Dietary L-arginine inhibits intestinal *Clostridium perfringens* colonisation and attenuates intestinal mucosal injury in broiler chickens

Beibei Zhang, Zengpeng Lv, Huixian Li, Shuangshuang Guo, Dan Liu and Yuming Guo*

State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing 100193, People's Republic of China

Abstract

We investigated the effects of dietary L-arginine level and feeding duration on the intestinal damage of broilers induced by *Clostridium perfringens* (CP) *in vivo*, and the antimicrobial effect of its metabolite nitric oxide (NO) *in vitro*. The *in vivo* experiment was designed as a factorial arrangement of three dietary treatments × two challenge statuses. Broilers were fed a basal diet (CON) or a high-arginine diet (ARG) containing 1·87 % L-arginine, or CON for the first 8 d and ARG from days 9 to 28 (CON/ARG). Birds were co-infected with or without *Eimeria* and CP (EM/CP). EM/CP challenge led to intestinal injury, as evidenced by lower plasma D-xylose concentration ($P < 0.01$), higher paracellular permeability in the ileum ($P < 0.05$) and higher numbers of *Escherichia coli* ($P < 0.05$) and CP ($P < 0.001$) in caecal digesta; however, this situation could be alleviated by L-arginine supplementation ($P < 0.05$). The intestinal *claudin-1* and *occludin* mRNA expression levels were decreased ($P < 0.05$) following EM/CP challenge; this was reversed by L-arginine supplementation ($P < 0.05$). Moreover, EM/CP challenge up-regulated ($P < 0.05$) *claudin-2*, interferon-γ (*IFN-γ*), toll-like receptor 2 and nucleotide-binding oligomerisation domain 1 (*NOD1*) mRNA expression, and L-arginine supplementation elevated ($P < 0.05$) *IFN-γ*, *IL-10* and *NOD1* mRNA expression. *In vitro* study showed that NO had bacteriostatic activity against CP ($P < 0.001$). In conclusion, L-arginine supplementation could inhibit CP overgrowth and alleviate intestinal mucosal injury by modulating innate immune responses, enhancing barrier function and producing NO.

Key words: **L-Arginine: *Clostridium perfringens*: Intestinal barrier function: Intestinal microflora: Immune response**

Clostridium perfringens (CP) is a gram-positive, anaerobic and opportunistic pathogen. CP overgrowth and the exotoxins it produces are responsible for the outbreak of necrotic enteritis (NE) in some cases. NE is an inflammatory disease of the small intestine affecting poultry, resulting in the loss of over 2 billion dollars annually in the international broiler industry[1]. In recent years, NE incidence has been increasing because of the limited use or removal of antibiotics. As reviewed by Shojadoost *et al.*[2], several risk factors predispose an individual to the onset of NE, including coccidial infection, barley- or wheat-based diet, high animal protein diet and a novel pore-forming toxin, NE B-like toxin. The most important known predisposing factor for NE is intestinal damage caused by coccidial pathogens[2,3]. NE manifestation includes acute clinical disease and subclinical disease[2,4]. Unlike clinical infection course with typical symptoms and high mortality, subclinical infection is difficult to detect and results in greater economic losses by chronic intestinal mucosal damage[4].

Previous studies have demonstrated that chickens challenged with both CP and coccidial vaccine have higher numbers of CP in the intestine than healthy chickens or those administered either CP or coccidial vaccine alone[5,6]. CP overgrowth is known to stimulate gene transcripts encoding T helper 1 (Th1) and Th2 cytokines such as *IL-1β*, *IL-10*, *IL-4*, interferon-α (*IFN-α*) and *IFN-γ*[6,7]. Furthermore, cytokines mediate tight junction (TJ) barrier function disorders, resulting in immune perturbation and inflammatory response, ultimately promoting the initiation and/or development of intestinal and systemic diseases[8]. CP infection has been reported to decrease the mRNA expression of intestinal TJ[9,10] and the infection is associated with proteolytic capacity towards TJ proteins[11]. However, factors such as nutrients also influence intestinal integrity, and the use of arginine has received increasing attention because of its role in immune regulation and its ability to restore mucosal integrity.

L-Arginine is a functional amino acid that plays a significant role in several physiological processes via its metabolites such as nitric oxide (NO) or polyamine. Dietary arginine supplementation reinforces the immune status of animals, consequently lowering morbidity and mortality in infectious disease[12,13]. L-arginine is known to promote the proliferation of lymphocytes in

Peyer's patches, balance pro-inflammatory (*IFN-γ* and *IL-2*) and anti-inflammatory (*IL-4* and *IL-10*) cytokines and increase the secretory IgA (sIgA) level in burn-injured mice[14]. Moreover, studies in several animal models have revealed that ʟ-arginine supplementation is capable of reducing intestinal mucosal lesions, maintaining intestinal barrier function, ameliorating mucosal morphology and promoting enterocyte proliferation[15–18].

The benefits of ʟ-arginine supplementation on animals seem to be dose dependent. Peck *et al.*[19] fed mice with severe protein malnutrition normal protein diets with different ʟ-arginine concentrations and studied the mortality of these mice following *Salmonella typhimurium*. Their results revealed that mortality of mice after infection did not reduce by supplementation with 10–20 g/kg ʟ-arginine, but mice mortality was aggravated by supplementation with 50 g/kg ʟ-arginine. Zhan *et al.*[20] reported that a lower level of dietary arginine (1·52 %) improved microvascular development in the small intestines of early-weaned pigs, but at a higher concentration of dietary arginine (2·02 %) adverse effects such as gut dysfunction were observed. However, it remains to be clarified whether or not dietary ʟ-arginine concentrations higher than the National Research Council (NRC, 1994)[21]-recommended level would benefit NE broilers. Therefore, we carried out the present study to investigate the effects of dietary ʟ-arginine level higher than those recommended by the NRC (1994)[21] on the mucosal integrity of intestines, microbial flora and mucosal immunity of broilers in a subclinical NE model.

Methods

In vivo study

Experimental design and diets. All experimental procedures were approved by the Animal Care and Use Committee of China Agricultural University. A total of 210 1-d-old Arbor Acres female broiler chicks were randomly allotted to six treatment groups (*n* 35/group). A 3 × 2 factorial randomised complete block design was used to determine the effects of dietary treatments (dietary ʟ-arginine level and feeding duration), pathogen exposure (co-infected with or without *Eimeria* and CP) and their interactions. The birds were divided into the following three dietary treatment groups: CON, birds in this group were fed a basal diet from days 1 to 28; CON/ARG, birds were given a basal diet for the first 8 d and a high-arginine diet from days 9 (1 d before challenge) to 28; ARG, birds received a high-arginine diet from days 1 to 28. In the high-arginine diet, the amount of ʟ-arginine added to the basal diet accounted for 1·87 % of the diet, and the alanine concentration in the diet was reduced for compensation. Wheat-based meal diets were formulated to meet or exceed the NRC (1994)[21] requirements. The birds were fed *ad libitum* and each treatment group was housed in a separate rearing isolator. Each isolator had a floor space of 11 200 (160 × 70) cm^2, and was equipped with two nipple drinkers and one feeder. Table 1 presents the ingredients and nutrition level of the diet. The arginine used was of reagent grade with a purity of over 98 % (A5006; Sigma-Aldrich Co.). Dietary amino acids were analysed by HPLC. All diets were pelleted and crumbled.

