hours, and then the feed intake and body weight (BW) of the birds in each replicate were measured. The average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratios (FCR, feed intake/BW gain.) of the birds were calculated for days 1–21, 22–42, and 1–42, respectively.

Sample collection

At day 42, one bird per replicate was randomly selected, for blood samples collected by wing vein puncture. Serum was separated by centrifugation at 3000 rpm for 10 min at 4°C. After the birds were euthanized by jugular exsanguination, approximately 1 cm long jejunal samples between Meckel's diverticulum to the proximal of jejunum were collected and snap-frozen in liquid nitrogen; approximately 2 cm long jejunal samples in length midway between the endpoint of the duodenal loop and Meckel's diverticulum were collected, flushed, and fixed with 10% neutral buffered formalin solution for morphological analysis [26]. Jejunal mucosa were scraped from the posterior part half of jejunum. Cecal content samples were aseptically collected and snap-frozen in liquid nitrogen. Serum, cecal content samples, and all of the tissues were stored at –80°C until analysis.

Plasma D-xylose concentration

Plasma D-xylose concentration was measured as Zhang et al.[24]. Briefly, at day 42, the other one feed-deprived bird per replicate was randomly selected, weighed, and administered D-xylose (X1500; Sigma-Aldrich) solution at a dose of 0.1 g/kg body weight (infused with 10 % D-xylose) by oral gavage. After 1 hour, blood

samples were collected into heparinized vacuum tubes by wing vein puncture. Plasma was separated by centrifugation at 3000 rpm for 10 min at 4°C and stored at –80°C. D-xylose concentration in plasma was determined using the D-xylose assay kit (A035; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

Serum biochemical assay

The concentrations of endotoxin lipopolysaccharide (LPS), procalcitonin (PCT), C-reactive protein (CRP), and interleukin 6 (IL-6) were determined using chicken ELISA kits (YM-3864, YM-S0818, YM-3783, Shanghai YuanMu Biological Technology Co. Ltd. H007; Nanjing Jiancheng Bioengineering Institute); the activities of myeloperoxidase (MPO) and diamine oxidase (DAO) were determined using commercially available assay kit (A088, A044; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions, respectively.

Intestinal morphology

Fixed jejunal tissues were embedded in paraffin, then tissue rings were sliced into 5-μm thickness, deparaffinized in xylene, rehydrated, and mounted on glass slides [25, 26]. Sections were stained by haematoxylin and eosin (H&E). The slides were photographed on a microscope slide scanner (3D HISTECH Ltd, Budapest, Hungary, Model Panoramic MIDI). At least nine villi per section and two sections each sample were measured to evaluate villus height (VH), and crypt depth (CD) using CaseViewer (V 2.43). The means of villus height and crypt depth were calculated and used to obtain the villus height/crypt depth (V/C).

Intestinal mucosa enzyme activities

The activities of inducible nitric oxide synthase (iNOS), lysozyme (LZM), superoxide dismutase (SOD), and catalase (CAT), while the concentration of malondialdehyde (MDA) in jejunal mucosa were determined using commercially available assay kits (A014, A050, A001, A007, and A003; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions, respectively.

Intestinal immune and tight junction-related genes expression

Total RNA was extracted from jejunal tissues using Eastep® Super Total RNA Extraction Kit (15596018; Promega Bingjing Biotech Co., Ltd) according to the manufacturer's instructions. The concentration and purity of total RNA were determined on an Ultra-micro spectrophotometer (IMPLEN, NanoPhotometer® N60). Total RNA from each sample was reverse-transcribed into complementary DNA using a TRUEscript RT Kit (+gDNA eraser) (PC5402; Aidlab Biotechnologies CO. Ltd). Two-step quantitative real-time PCR was performed with a Sybr Green qPCR Mix (PC3302; Aidlab Biotechnologies CO. Ltd) on a Real-Time PCR Detection Systems (Bio-Rad, CFX Connect™) according to the manufacturer's instructions. Oligonucleotide primers of inflammatory mediator genes (*TNF-α*, *IL-1β*, *IL-8*, *IFN-γ*, *TGF-β*, and *iNOS*), inflammation-related signaling pathway genes (*TLR2*, *TLR4*, *MyD88*, *NF-κB*, *JAK1*, *JAK2*, *JAK3*, *STAT1*, and *STAT6*), and tight junction-related genes (*ZO-1*, *occludin*, and *claudin-2*) for chicken were designed based on databases of National Center for Biotechnology Information (NCBI) using Oligo (V 7.0) and synthesized by Sango Biotech Co., Ltd (Shanghai, China). Table S2 lists the quantitative real-time PCR primers used in this study. The relative mRNA expression levels of each target gene were calculated based on the expression of the housekeeping gene β-actin using the $2^{-\Delta\Delta C_t}$ method [27].

Cecal microbiota pyrosequencing and analysis

Bacterial DNA was extracted from cecal content samples using a QIAamp DNA Stool Mini Kit (51504; Qiagen Inc.) according to the manufacturer's instructions. The concentration and purity of total DNA were determined on an Ultra-micro spectrophotometer (IMPLEN, NanoPhotometer® N60). V4 region of bacterial 16S rRNA gene was amplified with the barcoded primer pair 515F/806R (515F: 5'-GTG CCA GCM GCC GCG GTA A-3', 806R: 5'-GGA CTA CHV GGG TWT CTA AT-3') using PCR, then PCR products run on a 2% agarose gel and were purified using a QIAquick Gel Extraction Kit (28706; Qiagen Inc.) according to the manufacturer's instructions. Pyrosequencing for 16S rDNA was performed on a high-throughput sequencing platform (Illumina, HiSeq® 2500 Miseq PE250).

Sequencing results were merged using FLASH (V 1.2.7), filtered using QIIME (V 1.9.1), and the chimera sequences were excluded based on Silva database using UCHIME (V 4.1) to obtain effective tags finally. The effective tags with $\geq 97\%$ similarity were assigned to the same OTUs using Uparse (V 7.0.1001), and the taxonomic information of each operational taxonomic unit (OUT) was annotated based on Silva Database using Mothur (V 1.35.1). Multiple sequence alignment was conducted using MUSCLE (V 3.8.31) to analyse the phylogenetic relationship of different OTUs and the difference of the dominant species among different treatment groups. OTUs abundance information was normalized based on the sample with the most minimal sequences for subsequent analysis.

Venn diagram, rarefaction curve, box plot analysis, principal co-ordinates analysis (PCoA), and bacteria relative abundance were created with R software (V 2.15.3). Alpha diversity, including ACE, Chao1, Simpson, and Shannon index, were calculated using QIIME (V 1.9.1). Beta Diversity was calculated from bray_curtis distance using QIIME (V 1.9.1). Line discriminant analysis effect size (LEfSe) analysis was used to determine the significance of the difference between treatments.

All of the procedures were conducted by Novogene Bioinformatics Technology Co. Ltd (Beijing, China).

Statistical analysis

Data was analysed using GraphPad Prism (V 8.0.1). As a 2×2 factorial arrangement, two-way ANOVA was used to determine the main effects of dietary EA level and *C. perfringens* challenge, and their interaction, Tukey's multiple comparison was used to separate means when interactive effects significantly different or had a trend of difference [24, 26]. Results are presented as the means \pm SEMs. All statements of significance were based on $P < 0.05$, and P value between 0.05 and 0.10 was classified as a tendency [26].

Results

Growth performance

The growth performances of broilers on BW, ADG, ADFI, and FCR were shown in Table 1. *C. perfringens* challenge decreased BW and ADG of broilers during day 1 to 21, 22 to 42, and 1 to 42 ($P < 0.05$), while increased FCR during day 22 to 42 and 1 to 42 ($P < 0.05$). The dietary supplement of EA heightened ADG of broilers during day 22 to 42 ($P < 0.05$) and lowered FCR during day 22 to 42 and 1 to 42 ($P < 0.05$).

Table 1
Effect of *C. perfringens* challenge and dietary EA levels on the growth performance of broilers

Dietary EA levels	0 mg/kg		500 mg/kg		P-values			
<i>C. perfringens</i> challenge	-	+	-	+	SEM	<i>C. perfringens</i> challenge	Dietary EA level	Interaction
day 1-21								
BW, g	563.10	533.18	555.95	535.05	6.21	0.0466*	0.8277	0.7106
ADG, g	24.89	23.47	24.55	23.56	0.29	0.0470*	0.8284	0.7111
ADFI, g	39.73	40.34	38.82	38.10	0.65	0.9669	0.2517	0.6261
FCR, g/g	1.60	1.72	1.58	1.63	0.03	0.1962	0.4186	0.5772
day 22-42								
BW, g	1695.93	1589.26	1758.52	1662.96	24.00	0.0313*	0.1342	0.9000
ADG, g	53.94	50.29	57.27	53.71	0.91	0.0367*	0.0493*	0.9756
ADFI, g	102.16	103.95	103.69	100.17	1.08	0.7002	0.6155	0.2426
FCR, g/g	1.89	2.07	1.82	1.87	0.03	0.0327*	0.0146*	0.2526
day 1-42								
BW, g	1695.93	1589.26	1758.52	1662.96	24.00	0.0313*	0.1342	0.9000
ADG, g	39.42	36.88	40.91	38.63	0.57	0.0314*	0.1339	0.9003
ADFI, g	70.94	72.15	71.25	69.13	0.79	0.7797	0.4133	0.3176
FCR, g/g	1.80	1.96	1.74	1.80	0.03	0.0467*	0.0460*	0.3100
All values are expressed as the means (n = 6). * Significant main effect ($P < 0.05$) of <i>C. perfringens</i> challenge or dietary EA level. BW, body weight. ADFI, average daily feed intake. ADG, average daily gain. FCR, feed conversion ratios = g of feed intake / g of BW gain, g/g.								