Table 1. Composition and nutrient levels of experimental diets

Items (%)	Basal diet	ʟ-Arginine diet
Ingredients		
Wheat	68·87	68·87
Soyabean meal (44 % CP)	11·50	11·50
Maize gluten meal	9·00	9·00
Rapeseed meal	2·08	2·08
Soyabean oil	2·50	2·50
Calcium hydrophosphate	2·22	2·22
Limestone	1·00	1·00
ʟ-Lys-HCl	0·70	0·70
NaCl	0·30	0·30
Choline chloride (50 %)	0·25	0·25
ʟ-Thr	0·18	0·18
ᴅʟ-Met	0·15	0·15
ʟ-Ala	0·72	0·11
ʟ-Arg (98 %)	0·28	0·89
Trace mineral*	0·20	0·20
Vitamin premix†	0·03	0·03
Nutrient and energy concentration		
Metabolisable energy (MJ/kg)‡	12·55	12·55
Crude protein‡	21·19	21·19
Ca‡	1·02	1·02
Available P‡	0·50	0·50
Lys§	1·19	1·19
Met‡	0·51	0·51
Thr§	0·70	0·72
Arg§	1·25	1·87

* Supplied the following per kg complete feed: Cu, 8 mg; Zn, 75 mg; Fe, 80 mg; Mn, 100 mg; Se, 0·15 mg; I, 0·35 mg.
† Supplied the following per kg complete feed: retinyl acetate, 24 mg; cholecalciferol, 6 mg; menadione, 2·65 mg; thiamine, 2 mg; riboflavin, 6 mg; cyanocobalamin, 0·025 mg; *a*-tocopheryl acetate, 20 mg; biotin, 0·0325 mg; folic acid, 1·25 mg; pantothenic acid, 12 mg; niacin, 50 mg.
‡ Calculated value.
§ Analysed concentrations.

Eimeria and Clostridium perfringens co-infection procedures. The *Eimeria* and *C. perfringens* (EM/CP) challenge was performed on the basis of the reports of Collier *et al.*[7] and Liu *et al.*[22], with some modifications. Briefly, 10-d-old chickens in the infected groups were each challenged with live coccidiosis vaccine (2 × 10^4 oocysts containing a mixture of *Eimeria acervulina*, *Eimeria maxima* and *Eimeria tenella*) suspended in 500 μl of normal saline by oral gavage. From days 14 to 20, the infected groups were orally challenged with 1 ml of *C. perfringens* type A culture broth at 1 × 10^8 colony-forming units (CFU)/bird per d. Chickens in the uninfected group received the same amount of normal saline and sterile broth culture medium at the corresponding times. Coccidiosis vaccine was provided by Zhengdian Biotechnology Company, and the CP strain (CVCC2027) was obtained from the China Veterinary Culture Collection Center.

Sample collection. On days 21 and 28, eight birds were randomly selected per treatment and killed by jugular exsanguination. The midregions of the jejunum and ileum (approximately 1 cm) were collected, rapidly frozen in liquid N$_2$ and stored at –80°C for mRNA analysis. The digesta from the ileum (from the midpoint of the ileum to 1 cm proximal to the ileocaecal junction) and caecum were aseptically collected and stored at –20°C for bacteria counting. In addition, mucosa for the total sIgA examination was scraped from the bottom half of the jejunum and ileum, and stored at –20°C.

Table 2. 16S rDNA real-time PCR primers used for microflora analysis

Targets	Amplicon length (bp)	Sequence (5' → 3')	References
Lactobacillus spp.	341	F: AGCAGTAGGGAATCTTCCA	[26]
		R: CACCGCTACACATGGAG	
Escherichia coli	340	F: GTTAATACCTTTGCTCATTGA	[27]
		R: ACCAGGGTATCTAATCCTGT	
Clostridium perfringens	279	F: AAAGATGGCATCATCATTCAAC	[28]
		R: TACCGTCATTATCTTCCCCAAA	

F, forward; R, reverse.

Plasma D-xylose concentrations. On days 21 and 28, another eight feed-deprived broilers from each group were individually weighed and administered D-xylose (X1500; Sigma-Aldrich Co.) solution at a dose of 0·1 g/kg body weight (infused with 10 % D-xylose at 1 ml/kg body weight) by oral gavage, as described by Hou et al.[23]. A period of 1 h later, blood samples were collected into heparinised vacuum tubes by wing vein puncture. Plasma was separated by centrifugation at 3000 rpm for 10 min at 4°C and then stored at –20°C. The D-xylose concentration was determined using the D-xylose assay kit (A035; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

Ussing chamber experiment. The Ussing chamber procedures were performed as described by Clarke[24] with some modifications. On days 21 and 28 of the trial, after blood samples were obtained from the D-xylose-treated birds, they were then anaesthetised with 5 % (w/w) pentobarbital Na solution injection into the wing vein. The stomachs were then ripped open, and 5-cm-long ileal fragments proximal to the ileocaecal junction were rapidly separated, opened longitudinally and washed in ice-cold oxygenated (95 % O_2/5 %CO_2) Krebs–Ringer buffer[24]. Serosal layers were stripped off, and the ileal mucosa was mounted on the Ussing chamber system (model VCC MC6; Physiologic Instruments) with an exposed area of 0·2 cm². Mucosal and serosal sides were equilibrated in 5 ml of oxygenated (95 % O_2/5 % CO_2) Krebs–Ringer buffer maintained at 37°C for 20 min, and then the medium was removed from both chambers. Fluorescein isothiocyanate dextran 4 kDa (FD4, 4000 MW; Sigma-Aldrich Co.) was diluted with 5 ml of preheated (37°C) Krebs–Ringer buffer to 2 mg/ml and added to the mucosal side. The serosal side was replaced with the diluent. FD4 transportation was monitored by sampling 100 µl of solution from the serosal compartment after 30 min. FD4 concentration was measured in a fluorescence microplate reader (FLx800; Bio-Tek Instruments, Inc.), and mucosal-to-serosal flux of FD4 was expressed as µg/cm² per·h.

Measurement of secretory IgA in intestinal mucosa. Mucosal samples (0·3 g) were placed in tubes containing 2·7 ml of normal saline, and the mixtures were homogenised. The supernatants were collected by centrifugation at 4000 rpm for 10 min at 4°C. The total sIgA concentration was measured using a Chicken IgA ELISA Quantification Set (E30-103; Bethyl Laboratories Inc.) according to the manufacturer's instructions. The protein concentration in the supernatant was measured

using a BCA protein quantification kit (CW0014S; CW Bio). Values were expressed as the Ig level per gram of protein.

Ileal and caecal microflora analysis. The intestinal populations of lactobacilli, *Escherichia coli* and CP were analysed using 16S rDNA primers by quantitative PCR, as described previously[25]. In brief, genomic DNA from the intestinal contents was used as a template for PCR amplification using SYBR® Premix Ex Taq™ (RR420A; TaKaRa) and an ABI 7500 fluorescence quantitative PCR instrument (Applied Biosystems). Absolute quantification was achieved using standard curves constructed by amplification of known amounts of target plasmid DNA. The mathematical formula to calculate the number of target gene copies was referred to the method described by Du et al.[25]. Bacterial DNA was extracted using a QIAamp DNA Stool Mini Kit (51604; Qiagen), according to the manufacturer's instructions. The targeted groups, primer sequences, amplicon sizes and literature referenced are shown in Table 2. The CT of unknown samples was determined and compared with the standard curves. The results were expressed as $\log_{10}$ gene copies per gram (wet weight) of intestinal contents.

Real-time quantitative PCR. Total RNA was extracted from intestinal tissues using Trizol reagent (15596018; Invitrogen Life Technologies) according to the manufacturer's protocol. The concentration and purity of RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). In brief, 1 µg of total RNA from each sample was reverse-transcribed into complementary DNA (cDNA) using a Prime-Script RT reagent kit with cDNA eraser (RR036A; TaKaRa). One-step real-time PCR was performed with the SYBR® Premix Ex Taq™ (RR420A) using an ABI 7500 fluorescence quantitative PCR instrument in accordance with the manufacturer's guidelines. Table 3 lists the quantitative real-time PCR primers used in our study. The relative mRNA expression levels of each target gene were calculated based on the expression of the housekeeping gene GAPDH using the $2^{-\Delta\Delta C_t}$ method[29].

In vitro study

Determination of antimicrobial activity of nitric oxide against Clostridium perfringens. We measured the direct antimicrobial action of NO towards CP according to the previous study[25], with some modifications. In brief, 1×10^5 CFU of CP was mixed with 1 mL of fluid thioglycollate (FT) medium (CM801; Beijing Luqiao technology Co., Ltd) in sterile centrifuge

Table 3. Primers used for quantitative real-time PCR

Target genes	Accession number	Primer sequence (5′ → 3′)	Product size (bp)	Efficiency (%)
Claudin-1	NM_001013611·2	F: CATACTCCTGGGTCTGGTTGGT	100	103·5
		R: GACAGCCATCCGCATCTTCT		
Claudin-2	NM-001277622·1	F: CCATGGTCTCTATGGGACTCC	690	104·9
		R: GCTTCTACACGTATCCCGTC		
Occludin	NM-205128·1	F: ACGGCAGCACCTACCTCAA	123	107·8
		R: GGGCGAAGAAGCAGATGAG		
ZO-1	XM-015278975·1	F: CTTCAGGTGTTTCTCTTCCTCCTC	131	107·8
		R: CTGTGGTTTCATGGCTGGATC		
IFN-γ	NM_205149·1	F: GCTCCCGATGAACGACTTGA	63	101·8
		R: TGTAAGATGCTGAAGAGTTCATTCG		
IL-10	NM_001004414·2	F: GCTGCCAAGCCCTGTT	126	91·5
		R: CCTCAAACTTCACCCTCA		
TLR2	AB046119·2	F: GGGGCTCACAGGCAAAATC	102	105·7
		R: AGCAGGGTTCTCAGGTTCACA		
NOD1	JX465487·1	F: AGCACTGTCCATCCTCTGTCC	214	103·2
		R: TGAGGGTTGGTAAAGGTCTGCT		
GAPDH	NM_204305·1	F: TGCTGCCCAGAACATCATCC	142	98·2
		R: ACGGCAGGTCAGGTCAACAA		

ZO-1, zonula occludens 1; *IFN-γ*, interferon-γ; *TLR2*, toll-like receptor 2; *NOD1*, nucleotide-binding oligomerisation domain 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

tubes containing 0, 125, 250, 500, 1000, 2000 or 4000 μmol/l S-nitroso-N-acety1-N-DL-penicillamine (SNAP, N3398; Sigma-Aldrich Co.) as the NO source. The negative control group consisted of 1 ml of FT medium without CP and SNAP. After anaerobic incubation at 37°C for 24 h, 200 μl of culture medium was added to the microwell plate. The bacteria were counted by measuring the optical density of the culture at 600 nm using a microwell plate reader (ELx800; Bio-Tek Instruments, Inc.).

Statistical analysis

In vivo data were analysed using the General Linear Model procedure in SPSS version 18.0 (SPSS Inc.), and subjected to two-way ANOVA in a 3×2 factorial arrangement to analyse the main effects of dietary treatments and challenge, and their interaction. One-way ANOVA and Duncan's multiple comparison were used when a significant interaction was observed. When the main effect of dietary treatments was significant, polynomial contrasts were performed over the main effect means for dietary treatments (averaged over CP challenge). *In vitro* data were analysed with one-way ANOVA and Duncan's multiple comparison analysis. Results are presented as the means with pooled standard errors. All statements of significance were based on $P < 0.05$.

Results

Plasma D-xylose concentration and ileal fluorescein isothiocyanate dextran 4 kDa flux

As shown in Fig. 1(A) and (B), the plasma D-xylose concentration was significantly lower in the pathogen-challenged groups than in the uninfected groups on days 21 and 28 ($P < 0.01$). Furthermore, the plasma D-xylose concentration was significantly higher in the ARG group than in the CON group on day 21, regardless of pathogen challenge ($P < 0.05$). There was no interaction between EM/CP co-infection and dietary treatments on the plasma D-xylose concentration on days 21 and 28 ($P > 0.05$). EM/CP co-infection enhanced the ileal FD4 flux on days 21 and 28 ($P = 0.032$ and 0·071, respectively; Fig. 1(C) and (D)). Dietary L-arginine supplementation significantly reduced the ileal FD4 flux on day 21 ($P < 0.05$).

Intestinal secretory IgA level

The ileal sIgA level on day 21 was obviously higher in infected birds than in uninfected birds ($P < 0.05$); however, they decreased in the infected groups on day 28 ($P < 0.05$; Fig. 2). In addition, the jejunal sIgA level on day 28 was significantly higher in the ARG group than in the CON and CON/ARG groups ($P = 0.01$). No interactions on the sIgA levels were found between dietary treatments and pathogens challenge on days 21 and 28 ($P > 0.05$).