Intestinal morphology

As depicted in Fig. 1(A), the jejunum of broilers in Control group demonstrated the normal appearance of intestinal villus. In contrast, the jejuna of CP group showed severe pathological changes with the disappearance of the typical villus architecture and damages of epithelial cells. Compared to Control group, the jejunal morphologies in EAXCP and EA groups exhibited no noticeable appearance change. As shown in Fig. 1(B), *C. perfringens* challenge increased jejunal CD ($P < 0.05$) and decreased V/C values ($P < 0.05$) of broilers. On the contrary, the supplement of EA in diet exhibited a decreased effect on CD ($P < 0.01$), while an increased effect on VH and V/C values ($P < 0.01$) in jejunum of broilers.

Systemic inflammation

As presented in Fig. 2 (A, B, C, D, and E), the systemic inflammatory response intensity was evaluated by measuring the concentrations of inflammation biomarkers LPS, IL-6, CRP, PCT, and the activity of MPO in serum of broilers. The infection of *C. perfringens* caused a heightened tendency on the concentrations of LPS, IL-6, CRP, and PCT ($0.05 < P < 0.10$) in serum of broilers, while an increase on the activity of MPO ($P < 0.05$). The addition of EA in diet reduced the concentrations of CRP and PCT ($P < 0.05$), furthermore, resulted in an extreme decrease on the concentrations of LPS and IL-6 ($P < 0.01$), as well as the activity of MPO ($P < 0.01$) in serum of broilers. There was an interacting effect between dietary EA levels and *C. perfringens* challenge on the concentrations of IL-6 and CRP ($P < 0.05$), as well as a high interaction on the activity of MPO ($P < 0.01$) and the concentrations of LPS and PCT ($P < 0.01$) in serum of broilers. Multiple comparisons indicated that the concentrations of LPS, IL-6, CRP, PCT, and the activity of MPO in the serum of CP group were higher ($P < 0.05$) compared with those in the other three groups. Moreover, the serum of birds in EAXCP group possessed lower MPO activity ($P < 0.05$) than those in Control group.

Intestinal permeability

As shown in Fig. 2(F), intestinal permeability was assessed by determining the concentration of D-xylose in plasma of broilers. The plasma D-xylose concentration emerged a decreased tendency ($0.05 < P < 0.10$) in birds with *C. perfringens* infection. On the contrary, the supplement of EA in diet enhanced its concentration ($P < 0.05$). Meanwhile, an extreme interaction ($P < 0.01$) on plasma D-xylose concentration was shown between dietary EA levels and *C. perfringens* challenge. Furthermore, birds in CP group displayed a decrease on the concentration of plasma D-xylose ($P < 0.05$) relative to those in the other three groups.

Intestinal mucosa integrity and barrier-related enzyme activities

Serum DAO activity was measured to reflect the intestinal mucosa integrity of broilers, as well as the activities of iNOS and LZM in jejunal mucosa to evaluate barrier function. As described in Fig. 3(A, B, and C), the infection of *C. perfringens* enormously increased the activities of serum DAO, as well as iNOS and LZM ($P < 0.01$) in jejunal mucosa of broilers, however, dietary EA supplement decreased iNOS and LZM activities ($P < 0.01$). Furthermore, a highly interacting effect on iNOS activity ($P < 0.01$) was observed between dietary EA levels and *C. perfringens* challenge. As the results of multiple comparisons, birds in CP group showed higher iNOS activity than those in the other three groups ($P < 0.05$).

Tight junction-related gene expression in jejunal mucosa

Figure 3(D, E, and F) respectively exhibited the relative mRNA expressions of tight junction-related gene *ZO-1*, *occludin*, and *claudin-2* in jejunal mucosa of broilers. *C. perfringens* challenge elevated the mRNA expression of *claudin-2* ($P < 0.01$) in jejunal mucosa of broilers, yet down-regulated the mRNA expressions of *ZO-1* ($P < 0.01$), while caused a reduced tendency on the mRNA abundances of *occludin* ($0.05 < P < 0.10$). The dietary EA supplement increased the mRNA expression of *occludin* ($P < 0.01$) and lowered mRNA expression of *claudin-2* ($P < 0.01$) in jejunal mucosa of broilers. In addition, dietary EA levels and *C. perfringens* challenge exerted an interacting effect on the relative mRNA expressions of *occludin* ($P < 0.05$) and *claudin-2* ($P < 0.01$) in jejunal mucosa. In contrast to Control group, the mRNA abundance of *claudin-2* in jejunal mucosa was increased ($P < 0.05$) for birds in CP group. Meanwhile, the birds only fed EA diet possessed higher mRNA expression of *occludin* ($P < 0.05$) in jejunal mucosa than those with the other three treatments.

Intestinal antioxidant capability

As shown in Fig. 3(G, H, and I), we determined the concentration of the lipid peroxidation product MDA and the activities of antioxidant enzyme SOD and CAT in jejunal mucosa to assess the degree of oxidative damages in broilers. The infection of *C. perfringens* increased the concentration of MDA ($P<0.01$), decreased the activity of CAT ($P<0.01$), and had a downward tendency on the activity of SOD ($0.05<P<0.10$) in jejunal mucosa of broilers, whereas adding EA to diet declined the concentration of MDA ($P<0.01$), while heightened the activities of SOD and CAT ($P<0.01$). An extreme interaction effect on the activity of SOD ($P<0.01$) in jejunal mucosa was observed between dietary EA levels and *C. perfringens* challenge. Furthermore, multiple comparisons indicated that birds in CP group showed a decrease on jejunal SOD activity ($P<0.05$) compared with those in the other three groups.

Intestinal inflammation-related cytokine and pathway genes expression

The relative mRNA expressions of various inflammatory mediators genes *TNF- α* , *IL-1 β* , *IL-8*, *iNOS*, *TGF- β* , and *IFN- γ* in jejunal mucosa were exhibited in Fig. 4 (A, B, C, and D) and S1 (A and B), respectively. *C. perfringens* challenge caused up-regulations on the mRNA expressions of *TNF- α* ($P<0.01$), *IL-1 β* , and *iNOS* ($P<0.05$) in jejunal mucosa, but the EA diets down-regulated the mRNA abundances of *IL-1 β* , *iNOS* ($P<0.01$), *TNF- α* , and *IL-8* ($P<0.05$). Between dietary EA levels and *C. perfringens* challenge, an interaction effect was presented on the mRNA expression of *TNF- α* ($P<0.01$), as well as a tendency on *IL-1 β* , *iNOS*, and *TGF- β* ($0.05<P<0.10$). Birds in CP group exhibited higher mRNA expressions of *TNF- α* , *IL-1 β* , and *iNOS* ($P<0.05$) than those in the other three groups. But no significant difference was seen on the mRNA expressions of *TGF- β* and *IFN- γ* ($P>0.05$) in jejunal mucosa of birds.

Inflammation-related signaling pathway *TLR/NF- κ B* genes *TLR-2*, *TLR-4*, *NF- κ B*, and *MyD88* relative mRNA expressions in jejunal mucosa were shown in Fig. 4 (E, F, and G) and S1 (C), respectively. The infection of *C. perfringens* highly increased the mRNA expressions of *TLR-2*, *TLR-4*, and *NF- κ B* ($P<0.01$) in jejunal mucosa, whereas the addition of EA in diets decreased the mRNA abundances of *TLR-2*, *NF- κ B* ($P<0.01$), and *TLR-4* ($P<0.05$). Meanwhile, an interacting effect was presented on *TLR-4* expression ($P<0.01$) between dietary EA levels and *C. perfringens* challenge. The results of multiple comparisons indicated birds in CP group shown an up-regulated mRNA expressions of *TLR-4* ($P<0.05$) compared with those in the other three groups. However, no significant difference was found on the relative mRNA expression of *MyD88* ($P>0.05$) in jejunal mucosa of birds.

Figure 4 (H and I) and Fig. S1 (D, E, and F) respectively exhibited the relative mRNA expressions of inflammation-related signaling pathway *JAK/STAT* genes *JAK3*, *STAT6*, *JAK1*, *JAK2*, and *STAT1* in jejunal mucosa of broilers. *C. perfringens* challenge resulted in an up-regulation on the mRNA expressions of *JAK3* ($P<0.05$) and *STAT6* ($P<0.01$), but the supplement of EA in diet led a down-regulation on the mRNA expression of *STAT6* ($P<0.01$) and a downward trend on *JAK3* ($0.05<P<0.10$) in jejunal mucosa. Moreover, dietary EA levels and *C. perfringens* challenge caused an interacting effect on the mRNA abundances of *STAT6* ($P<0.05$) and a tendentious interaction on *JAK1* ($0.05<P<0.10$) in jejunal mucosa. As the results of multiple comparisons, an up-regulated mRNA expression of *STAT6* ($P<0.05$) was shown for birds in CP group compared

with those in Control group. Nevertheless, no significant difference was seen on the mRNA expressions of *JAK1*, *JAK2*, and *STAT1* ($P>0.05$) in jejunal mucosa of birds.