Quantification of ileal and caecal microbiota

Birds co-infected with EM/CP had higher populations of *E. coli* and CP in the caecal contents on day 21 ($P < 0.05$ and $P < 0.001$, respectively; Fig. 3). Regardless of pathogen challenge, addition of L-arginine (ARG and CON/ARG groups) significantly decreased the population of CP in the ileum ($P < 0.001$). On day 21, dietary treatments and EM/CP co-infection exerted an interactive effect on the population of CP in the ileal contents ($P < 0.05$). In unchallenged birds, the CP population was decreased in the CON/ARG and ARG groups compared with the CON group, whereas in challenged birds it was the lowest in the ARG group with no significant differences between the other two groups. Moreover, there was an interaction between dietary treatments and pathogen challenge on the caecal CP population ($P < 0.05$): L-arginine supplementation significantly decreased the CP population in the caecum of EM/CP-challenged birds. However, different dietary treatments, pathogen challenge or interaction of these two factors did not result in changes in the *Lactobacillus* populations ($P > 0.05$).
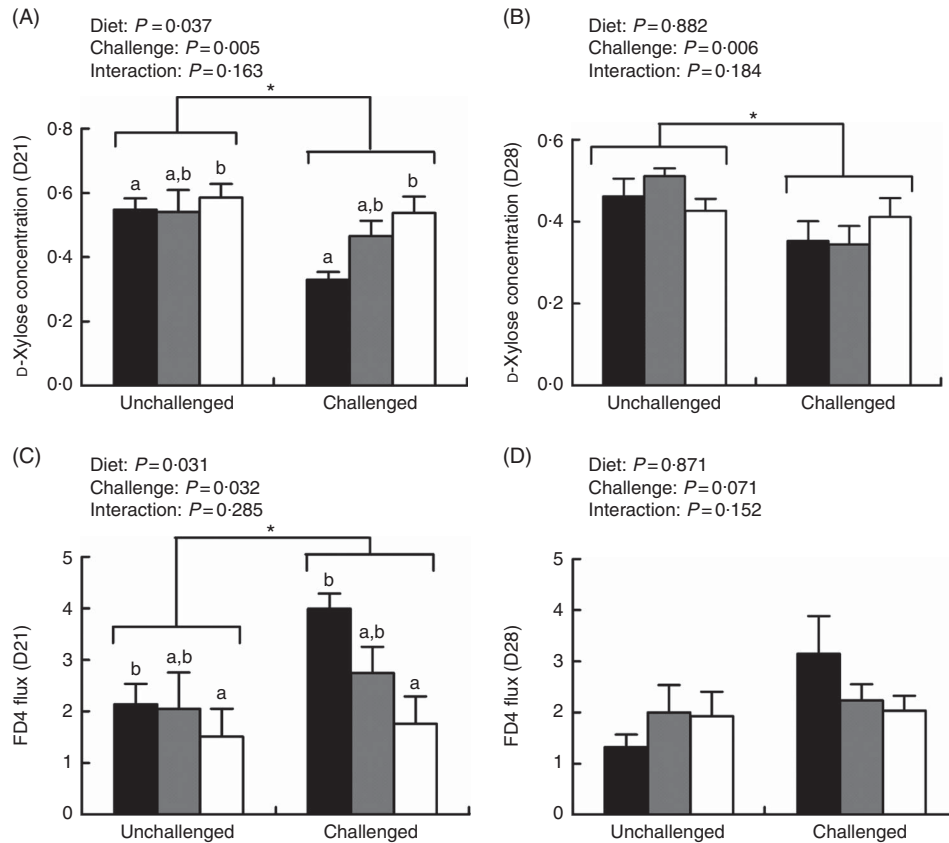
Fig. 1. Effect of dietary L-arginine level and feeding duration on plasma D-xylose concentration (mmol/l, A and B) and ileal fluorescein isothiocyanate dextran 4kDa (FD4) flux (μg/cm^2·per h, C and D). Values are means (n 8), with their standard errors represented by vertical bars. Unchallenged, chickens without EM/CP co-infection; challenged, chickens with EM/CP co-infection; ■, CON, basal diet from days 1 to 28; ▨, CON/ARG, basal diet from days 1 to 8 and L-arginine diet from days 9 to 28; □, ARG, L-arginine diet from days 1 to 28. ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$). * Significant main effect ($P < 0.05$) of *Emeria* and *Clostridium perfringens* (EM/CP) co-infection.

Gene expression of tight junction proteins

On day 21, EM/CP challenge significantly down-regulated the mRNA expression levels of jejunal and ileal *claudin-1* and jejunal *occludin* ($P < 0.05$), but increased the ileal *claudin-2* mRNA expression ($P < 0.05$; Tables 4 and 5). In addition, L-arginine expression significantly increased the jejunal *claudin-1* expression on day 21 and the ileal *occludin* expression on day 28, regardless of challenge ($P < 0.05$). Furthermore, the interactions of dietary treatments and pathogen challenge were found to significantly affect jejunal *claudin-2* and ileal *occludin* expression on day 28 ($P < 0.05$). In the co-infected birds, the mRNA expression of *claudin-2* and *occludin* was elevated with L-arginine supplementation ($P < 0.05$). However, no significant differences were seen on the expression of zonula occludens 1 (*ZO-1*) by the main effects and their interaction on days 21 and 28.

Inflammation-related gene expression

As shown in Tables 6 and 7, co-infection with EM/CP significantly elevated the mRNA expression levels of jejunal *IFN-γ* on days 21 and 28 ($P < 0.05$), ileal *IFN-γ* on day 28 ($P < 0.01$), jejunal toll-like receptor 2 (*TLR2*) on days 21 and 28 ($P < 0.05$) and jejunal nucleotide-binding oligomerisation domain 1 (*NOD1*) on day 28 ($P < 0.05$). Regardless of challenge,

L-arginine supplementation increased the mRNA expression levels of jejunal and ileal *IFN-γ* on day 28 ($P < 0.001$ and $P < 0.01$, respectively), jejunal *IL-10* on day 28 ($P < 0.05$) and ileal *IL-10* on day 21 ($P < 0.05$).

There was a significant interaction between dietary treatments and EM/CP challenge on the mRNA expression of *IFN-γ* in the ileum on day 28 ($P < 0.05$). The *IFN-γ* mRNA expression in challenged birds was significantly increased with L-arginine supplementation ($P < 0.05$). Furthermore, the interaction between dietary treatments and EM/CP challenge significantly influenced *NOD1* mRNA expression in the jejunum on day 21 and in the ileum on day 28 ($P < 0.05$). L-Arginine supplementation significantly increased the *NOD1* mRNA expression in chickens challenged by EM/CP ($P < 0.05$). In addition, jejunal *IL-10* mRNA expression was significantly influenced by the interaction between dietary treatments and pathogen challenge on day 28 ($P < 0.05$). The *IL-10* mRNA expression was the lowest in the CON group co-infected with EM/CP and the highest in the ARG groups ($P < 0.05$).