Cecal microbiota

A total of 2,401,670 pairs of reads were generated after 16S rRNA sequencing of 23 cecal digesta samples (Because one sample was damaged, there were only five replicates in EAXCP Group). After splicing, filtering, and removing chimeras, we obtained 1,488,682 effective Tags or $64,725 \pm 613$ effective Tags for each sample. Based on 97% sequence similarity, Tags were clustered into 1,637 OTUs, of which four groups shared 888 OTUs, and only 76, 92, 129, and 100 OTUs were exclusive in Control, CP, EA, and EAXCP groups, respectively (Fig. S2 (A)). The Good's coverage estimators (Table S3) and the rarefaction curves (Fig. S2 (B)) indicated that sufficient sequencing coverage was achieved.

The alpha diversity of cecal microbiota was shown in Table S4, which exhibited the supplement of EA in diet increased observed species and Shannon index ($P<0.05$). The beta diversity analysis was illustrated via Box and PCoA plots in Fig. S2 (C and D), showing no difference of the microbial community structure among groups.

The most abundant (top 10) phyla and genus of cecal microbiota were presented in Fig. S3. At the phylum level, the cecal microbiota was dominated by *Firmicutes* (36~53%), *Bacteroidota* (26~35%), *Verrucomicrobiota* (5~12%), and *Euryarchaeota* (3~7%), together making up over 86% of the total sequences. *C. perfringens* challenge increased the relative abundance of *Firmicutes* ($P<0.05$) and decreased the relative abundance of *Desulfobacterota* ($P<0.05$). Similarly, EA increased the relative abundance of *Firmicutes* ($P<0.05$) and showed a trend of lowering the relative abundance of *Desulfobacterota* and *Campilobacterota* ($0.05<P<0.10$). Meanwhile, dietary EA levels and *C. perfringens* challenge led an interacting effect on the relative abundances of *Firmicutes* ($P<0.05$) and a trend on *Elusimicrobia* ($0.05<P<0.10$). Multiple comparisons showed that the cecal microbiota in EAXCP group possessed higher the relative abundance of *Firmicutes* ($P<0.05$) than those in the other three groups. At the genus level, only the main effect of *C. perfringens* challenge showed a heightening trend on the relative abundance of *[Ruminococcus]_torques_group* ($0.05<P<0.10$).

LEfSe analysis was used to determine the statistically difference between groups. Compared with cecal microbiota in Control group, *Butyricicoccaceae*, *Gordonibacter*, *Gordonibacter_pamelaeae*, and *Oscillospiraceae* were higher in CP group (Fig. 5A); *Peptostreptococcaceae*, *Peptostreptococcales_Tissierellales*, *Romboutsia*, *Romboutsia_ilealis*, *Erysipelotrichales*, *Erysipelotrichaceae*, *Turicibacter*, and *Turicibacter_sp_H121* were enhanced in EA group (Fig. 5B); *Rhodobacteraceae*, *Sellimonas*, *Rhodobacterales*, *Bacteroidales*, *Bacteroidia*, *Barnesiellaceae*, *Monoglobus*, *Monoglobales*, *Monoglobaceae*, *Parabacteroides_goldsteinii*, RF39, *Intestinimonas*, *Oscillospiraceae*, *Erysipelotrichaceae*, *Clostridia*, and *Firmicutes* were more abundant, while *Negativicutes*, *Desulfovibrionia*, *Desulfovibrionaceae*, *Desulfovibrionales*, *Desulfobacterota*, *Selenomonadaceae*, *Megamonas*, *Veillonellales_Selenomonadales*, *Bacteroides_coprophilus*, *Opitutales*, *Puniceicoccaceae*, *Cerasicoccus*, *Acidaminococcales*, *Acidaminococcaceae*, *Phascolarctobacterium*, *Synergistales*, *Synergistia*, *Synergistaceae*, *Synergistes*, and *Synergistota* were lower in EAXCP group (Fig. 5C). In contrast to cecal microbiota in EAXCP group, those in CP group had more abundance in *Lachnospiraceae_NK4A136_group*, *Desulfobacterota*, *Desulfovibrionia*, *Desulfovibrionaceae*, *Desulfovibrionales*, *Acidobacteriota*, but less abundance in *Marvinbryantia*, *Monoglobus*, *Monoglobales*,

Monoglobaceae, *Gordonibacter*, *Gordonibacter_pamelaeae*, *Bacteroides_clarus*, *Bifidobacterium_breve*, *Bifidobacteriaceae*, *Bifidobacterium*, *Bifidobacteriales*, *Clostridia_vadinBB60_group*, *Turicibacter_sp_H121*, *Turicibacter*, *Faecalibacterium*, *Clostridia*, and *Firmicutes* (Fig. 5D); cecal microbiota in EA group possessed more enriched *Desulfovibrionia*, *Desulfovibrionaceae*, *Desulfovibrionales*, *Desulfobacterota*, *unidentified_Elusimicrobia*, *Elusimicrobiaceae*, *Elusimicrobium*, *Elusimicrobiales*, *Elusimicrobia*, *Proteobacteria*, *Gammaproteobacteria*, *Enterobacteriaceae*, *Enterobacteriales*, *Escherichia_Shigella*, *Escherichia_coli*, *Bacteroides_salanitronis*, *Eubacterium_xylanophilum_group*, *Muribaculaceae*, *Lachnospiraceae_NK4A136_group*, while less *Ruminococcus*, *Fusobacterium_mortiferum*, *Fusobacteriales*, *Fusobacteriia*, *Fusobacteriaceae*, *Fusobacterium*, *Fusobacteriota*, *Chloroflexi*, *Intestinimonas*, *Subdoligranulum*, *Lactobacillaceae*, *Lactobacillus*, *Lactobacillales*, *Bacilli*, *Clostridia*, and *Firmicute* (Fig. 5E).

Discussion

According to our results, *C. perfringens* challenge caused damages on jejunal barrier of broilers and increased the permeability of jejunal mucosa, allowing antigenic substances (LPS, etc.) to enter the blood and internal environment, which in turn triggered jejunal inflammation and oxidative stress, as well as systemic inflammation, reducing the ability of intestinal digestion and absorption, finally impaired the growth performance of broilers. However, dietary EA supplementation exerted anti-inflammatory and antioxidant effects in the jejunal mucosa, which protected and improved the intestinal barrier, preventing the invasion of antigenic substances, and finally improved the growth performance of broilers. Meanwhile, the supplementation of dietary EA also relieved the cecal microbiota imbalance caused by the *C. perfringens* challenge, protecting the health of broilers (Fig. 6).

Toll-Like Receptors (TLRs) are important members of pattern recognition receptors, TLR4 could recognize LPS, which is unique to Gram-negative bacteria, and TLR2 could recognize peptidoglycans (PGN), which is abundant in Gram-positive bacteria [28]. TLRs can trigger subsequent inflammatory responses through MyD88 dependent or independent signaling pathways that activate NF- κ B and finally lead to the release of pro-inflammatory mediators, including TNF- α , IL-1 β , IL-6, IL-8, and iNOS [28]. In inflammatory bowel disease, LPS or cytokines (E.g IL-6 and IFN- γ) can activate JAK/STAT signaling pathway to regulate the expression of pro-inflammatory mediators, including Claudin-2 and iNOS [29]. In our results, *C. perfringens* challenge increased the mRNA expression of TLR4, TLR2, NF- κ B, JAK3, and STAT6, while dietary EA supplement relieved these adverse effects. Due to the deficiency of appropriate antibodies available for use in studies of chickens, we did not determine the protein levels and phosphorylation status of components in these signaling pathways. A series of studies [7, 12, 30] have reported the activation process of TLR/NF- κ B or JAK/STAT signaling pathways in broilers with *C. perfringens* challenge. Similar to our results, EA was proved to possess a protective effect on concanavalin A-induced hepatitis in mice via decreasing the expressions of TLR2 and TLR4 and suppressing NF- κ B signaling pathway [19]. *C. perfringens* challenge in this study has no obvious effect on the mRNA expression of *MyD88*, which may indicate TLRs activate NF- κ B through MyD88 independent signaling pathways. Some studies [29, 31] have reported EA inhibited the phosphorylation of JAK1, JAK2, STAT1, and STAT3 to exert anti-inflammatory effects in keratinocytes or rats, but no report has been found on the impact of EA on JAK3/STAT6 in any animals. In human and mice, the activation of JAK3/STAT6 pathway was related to the differentiation of monocytes and the enhancement of Th2 inflammatory response (the release of IL-4, IL-5,

and IL-13) [32]. It means that *C. perfringens* challenge may trigger the Th2 inflammatory response related to the JAK3/STAT6 pathway in jejunal mucosa of broilers, while EA relieves this hazard in this pathway.

During the inflammatory response, the activation of TLR/NF- κ B and JAK/STAT signaling pathways can induce the release of a variety of pro-inflammatory cytokines, which will lead to the activation of immune cells and the production of more cytokines [33]. TNF- α and IL-1 β are pleiotropic pro-inflammatory cytokines, whose dysregulations are linked with a wide range of pathological conditions, such as infection, metabolic syndrome, and inflammatory bowel disease [33]. IL-8 is a very potent trigger to immune cells' migration and proliferation, which guides neutrophils to the direction of inflammation [33]. TGF- β and IFN- γ also play an important role in a variety of inflammation-related diseases; C-reactive protein promotes the inflammatory response of atrial fibrillation through the overexpression of TGF- β related to the TLR4/NF- κ B/TGF- β pathway in HL-1 cells, which is related to heart arrhythmia [34], while IFN- γ was reported to contribute to the hepatic inflammation in HFD-induced nonalcoholic steatohepatitis by STAT1 β /TLR2 signaling pathway in mice [35]. iNOS is related to immune response via macrophage defence mechanism, its expression and the increase of NO levels can cause various inflammation-related pathophysiological conditions, the cell wall components of bacteria (mainly through LPS) can activate the JAK/STAT signaling pathway and subsequently activate NF- κ B to initiate iNOS transcription [36]. In our study, *C. perfringens* challenge caused up-regulations on the mRNA expressions of pro-inflammatory mediator genes *TNF- α* , *IL-1 β* , and *iNOS* in jejunal mucosa, while the EA diets down-regulated the mRNA abundances of *TNF- α* , *IL-1 β* , *IL-8*, and *iNOS*. A series of studies [12, 24, 30] have proved that *C. perfringens* challenge can cause an up-regulation on pro-inflammatory mediator genes in the intestine of broilers, including *TNF- α* , *IL-1 β* , *IL-8*, *TGF- β* , *IFN- γ* , and *iNOS*. Meanwhile, the alleviating effect of EA on inflammatory mediators, including TNF- α , IL-1 β , IL-8, and iNOS has been widely reported in mice or rats [15, 16], which are in line with our results and further indicated that EA reduced inflammatory mediators in broilers may be through NF- κ B and STAT signaling pathways. However, the *C. perfringens* challenge or dietary EA levels show no significant effect on *TGF- β* and *IFN- γ* in our results, which may be related to the difference on *C. perfringens* strains and frequency of the challenge.

The activation of inflammatory pathways and the release of inflammatory mediators can affect the antioxidant, barrier, and absorption functions of the jejunum, which are vital to the growth performance and health of broilers.

Oxidative stress plays an important role in NE. SOD can convert $O_2^{\cdot\cdot}$ into H_2O_2 , CAT then transforms the generated H_2O_2 into H_2O , thus preventing the harmful effects of oxidative radicals [37]. In our results, SNE induced by *C. perfringens* decreased the antioxidant capacity of jejunal mucosa by reducing the activities of SOD and CAT, resulting in an increase on MDA concentration, while the dietary EA supplementation relieved these adverse effects. EA itself has good antioxidant capacity [14], which may jointly explain the antioxidant mechanism of EA in SNE induced by *C. perfringens*. Moreover, in the oxidized fish oil-induced oxidative stress of mice, the supplementation of EA in diet increased the total antioxidant capacity (T-AOC) and the activities of the glutathione peroxidase (GSH-Px) and SOD, while decreased the MDA concentration in the intestine [38]. Another report demonstrated that EA exerted anti-inflammatory and antioxidant functions against streptozotocin-induced diabetic nephropathy in rats via reducing the activation of NF- κ B and increasing the nuclear translocation of Nrf2 to up-regulate GSH, γ -GCL, and SOD activities [18].

Tight junction proteins are vital structures of the physical barrier in jejunal mucosa, which form a seal between intestinal epithelial cells and prevent the transmission of macromolecules [7]. In the present study, *C. perfringens* challenge decreased the jejunal mRNA expressions of *ZO-1* and *occludin* in broilers and increased the mRNA expression of *claudin-2*, the dietary supplementation of EA relieved these adverse effects. *ZO-1* and *occludin* are barrier-forming proteins, whose reduction mean damage of tight junctions; whereas *claudin-2* is a pore-forming protein, whose increase can increase the permeability of intestinal barrier [24]. As many studies reported [9, 12], the infection of *C. perfringens* can reduce the mRNA expression of *ZO-1* and *occludin* in broilers through the activation of NF- κ B. While pomegranate and pomegranate leaf, which rich in EA, can relieve the decrease of *ZO-1* and *occludin* caused by alcoholic liver disease or hyperlipidemia in the intestine of mice [17]. The infection of *C. perfringens* can increase the expression of *claudin-2* in intestine of broilers [24], which may be explained as a result of 'cross-talk' caused by IL-6 between JAK/STAT, SAP/MAPK, and PI3K signaling pathways [39]. Interestingly, the mRNA expression of *occludin* was increased in the broilers only fed the diet with EA supplementation; in another study [12], thymol and carvacrol supplementation demonstrated a similar effect on the mRNA expression of *occludin* in broilers challenged with *C. perfringens*.

D-xylose crosses the intestinal mucosa via a Na⁺-dependent mobile-carrier mechanism, in the case of malabsorption syndrome, the entry of D-xylose from the gut lumen to the portal vein is damaged, resulting in reduced concentrations of D-xylose in blood [24]. DAO is an intracellular enzyme in the small intestinal epithelia and released into the peripheral circulation as a result of intestinal villi damage, so the level of serum DAO could reflect the severity of intestinal mucosal injury [40]. In our study, the decrease of plasma D-xylose concentration indicated that *C. perfringens* challenge had impaired the intestinal absorption function, while the increase of DAO activity in serum may imply the relation to the impaired intestinal epithelium. The supplement of dietary EA alleviated the decrease of plasma D-xylose concentration, but had no effect on DAO activity in serum. Similar to our results, the arginine additive alleviated an increase on plasma D-xylose concentration caused by the *C. perfringens* challenge [24]. LZM can cleave peptidoglycan of the cell wall in Gram-positive bacteria, resulting in the loss of cellular membrane integrity and cell death [26]. In our results, *C. perfringens* infection increased the activities of iNOS and LZM in jejunal mucosa, while the supplement of EA in diet relieved these adverse effects. LZM was up-regulated in the gastrointestinal tract of patients affected by chronic inflammation, which was related to the LZM-mediated processing of luminal bacteria in the colon that triggered the pro-inflammatory response [41]. These up-regulations of iNOS and LZM in our result further explained the mechanism of chronic inflammation caused by SNE.

Damage of the intestinal barrier and absorption function was also reflected in the microstructure of jejunum. The results of jejunal morphology, including VH, CD, and V/C ratio by HE staining, were important indexes that intuitively reflected jejunal health and absorption surface. *C. perfringens* challenge seriously destroyed the villi structure and reduced the absorption surface of nutrient, which is in line with the results reported previously [12, 25]. On the contrary, the dietary EA supplementation alleviated the jejunal lesions, indicated the good condition of enterocytes and efficient ability of nutrient absorption. In the mice model [38], EA effectively alleviated the intestinal damage caused by oxidized fish oil via significantly increasing the VH and V/C, while improving the mucous epithelium injury. Also, thymol and carvacrol alleviated the ileal lesion and improved V/C ratio in broilers with *C. perfringens* infection [12]. Furthermore, the antioxidant and anti-inflammatory effects of EA may explain the mechanism that is beneficial to the health of intestine and villus-crypt architecture.

Intestinal NE lesions and mucosal atrophy greatly compromises epithelial permeability and mucosal barrier function, which may result in adverse effects on internal environment homeostasis and production performance of broilers, therefore, these serum inflammation biomarkers were used to evaluate the systemic inflammatory response intensity of broilers. LPS is an endotoxin produced by Gram-negative bacteria, its increase in blood reflected the bacteria translocation to liver, spleen, and blood [42]. IL-6 is an important cytokine of inflammatory bowel diseases, which can activate the JAK/STAT signaling pathway and promote the release of various inflammatory factors [29]. CRP is synthesized in liver, mainly in response to IL-6, and can be combined with the pathogen LPS to activate the classical complement pathway [43]. PCT is a diagnostic marker of bacterial infection, which is produced by LPS, TNF- α , and IL-6 acting on neuroendocrine cells or special cells in the liver and spleen[44]. MPO is a sign of neutrophil aggregation and inflammation, its activity is a marker of neutrophil infiltration into the intestine[44]. *C. perfringens* infection increased the concentrations or activities of LPS, IL-6, CRP, PCT, and MPO, causing a higher stress of systemic inflammatory response in broilers, while the supplement of EA in diet relieved these adverse effects. In line with our results, *Lactobacillus acidophilus* supplementation significantly decreased the serum LPS content in broilers with *C. perfringens* challenge [29], while EA treatment can decrease the mRNA expressions of TNF- α and IL-6 in the liver and intestine of oxidative stress mice [38]. In NE model caused by *C. perfringens*, probiotic powder containing *Lactobacillus plantarum* decreased the MPO activity in the ileum mucosa of broilers [37]. Overall, our study reflected that EA alleviated the systemic inflammatory response caused by *C. perfringens* challenge, possibly by protecting the integrity of intestinal mucosa and reducing the expression of inflammatory factors.