In vitro cytotoxicity of nitric oxide towards Clostridium perfringens

There were no significant differences in the optical density at 600 nm among the different treatment groups at the beginning
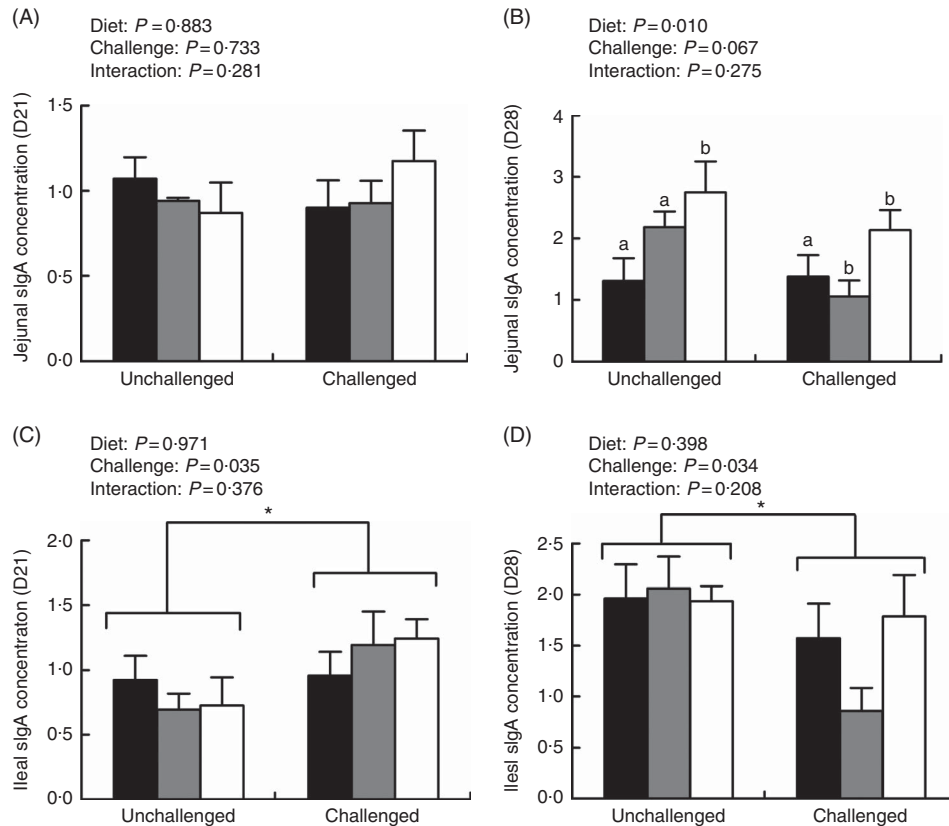
Fig. 2. Effect of dietary L-arginine level and feeding duration on secretory IgA (sIgA) concentration (mg/g protein) of jejunal (A and B) and ileal (C and D) mucosa. Values are means (*n* 8), with their standard errors represented by vertical bars. Unchallenged, chickens without EM/CP co-infection; challenged, chickens with EM/CP co-infection; ■, CON, basal diet from days 1 to 28; ▨, CON/ARG, basal diet from days 1 to 8 and L-arginine diet from days 9 to 28; □, ARG, L-arginine diet from days 1 to 28. a,b Mean values with unlike letters were significantly different (*P* < 0·05). * Significant main effect (*P* < 0·05) of *Emeria* and *Clostridium perfringens* (EM/CP) co-infection.

of the experiment (data not shown). After incubation for 24 h at 37°C, CP growth was completely inhibited by 2000 and 4000 μM of SNAP (*P* < 0·001, Fig. 4).

Discussion

Cereals with high level of NSP (wheat, rye and barley), and coccidial infection, favour the proliferation of CP and its toxin production[2]. Here, we induced experimental NE in broiler chickens using wheat-based diets and challenge with *Emeria* and CP. Although no obvious clinical signs of NE or NE-related mortality were observed, the challenge did induce the macroscopic pathological changes in the intestine, such as distinct hyperaemia, haemorrhage and focal necrosis or ulceration. In addition, pathogen challenge resulted in impaired intestinal absorption, increased ileal mucosal permeability and high colonisation rates of CP in the caecum, which were in accordance with the characteristics of subclinical NE established in previous studies[22,30].

The intact intestinal tract is not only essential for nutrition absorption but also functions as a physical barrier against the harmful components. D-Xylose absorption, mucosal permeability and expression of TJ proteins are the widely used indicators of intestinal epithelial health. As D-xylose crosses the