On the other hand, intestinal microbiota is involved in intestinal nutrition, defense, and immunity. The high diversity of intestinal microbiota is beneficial to maintain the stability of the intestinal microenvironment and defend against the invasion of pathogenic microorganisms [45]. In our study, only the dietary EA supplement increased the alpha diversity, including observed species and Shannon index, which may mean an improvement in intestinal health; but the beta diversity analysis showed no difference of the microbial community structure among groups, which may be related to the microbiota from different parts of the intestine and the time of sample collection. In broilers challenged by *C. perfringens* and *Eimeria* [46], the effects of dietary lauric acid supplement or the challenge on microbiota in the jejunum were distinct from those in the cecum, as well as the change of microbiota was more significant in jejunum; however, these treatments did not promote significant difference of taxa abundance and diversity in cecum, which may explain our results. In terms of microbial abundance, *C. perfringens* challenge increased the relative abundance of *Firmicutes* and decreased the relative abundance of *Desulfobacterota*. Similarly, EA increased *Firmicutes* relative abundance and showed a trend of lowering *Desulfobacterota* and *Campilobacterota* relative abundances. It has been reported that *Firmicutes* improved the utilization of energy in the diet and the ratio of *Firmicutes* to *Bacteroides* was often positively associated with weight gain [25]. However, both EA and *C. perfringens* challenge resulted in an increase in *Firmicutes* abundance and a decrease in *Desulfobacterota* abundance, which may be related to the longer time interval between challenge and sample collection, as well as the immune regulation of broilers, especially in the EAXCP group, which may mean that the challenge has played an immune-stimulating effect with the presence of EA. In rats with stress-induced depressive-like behavior [47], fecal microbiota transplantation ameliorates gut microbiota imbalance and intestinal barrier damage through increasing *Firmicutes* and decreasing *Desulfobacterota* and *Bacteroidetes* at phylum levels; this treatment also reduced the loss of villi and epithelial cells, suppressed the inflammatory cell infiltration, and increased the expression

of ZO-1 and occludin in the ileum. These results were amazingly similar to ours, which may indicate that the microbiota displayed a similar mechanism in the intestinal protection of broilers. *Campylobacter* was believed to be closely related to the zoonotic campylobacter disease [48], the EA-induced decrease of *Campilobacterota* relative abundance may have a protective effect on the health of broilers. At the genus level, only the main effect of *C. perfringens* challenge showed a trend of heightening *Ruminococcus*_torques_group abundance. The increase of *[Ruminococcus]_torques_group* abundance was reported in irritable bowel syndrome, which phylotype was associated with severity of bowel symptoms [49]. Another study showed that the *Ruminococcus*_torques_group seemed to be especially involved in controlling paracellular permeability [50], which may be another factor that SNE affects intestinal permeability of broilers in our result.

LEfSe analysis revealed the different phylotypes of cecal microbiota between groups. Compared with cecal microbiota in Control group, the increased abundance of *Oscillospiraceae* in CP group was thought to be linked to intestinal inflammation [51]. The effect of *Butyricicoccaceae* abundance on inflammation was lacking in reports, but it was thought to be an important butyrate producer [51], which may be beneficial to the recovery of the intestines. *Gordonibacter_pamelaeae* has been reported to have the function of transforming EA into urolithin [20], its high abundance was also observed in the EAXCP group, which may mean the transfer of the microbiota between the CP and EAXCP groups, because they were kept in the same pheasantry room. Compared with cecal microbiota in Control group, a main increase on the abundances of *Turicibacter_sp_H121* and *Romboutsia_ilealis* in EA group was found in our results. The increase on *Turicibacter_sp_H121* was also observed in cecal microbiota of EAXCP group compared with CP group, which may indicate that EA is beneficial for *Turicibacter_sp_H121*, but the effect of the increase is unclear. *Romboutsia_ilealis* is a beneficial intestine bacterium, whose decrease in the response to *Streptococcus_agalactiae* infection of zebrafish was considered harmful [52]. The cecal microbiota between EAXCP and other three groups were quite different. In EAXCP group, the increased abundance of *Sellimonas* has been reported as a potential biomarker of homeostasis gut recovery after dysbiosis events [53], *Bacteroidales* was thought to be involved in the synthesis of fatty acids and are beneficial to the health of the host [54], *Erysipelotrichaceae* was highly abundant in good FCR broilers [55], and mice fed with normal diet possessed more abundant than those fed with high fat diet on *Monoglobaceae* and RF39 [56]. *Rhodobacteraceae* is widely reported in aquatic animals or marine environments and has no adverse effects on host health. In Control group, the abundance of *Synergistes* was reported to be negatively correlated with the levels of IL-1 β , IL-6, and TNF- α in serum samples from piglets [57], but *Phascolarctobacterium* predominated among the *Clostridia* in low FCR birds [58]. Dietary supplementation with medium-chain α -monoglycerides can decrease the abundances of *Cerasicoccus*, and improve productive performance and egg quality in aged hens [58]. Comparing with birds in CP group, those in EAXCP group had higher abundance of *Faecalibacterium*, which was enriched in chickens with the higher BW [59]. It was speculated that *Clostridiales_vadinBB60_group* might also be beneficial bacteria in intestinal tract [7]. *Bifidobacterium_breve* was probiotic which has been verified [60]. Comparing with birds in EA group, those in EAXCP group had a higher abundance of *Subdoligranulum*, which was negatively correlated with CRP and IL-6 in human [61]. *Fusobacterium_mortiferum* was often reported in clinical infections of human, but its strains, which were isolated from poultry caeca, also can produce bacteriocin-like substances inhibiting *Salmonella enteritidis* [62]. For another, birds in EA group had higher abundance of *Muribaculaceae*, which negatively correlated with inflammatory markers in high fat-high sucrose diet-induced insulin resistant mice [63]. *Eubacterium_xylanophilum_group* was thought to be lactic acid- and SCFA-producing bacteria, which

enhanced intestinal homeostasis and ameliorated weaning stress in piglets [64]. *Escherichia coli* showed higher levels in broilers with smaller BW [59]. In rats, *Elusimicrobium* was thought to be beneficial bacteria, whose increase can protect the intestinal barrier [65]. Overall, *C. perfringens* challenge caused an adverse effect on the cecal microbiota of broiler chickens, dietary EA supplementation led to a small beneficial effect, while the simultaneous effect of dietary EA and challenge seems to stimulate the immune function of broilers and made them possess a better cecal microbiota. Furthermore, the cecal microbiota of the EAXCP group seems very different from other groups, which may explain the significant interaction between dietary EA level and *C. perfringens* challenge in our results.

Finally, growth performance is the most comprehensive indicator of commercial broiler quality. SNE induced by *C. perfringens* usually reduces the performance of broilers without serious clinical symptoms and high mortality [4, 6]. In this study, the main effect of *C. perfringens* infection lowered BW and ADG of flocks, as well as enhanced FCR. However, the addition of EA in diet increased ADG and decreased FCR in broilers. Previous studies [26, 42] reported that SNE challenge reduced ADG, body weight gain, and feed intake, while heighten FCR in broilers, which were similar to our results. The supplementation of plant extracts, including tannin and polyphenol compounds, have been proved to be effective against NE [12, 46], but the effect of EA on the growth performance of broilers has not been reported. In laying quails, EA improved the feed conversion and egg quality [66]. In addition, pomegranate extract was reported to have a positive effect on the growth and slaughter performances of broilers [67]. These improvements provided by EA may be explained by improving the intestinal barrier function and microbiota structure, thereby indirectly increasing the performance of broilers raised without anti-resistant diets [66].

Conclusion

In summary, we found that dietary ellagic acid ameliorated *C. perfringens*-induced SNE in broilers via regulating jejunal inflammation signaling pathways *TLR/NF- κ B* and *JAK3/STAT6*, relieving jejunal oxidative stress, and balancing cecal microbiota to inhibit intestinal barrier damage, prevent systemic inflammatory response, and improve nutrient absorption capacity, finally protect and enhance growth performance.

Abbreviations

NE: Necrotizing enteritis; SNE: subclinical necrotizing enteritis; EA: Ellagic acid; CP: Crude protein; ADFI: Average daily feed intake; BW: Body weight; ADG: Average daily gain; FCR: Feed conversion ratios; H&E: Haematoxylin and eosin; CD: Crypt depth; VH: Villi height; MPO: Myeloperoxidase; LPS: lipopolysaccharide; DAO: Diamine oxidase; IL-6: Interleukin 6; CRP: C-reactive protein; PCT: Procalcitonin; iNOS: Inducible nitric oxide synthase; LZM: Lysozyme; ZO-1: Zonula occludens 1; MDA: Malondialdehyde; CAT: Catalase; SOD: Superoxide dismutase; TLR: Toll-like receptors; NF- κ B: Nuclear factor kappa-B; MyD88: Myeloid differentiation factor 88; JAK: Janus kinase; STAT: signal transducer and activator of transcription; TNF- α : Tumor necrosis factor alpha; IL-1 β : Interleukin 1 beta; TGF- β : Transforming growth factor beta; IFN- γ : Interferon gamma; OUT: Operational taxonomic unit; PCoA: principal co-ordinates analysis; LEfSe: Line discriminant analysis effect size; SD: Standard deviation; SEM: Standard error of mean.

Declarations

Ethics approval and consent to participate

Broilers were cared for in accordance with the guidelines for the care and use of laboratory animals presented in the guide issued by the National Institute of Health and by China's Ministry of Agriculture. All experimental procedures were approved by the Animal Care and Use Committee of China Agricultural University (Approval NO.AW13301202-1-13).

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

All of the authors declare that they have no conflict of interest.

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Author Contributions

Formal analysis, G.L., Q.M., P. Z., and J.Z.; methodology, Y.T., Y.G., and L.Z.; project administration, Y.T., Y.W. and Y.G.; supervision, Q.M. and L.Z.; writing (original draft), Y.T. and X.Z.; writing (review and editing), L.Z.

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Figures

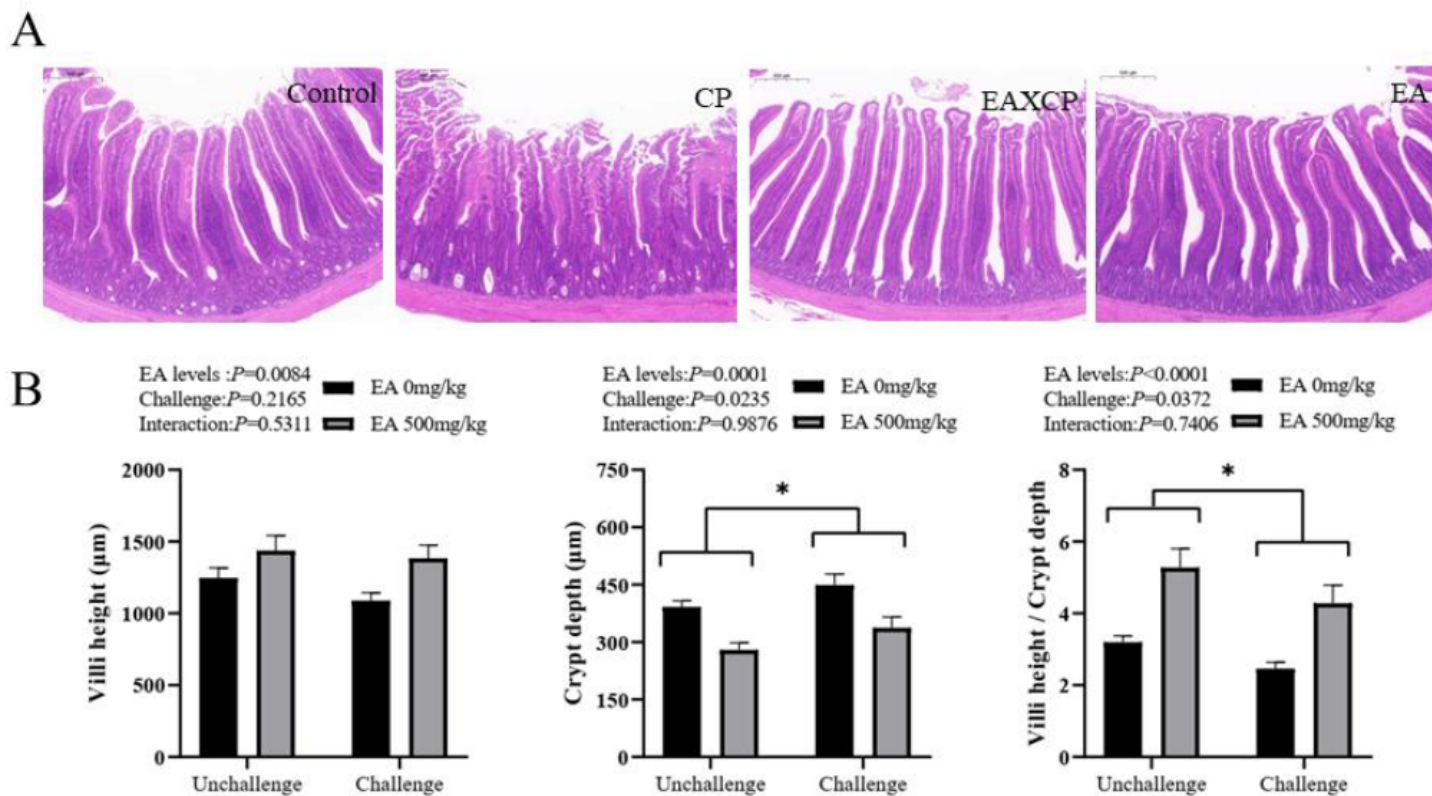


Figure 1

Effect of *C. perfringens* challenge and dietary EA levels on jejunal morphological parameters of broilers at day 42. (A) Representative photomicrographs of jejunal cross-section with HE staining. (B) Jejunal villi height (VH), crypt depth (CD), and villi height/crypt depth (V/C), respectively. Unchallenged, birds without *C. perfringens* infection; challenged, birds with *C. perfringens* infection. Values are means ($n = 6$) with their standard errors represented by vertical bars. * Significant main effect ($P<0.05$) of *C. perfringens* challenge.

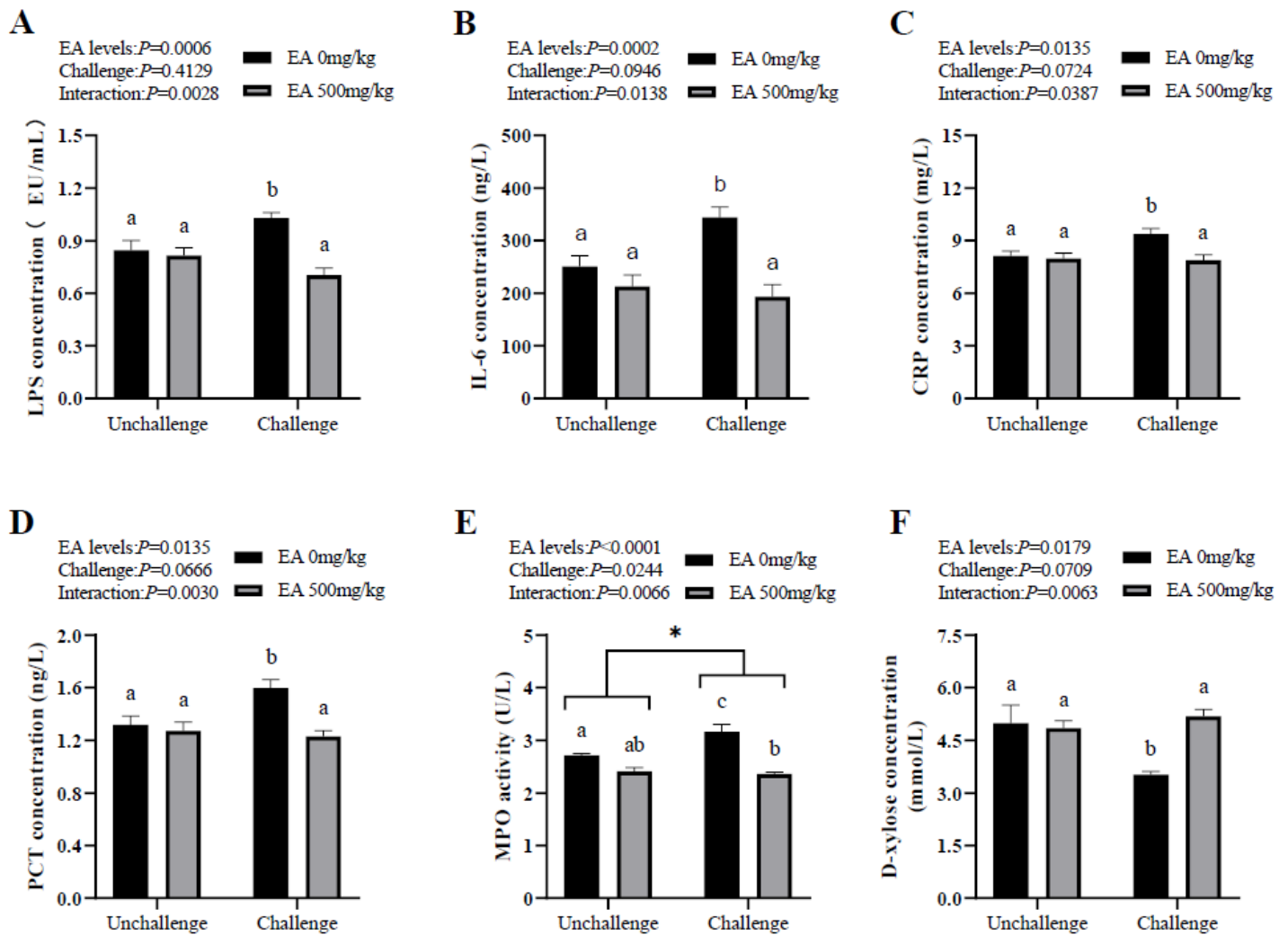


Figure 2

Effect of *C. perfringens* challenge and dietary EA levels on serum inflammation biomarkers and plasma D-xylose concentration of broilers at day 42. (A, B, C, D, and E) The concentrations of endotoxin lipopolysaccharide (LPS, EU/mL), interleukin 6 (IL-6, ng/L), C-reactive protein (CRP, mg/L), procalcitonin (PCT, ng/L), and the activity of myeloperoxidase (MPO, U/L) in serum. (F) Plasma D-xylose concentration (mmol/L). Unchallenged, birds without *C. perfringens* infection; challenged, birds with *C. perfringens* infection. Values are means ($n = 6$) with their standard errors represented by vertical bars. a, b, c Values with unlike letters were significantly different ($P<0.05$). * Significant main effect ($P<0.05$) of *C. perfringens* challenge.