intestinal mucosa via an Na+-dependent mobile-carrier mechanism, active absorption of D-xylose indicates active absorption of water and Na[31]. 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 the blood[31,32]. In the present study, decreased plasma D-xylose concentration in pathogen-challenged birds reflected malabsorption of the small intestine, which could be related to the impaired intestinal epithelium. The addition of L-arginine increased the plasma D-xylose concentrations regardless of EM/CP challenge, indicating that L-arginine could promote the ability of the small intestine to absorb water and Na. This was consistent with previous results in rats, which reported that L-arginine (1 mM) added to the oral rehydration solutions could improve intestinal water and Na absorption[33]. In this study, we used FD4 flux determined by the Ussing chamber to evaluate intestinal permeability. FD4 permeates the intestinal epithelium mainly via paracellular pathways[34]. When the intestinal barrier is impaired, FD4 flux from the mucosal to serosal side will increase. In this study, the remarkable elevation of FD4 flux in the ileum of challenged birds indicated impaired intestinal barrier function. L-Arginine administration reduced the ileal FD4 flux regardless of pathogen challenge, revealing that dietary L-arginine exerts a protective effect on intestinal barrier function.
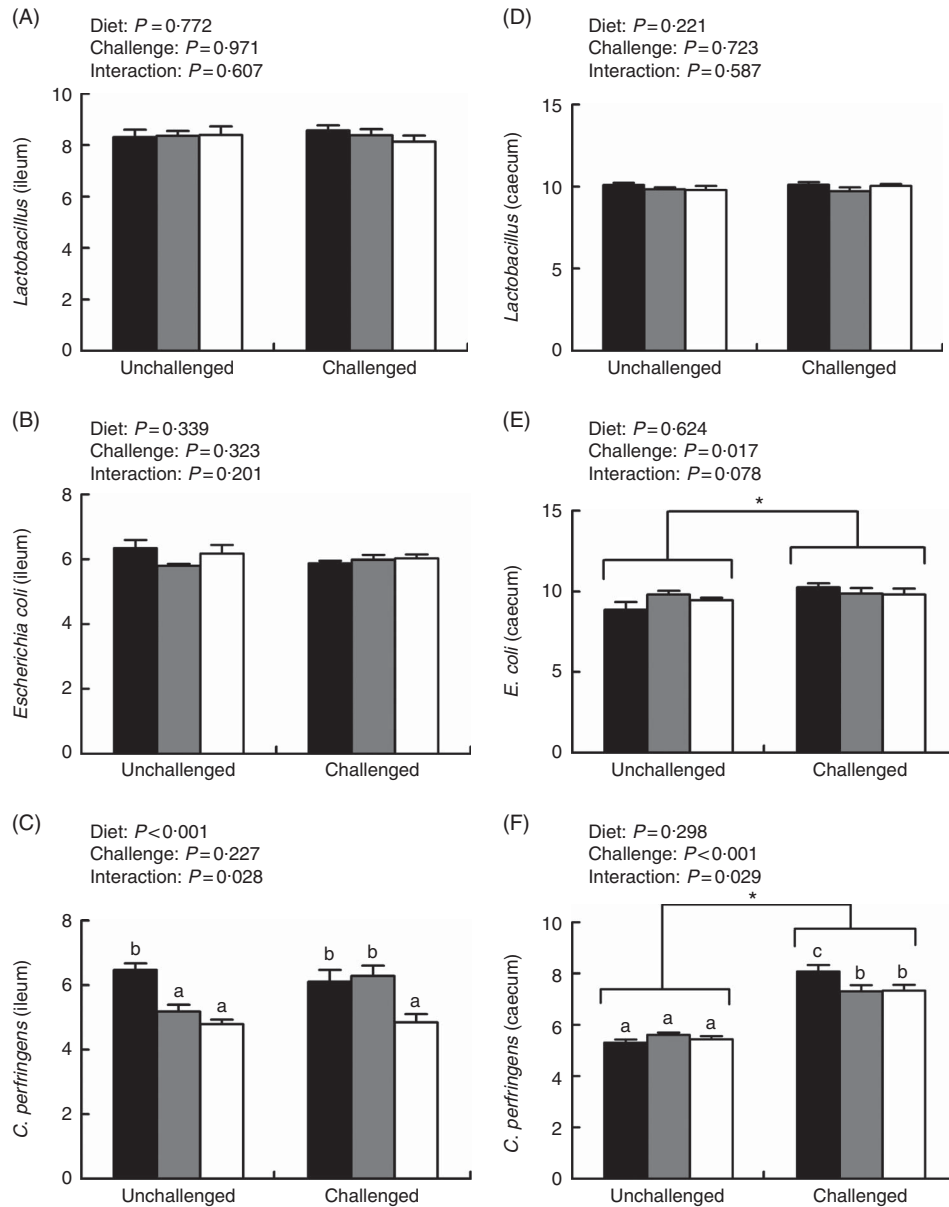
Fig. 3. Effect of dietary L-arginine level and feeding duration on microbial populations ($\log_{10}$ gene copies/g wet weight) in ileal (A, B and C) and caecal (D, E and F) contents on day 21. Values are means (*n* 8), with their standard errors represented by vertical bars. Unchallenged, chickens without EM/CP co-infection; challenged, chickens with EM/CP co-infection; ■, CON, basal diet from days 1 to 28; ▨, CON/ARG, basal diet from days 1 to 8 and L-arginine diet from days 9 to 28; ☐, ARG, L-arginine diet from days 1 to 28. [a,b,c] Mean values with unlike letters were significantly different ($P < 0.05$). * Significant main effect ($P < 0.05$) of *Emeria* and *Clostridium perfringens* (EM/CP) co-infection.

TJ proteins are vital for the regulation of gut barrier function. During inflammation, the disrupted TJ may result in increased mucosal permeability and pathogen invasion[35]. Following EM/CP challenge, changes were discovered in intestinal TJ expression, characterised by the reduced *claudin-1* and *occludin* mRNA expression levels, and even elevated *claudin-2* expressions. Claudin-1 and occludin are barrier-forming TJ proteins, whereas claudin-2 is a pore-forming TJ protein[8]. Thus far, the up-regulation of *claudin-2* expression has been only consistently reported in inflammatory bowel disease, and it is regarded as an indicator of inflammation[35]. Occludin exerts many regulatory functions and is sensitive to changes in the TJ[36]. The barrier created by occludin and the adjacent cells prevents macromolecules from passing through it, but allows small ions to pass through[37]. Our study revealed that L-arginine prevented the EM/CP-challenge-induced decrease in *claudin-1* mRNA expression and increased the *occludin* mRNA expression, which could be conducive to the restoration of intestinal permeability. This finding was similar to those reported by previous studies[38,39]. In the present study, an elevation of *claudin-2* expression in challenged birds fed a high-arginine diet unexpectedly did not lead to increased paracellular permeability, which could probably be attributed to increased expression of tightening TJ proteins in the meantime.

Table 4. Effect of dietary L-arginine level and feeding duration on the relative mRNA expression of the jejunal tight junction
(Mean values with their standard errors; *n* 8)

Items	Unchallenged			Challenged				Analysis by effect (*P*)		
	CON	CON/ARG	ARG	CON	CON/ARG	ARG	SEM	Arg	Challenge	Arg × Challenge
Day 21										
Claudin-1	1·019	1·069	1·245	0·888	0·911	1·058	0·035	0·043	0·019	0·939
Claudin-2	1·179	1·895	1·429	2·349	1·867	1·812	0·181	0·845	0·171	0·405
Occludin	1·040	1·098	1·037	0·980	0·802	0·992	0·034	0·670	0·049	0·226
ZO-1	1·015	1·088	0·982	0·946	0·895	1·082	0·026	0·708	0·306	0·096
Day 28										
Claudin-1	1·059	0·893	0·960	0·866	1·095	0·863	0·040	0·710	0·725	0·157
Claudin-2	1·009[b]	0·861[a,b]	1·219[b]	0·469[a]	1·110[b]	0·785[a,b]	0·070	0·252	0·081	0·036
Occludin	0·816	0·718	0·775	0·802	0·885	0·817	0·026	0·981	0·240	0·393
ZO-1	1·002	0·934	0·952	0·938	1·037	0·965	0·023	0·901	0·724	0·378

ZO-1, zonula occludens 1; unchallenged, chickens without *Eimeria* and *Clostridium perfringens* (EM/CP) co-infection; challenged, chickens with EM/CP co-infection; CON, basal diet from days 1 to 28; CON/ARG, basal diet from days 1 to 8 and high-arginine diet from days 9 to 28; ARG, high-arginine diet from days 1 to 28; Arg × Challenge, interaction between dietary L-arginine treatment and EM/CP co-infection.
[a,b] Mean values in the same row with unlike superscript letters were significantly different (*P* < 0·05).