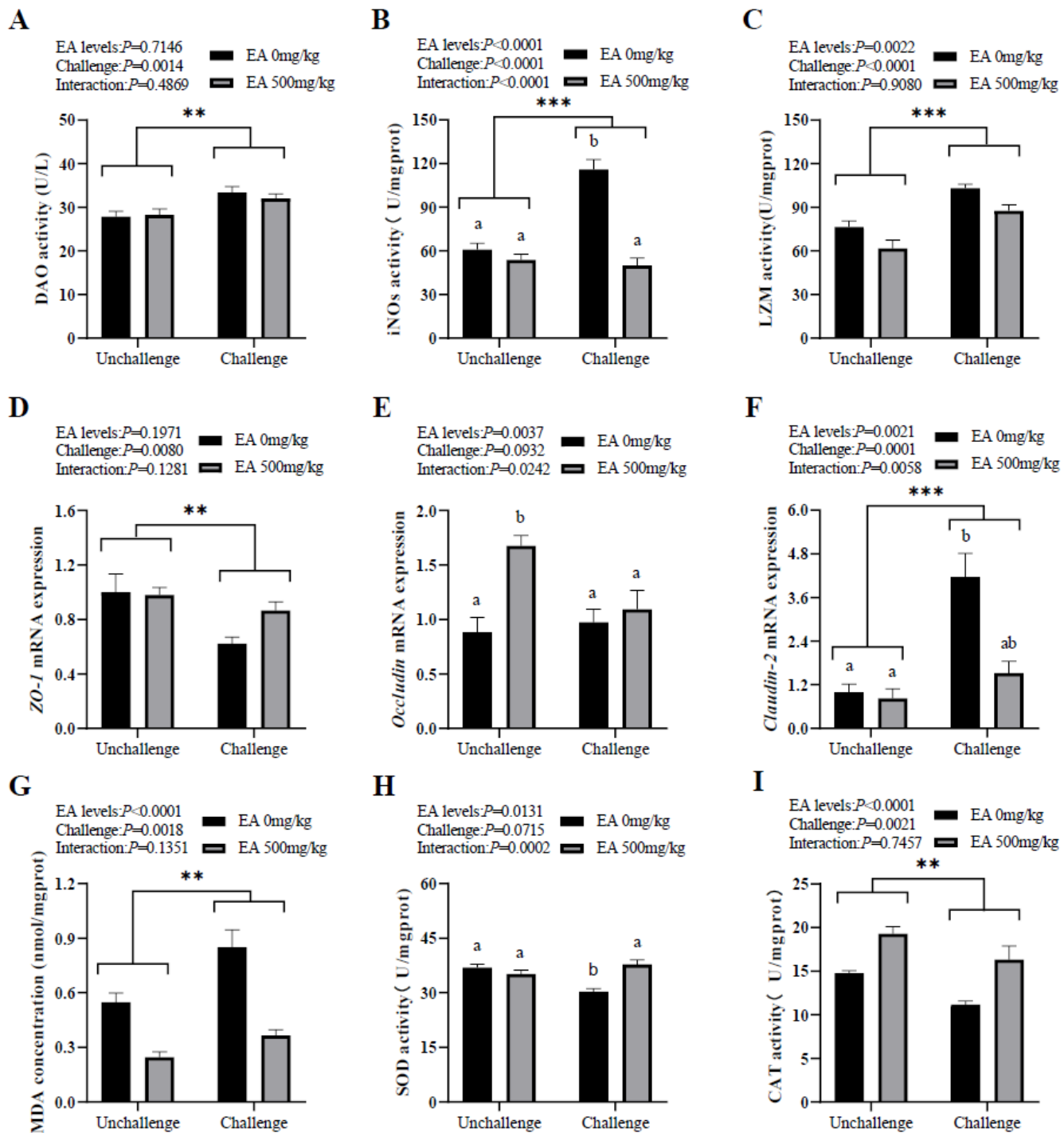


Figure 3

Effect of *C. perfringens* challenge and dietary EA levels on intestinal barrier-related biomarkers of broilers at day 42. (A) Diamine oxidase activity (DAO, U/L) in serum. (B and C) The activities of inducible nitric oxide synthase (iNOS, U/mgprot) and lysozyme (LYM, U/mgprot) in jejunal mucosa. (D, E, and F) Relative mRNA expressions of zonula occludens 1 (ZO-1), occludin, and claudin-2 in jejunal mucosa. (G, H, and I) The concentration of malondialdehyde (MDA, nmol/mgprot) and the activities of superoxide dismutase (SOD, U/mgprot) and catalase (CAT, U/mgprot) in jejunal mucosa. Unchallenged, birds without *C. perfringens* infection; challenged, birds with *C. perfringens* infection. Values are means ($n = 6$) with their standard errors

represented by vertical bars. a, b Values with unlike letters were significantly different ($P<0.05$). *, **, *** Significant main effect ($P<0.05$, $P<0.01$, $P<0.001$) of *C. perfringens* challenge.

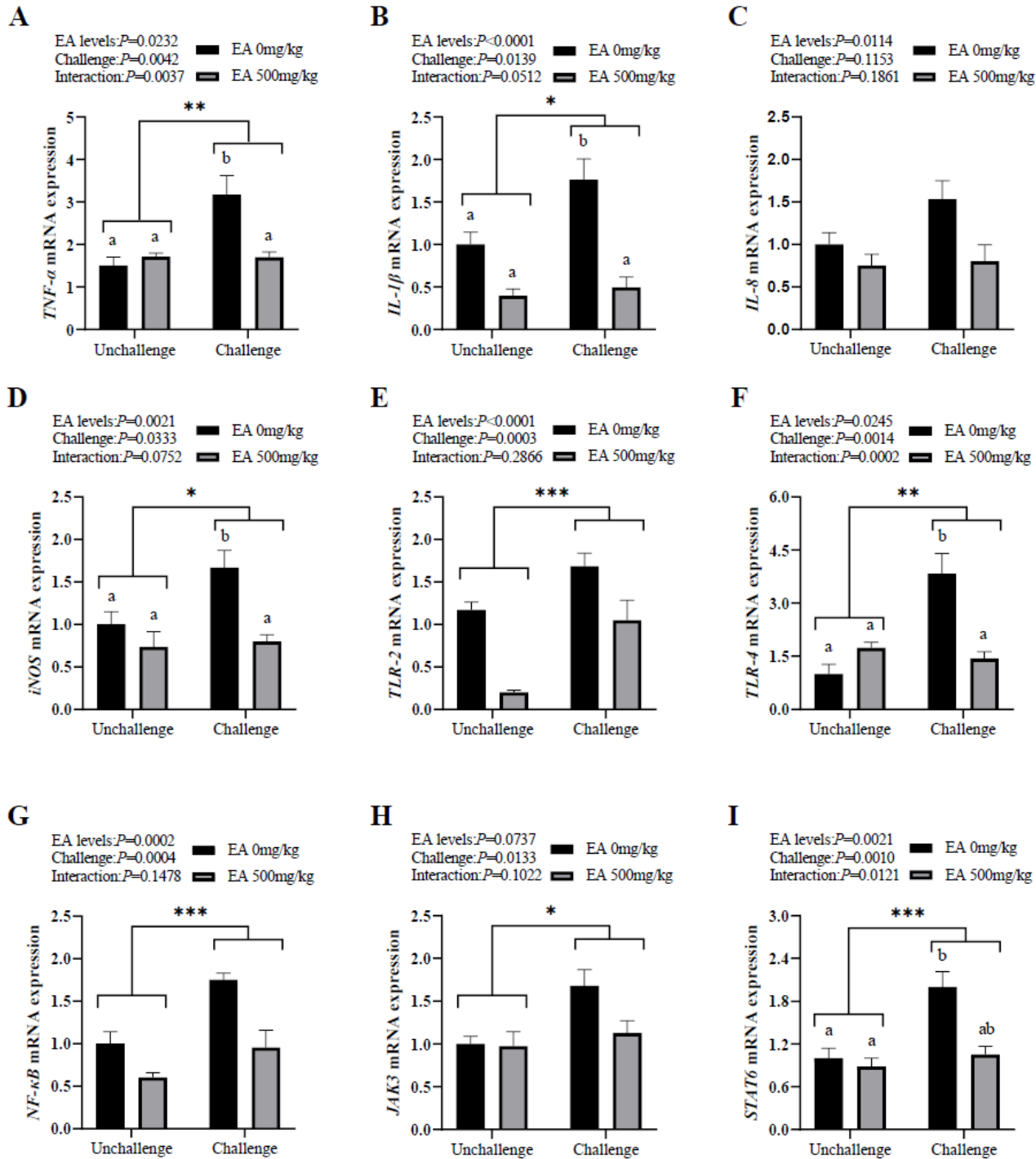


Figure 4

Effect of *C. perfringens* challenge and dietary EA levels on relative mRNA expression of inflammation-related pathway and cytokine genes in jejunal mucosa of broilers at day 42. (A, B, C, and D) The relative mRNA expressions of tumor necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β), interleukin 8 (IL-8), and inducible nitric oxide synthase (iNOS). (E, F, and G) The relative mRNA expressions of toll-like receptor 2 (TLR-2), toll-like receptor 4 (TLR-4), and nuclear factor kappa B (NF- κ B). (H and I) The relative mRNA expression of janus kinase

3 (JAK3) and signal transducers and activators of transcription 6 (STAT6). Unchallenged, birds without *C. perfringens* infection; challenged, birds with *C. perfringens* infection. Values are means (n = 6) with their standard errors represented by vertical bars. a, b Values with unlike letters were significantly different (P<0.05). *, **, *** Significant main effect (P<0.05, P<0.01, P<0.001) of *C. perfringens* challenge.

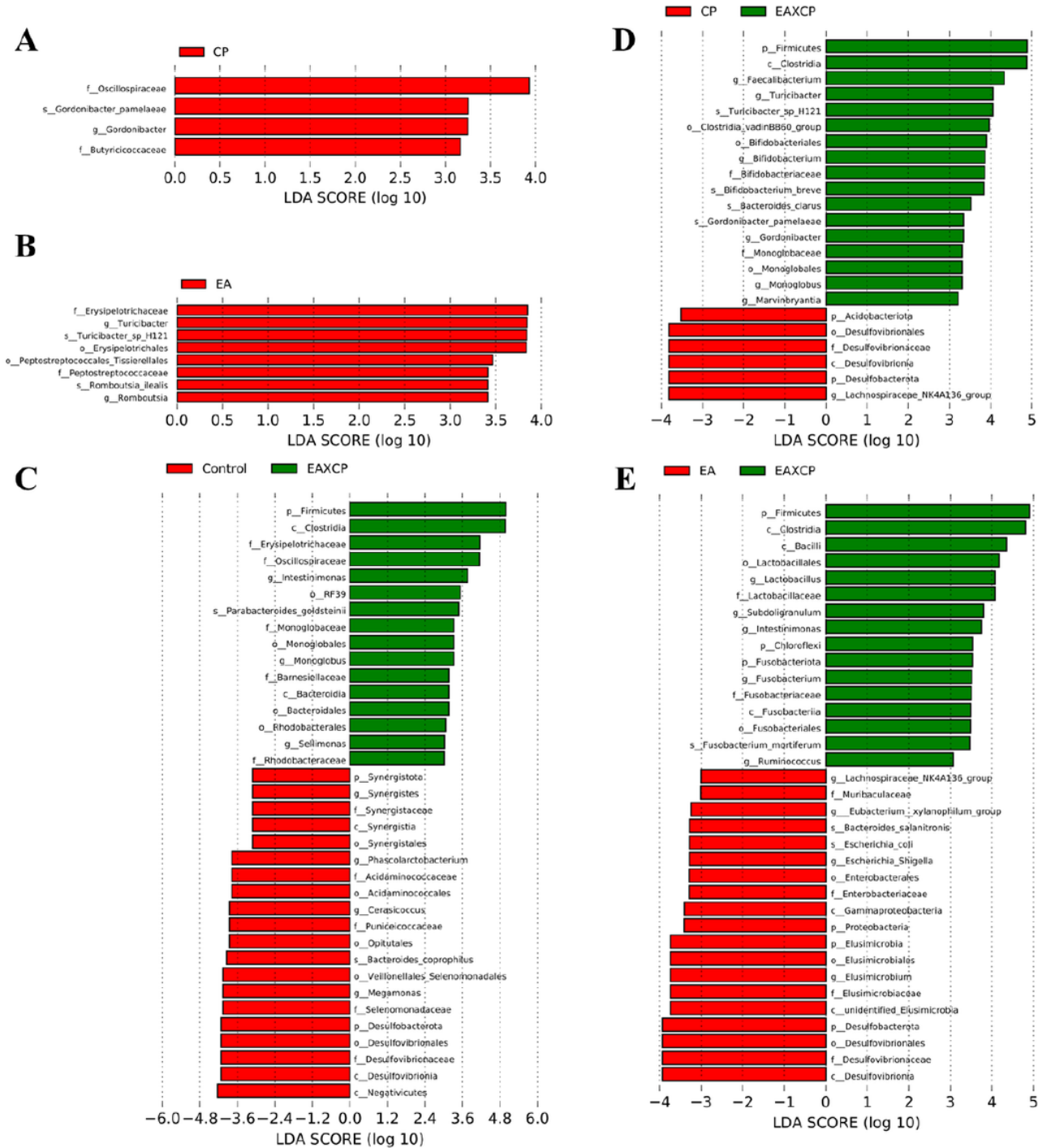


Figure 5

LEfSe analysis revealed the different phylotypes of cecal microbiota between groups. (A) Cecal microbiota between Control and CP groups. (B) Cecal microbiota between Control and EA groups. (C) Cecal microbiota between Control and EAXCP groups. (D) Cecal microbiota between CP and EAXCP groups. (E) Cecal microbiota

between EA and EAXCP groups. These Fig.s showed the bacteria of which the LDA Score is greater than the set value (the default setting was 3.0) between groups. The length of the histogram represents the size of the difference species (i.e., LDA Score), and the different colors represent the different groups.

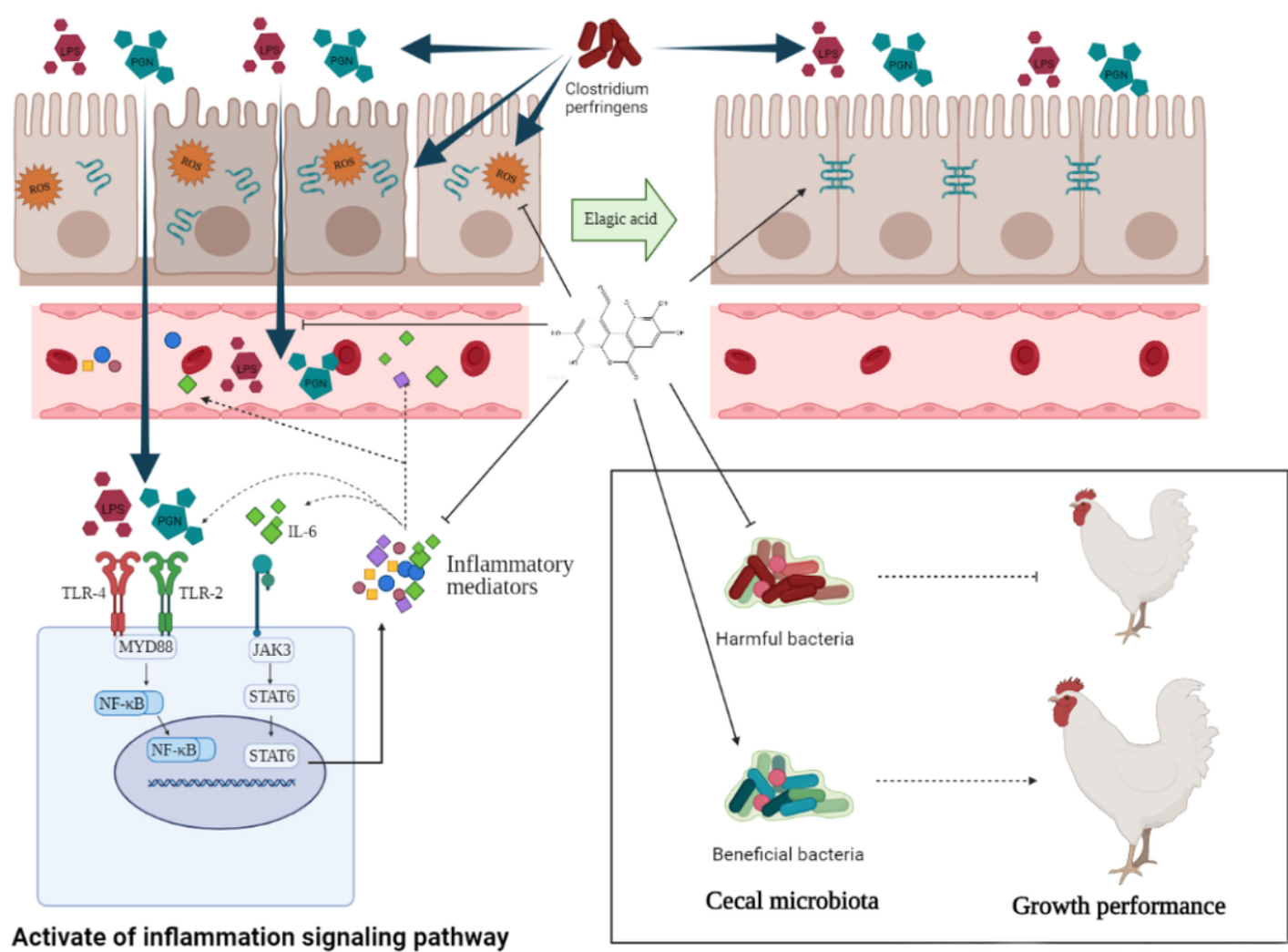


Figure 6

Dietary ellagic acid ameliorated *C. perfringens*-induced SNE in broilers via regulating inflammation signaling pathways TLR/NF- κ B and JAK3/STAT6, as well as cecal microbiota to inhibit intestinal barrier damage. \uparrow Arrows indicate the effect of stimulation; \neg Arrows indicate the effect of suppression.

Supplementary Files

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