Table 5. Effect of dietary L-arginine level and feeding duration on the relative mRNA expression of the ileal tight junction
(Mean values with their standard errors; *n* 8)

Items	Unchallenged			Challenged				Analysis by effect (*P*)		
	CON	CON/ARG	ARG	CON	CON/ARG	ARG	SEM	Arg	Challenge	Arg × Challenge
Day 21										
Claudin-1	1·070	1·258	1·224	0·995	0·943	1·085	0·044	0·514	0·048	0·504
Claudin-2	1·176	1·912	1·058	2·811	2·684	2·139	0·249	0·504	0·021	0·763
Occludin	1·027	0·892	0·891	0·946	0·925	0·862	0·025	0·190	0·608	0·638
ZO-1	1·024	1·048	0·921	1·142	1·032	1·005	0·029	0·252	0·296	0·624
Day 28										
Claudin-1	1·058	1·138	1·097	1·071	1·011	1·062	0·050	0·993	0·643	0·862
Claudin-2	1·025	0·864	0·899	0·721	0·993	0·554	0·058	0·325	0·132	0·168
Occludin	1·01[a,b]	0·923[a,b]	0·978[a,b]	0·825[a]	1·058[b,c]	1·249[c]	0·034	0·034	0·220	0·009
ZO-1	0·942	1·084	1·089	1·090	1·129	1·144	0·034	0·435	0·236	0·803

ZO-1, zonula occludens 1; unchallenged, chickens without *Eimeria* and *Clostridium perfringens* (EM/CP) co-infection; challenged, chickens with EM/CP co-infection; CON, basal diet from days 1 to 28; CON/ARG, basal diet from days 1 to 8 and high-arginine diet from days 9 to 28; ARG, high-arginine diet from days 1 to 28; Arg × Challenge, interaction between dietary L-arginine treatment and EM/CP co-infection.
[a,b,c] Mean values in the same row with unlike superscript letters were significantly different (*P* < 0·05).

Table 6. Effect of dietary L-arginine level and feeding duration on the relative mRNA expression of the jejunal tight junction and immune-related molecules
(Mean values with their standard errors; *n* 8)

Items	Unchallenged			Challenged				Analysis by effect (*P*)		
	CON	CON/ARG	ARG	CON	CON/ARG	ARG	SEM	Arg	Challenge	Arg × Challenge
Day 21										
IFN-γ	1·134	1·386	1·166	2·021	1·769	1·576	0·129	0·743	0·035	0·660
IL-10	1·063	2·099	2·084	1·706	1·444	1·606	0·129	0·288	0·534	0·092
TLR2	1·090	0·952	0·806	1·176	1·145	1·365	0·056	0·809	0·012	0·175
NOD1	1·055[a,b]	0·796[a,b]	0·755[a,b]	0·725[a]	0·908[a,b]	1·088[b]	0·046	0·806	0·660	0·011
Day 28										
IFN-γ	0·979	1·103	1·818	0·984	1·545	3·012	0·164	<0·001	0·037	0·172
IL-10	1·097[b,c]	0·800[a,b]	1·381[c]	0·502[a]	1·110[b,c]	1·365[c]	0·085	0·011	0·480	0·041
TLR2	0·965	0·766	0·886	1·219	1·199	1·311	0·089	0·839	0·045	0·900
NOD1	1·009	0·953	1·052	1·041	1·318	1·278	0·047	0·420	0·024	0·324

IFN-γ, interferon-γ; TLR2, toll-like receptor 2; NOD1, nucleotide-binding oligomerisation domain 1; unchallenged, chickens without *Eimeria* and *Clostridium perfringens* (EM/CP) co-infection; challenged, chickens with EM/CP co-infection; CON, basal diet from days 1 to 28; CON/ARG, basal diet from days 1 to 8 and high-arginine diet from days 9 to 28; ARG, high-arginine diet from days 1 to 28; Arg × Challenge, interaction between dietary L-arginine treatment and EM/CP co-infection.
[a,b,c] Mean values in the same row with unlike superscript letters were significantly different (*P* < 0·05).

As gut microbial composition is one of the indicators of gut health[40], bacteria can contribute to enhanced intestinal permeability if the gastrointestinal ecosystem is imbalanced[41]. Therefore, we estimated the populations of *Lactobacillus*, *E. coli* and CP in the digesta. Many specific members of microflora have been found to coexist with NE, of which the proportion of *E. coli* is reported to be one of the largest[7,42,43]. Similar to previous studies[22,25], we found that the significant

Table 7. Effect of dietary L-arginine level and feeding duration on the relative mRNA expression of the ileal tight junction and immune-related molecules
(Mean values with their standard errors; n 8)

Items	Unchallenged			Challenged				Analysis by effect (P)		
	CON	CON/ARG	ARG	CON	CON/ARG	ARG	SEM	Arg	Challenge	Arg × Challenge
Day 21										
IFN-γ	0·821	1·048	1·283	1·367	1·146	1·265	0·070	0·475	0·136	0·229
IL-10	1·037	1·665	1·957	1·297	1·240	1·655	0·102	0·039	0·424	0·324
TLR2	1·103	1·159	1·014	1·271	0·873	0·999	0·044	0·157	0·598	0·102
NOD1	1·019	1·165	0·950	0·757	0·947	1·067	0·041	0·196	0·128	0·119
Day 28										
IFN-γ	0·955[a]	0·814[a]	1·399[a,b]	0·813[a]	1·992[b]	1·899[b]	0·113	0·004	0·007	0·016
IL-10	1·063	1·087	0·995	0·673	1·195	1·061	0·088	0·447	0·701	0·433
TLR2	1·052	1·063	1·085	0·897	0·929	1·017	0·047	0·810	0·238	0·934
NOD1	1·020[a,b]	1·020[a,b]	1·108[b]	0·833[a]	1·293[b]	1·159[b]	0·039	0·020	0·526	0·039

IFN-γ, interferon-γ; TLR2, toll-like receptor 2; NOD1, nucleotide-binding oligomerisation domain 1; unchallenged, chickens without *Eimeria* and *Clostridium perfringens* (EM/CP) co-infection; challenged, chickens with EM/CP co-infection; CON, basal diet from days 1 to 28; CON/ARG, basal diet from days 1 to 8 and high-arginine diet from days 9 to 28; ARG, high-arginine diet from days 1 to 28; Arg × Challenge, interaction between dietary L-arginine treatment and EM/CP co-infection.
[a,b] Mean values in the same row with unlike superscript letters were significantly different (P < 0·05).
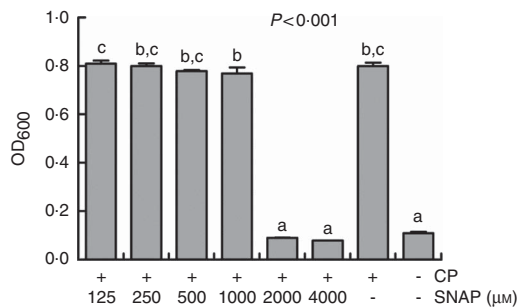
Fig. 4. Direct antimicrobial activity of nitric oxide towards *Clostridium perfringens* (CP). Bacteria were cultured in fluid thioglycollate medium in the presence of 0, 125–4000 μmol/l S-nitroso-N-acetylpenicillamine (SNAP), as described in the 'Methods' section. After 24 h, bacterial growth was monitored by measuring the optical density at 600 nm. Values are means (n 3), with their standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different (P < 0·05).

increase in the CP population was accompanied by a sharp increase in the *E. coli* populations in the caecal content. This may be because the challenge increases mucus production of birds owing to intestinal inflammation, and the mucus serves as a growth substrate for some caecal bacteria[40]. *Lactobacillus* is the well-known beneficial gut bacteria, which is predominant in the ileam and the fourth largest groups in the caecum[44]. In our study, the number of *Lactobacillus* bacteria in the ileum and caecum were not influenced following the EM/CP challenge. Similar results were observed in chickens infected with *C. perfringens*[25,42]. However, some investigators reported that *C. perfringens* challenge significantly increased the *Lactobacillus* population in the intestinal digesta[22,40]. The inconsistent results might be related to the strain, dosage and frequency of *C. perfringens* infection, which need further study. As reviewed by Hardy *et al.*[45], previous researches have reported the antimicrobial properties of L-arginine in humans and animals. To the best of our knowledge, there are no published studies on the effects of dietary arginine on the CP population in the guts of chickens. In this experiment, L-arginine supplementation reduced the CP population in the

ileum and caecum of broiler chickens. One of the reasons for this result could be that L-arginine stimulates intestinal epithelial cell proliferation and survival via the regulation of the mammalian target of rapamycin[46]. When the birds are infected with enteric pathogens, the restoration of normal epithelial cell turnover makes it challenging for the pathogens to maintain a stable niche for colonisation[47].

SIgA is the primary immunological barrier that prevents intraluminal pathogens from colonising to the intestinal mucosa, and maintains homoeostasis with the commensal microbiota[48]. The stimulation of mucosal IgA requires for large doses of bacteria, the extent of which depends on the total bacterial dose[49]. Our data illustrated that ileal sIgA levels increased with the enhanced colonisation of *C. perfringens* and *E. coli* in challenged birds 1 d after the challenge (day 21), which was corresponding with the results of previous studies[10,50]. Thus, we speculated that in the early period of infection large amounts of sIgA were induced by the overgrowth of enteropathogenic bacteria to defend against acute intestinal injury. Polosukhin *et al.*[51] observed that parents with chronic obstructive pulmonary disease had reduced sIgA in bronchoalveolar lavage. He stated that bronchial epithelial structural abnormalities led to localised sIgA deficiency[51]. Another research also reported that caecal sIgA production was decreased in dextran sulphate Na-induced chronic colitis[52]. Thus, we considered the reduction of ileal sIgA levels in challenged birds 8 d after the challenge (day 28) as an intestinal epithelial impairment. The present study showed that sIgA levels were increased by L-arginine supplementation in the jejunum on day 28, leading to protection of the intestinal mucosal surface[16].

Pattern recognition receptors (PRR) detect various microbial infections and trigger inflammation via specific signalling pathways to defend against invading pathogens. TLR and NOD-like receptors (NLR) are two major PRR families[53]. In the current experiment, we analysed the mRNA expression levels of intestinal *TLR2* and *NOD1*. TLR2 can detect peptidoglycan and lipoteichoic acid from gram-positive bacteria on the cell surface[53]. Our data showed that EM/CP challenge led to a

higher mRNA expression of *TLR2* in the jejunum of broilers. Similar result was reported by Du *et al.*[10]. NOD1 and NOD2 are well-known members of the NLR family, which recognise bacterial peptidoglycan in the cytoplasm in mammals[53]. As the nucleotide sequence of *NOD2* in chickens has not been uncovered yet, we only determined the changes of *NOD1* here. In the present study, EM/CP co-infection significantly increased the intestinal *NOD1* gene expression. Guo *et al.*[54] reported that CP could be recognised by NOD1 in chicken primary intestinal epithelial cells. *NOD1*$^{-/-}$ mice infected with *Chlamydophila pneumoniae* exhibited delayed bacterial clearance and delayed neutrophil recruitment with evidence for defective NO and blunted cytokine production[55]. In our study, the elevated intestinal mRNA expression of *NOD1* with L-arginine supplementation indicated that L-arginine promoted the recognition of invasive pathogenic components in the cytoplasm. However, how CP peptidoglycan is transferred to the cytosol to combine with NOD1 and how L-arginine stimulates NOD1 remain unclear.

To clarify the effects of L-arginine on gut pro- and anti-inflammatory responses in the NE model, we examined the *IFN-γ* and *IL-10* mRNA expression. Our results showed that addition of L-arginine elevated the mRNA expression levels of both *IFN-γ* and *IL-10* in the small intestine. Shang *et al.*[56] suggested that L-arginine increased the gene transcripts of both Th1 (*IL-2* and *IFN-γ*) and Th2 (*IL-4* and *IL-10*) cytokines in splenocytes of rats with sepsis induced by caecal ligation and puncture. Viana *et al.*[16] obtained similar results in the serum of mice with intestinal obstruction. Previous studies have demonstrated that IFN-γ evokes epithelial apoptosis and impairs intestinal barrier function[57,58]. However, Kominsky *et al.*[59] found that IFN-γ up-regulates cellular methylation pathways that play a protective role in intestinal epithelium. More recently, Kominsky *et al.*[60] demonstrated that IFN-γ specifically increased IL-10 receptor 1 (IL-10R1) expression, as well as up-regulating *IL-10* gene expression in the intestinal epithelium. Knockdown of *IL-10R1* led to increased intestinal permeability in mice. This discrepancy may be due to the differences in challenge model and experimental animals. IFN-γ secretion contributes to macrophage activation, improvement in microbicidal activity and enhanced expression of the epithelial receptor for the sIgA[61,62]. In addition, IL-10 has been previously reported to stimulate the production and secretion of sIgA[63]. Thus, our data showed that L-arginine elevated the mRNA expression of *IFN-γ* and *IL-10*, which might contribute to the clearance of pathogens and improvement of intestinal integrity[16].

NO has been reported to inhibit the growth of *Salmonella* species, *Actinobacillus* species, *Streptococcus pyogenes* and *Staphylococcus aureus*[64,65], but it does not affect certain bacterial strains such as group B streptococci[65]. Thus far, data regarding the bacteriostasis effect of NO against CP *in vitro* are scarce. In our experiment, culturing CP with over 2000 μM SNAP significantly retarded its growth, indicating that NO exerts direct antimicrobial action against CP. L-Arginine has been considered as the sole NO source in the host, and supplementation with L-arginine has been shown to increase the NO levels in both animals and cells[66,67]. Viana *et al.*[16] confirmed that the role of arginine in reducing bacterial translocation and stimulating

cytokines and sIgA in mice after intestinal obstruction were mediated by NO production. In our study, arginine treatment reduced CP populations, and improved intestinal permeability might be related to NO production.

In conclusion, L-arginine maintained intestinal integrity and accelerated pathogen clearance in broiler chickens, which might at least partly be associated with accelerated pathogen recognition, modulation of gut pro-inflammatory and anti-inflammatory cytokines, enhanced mRNA expression of TJ proteins and sIgA and NO production. In our study, diets including 1·87% L-arginine fed for 28 days could alleviate intestinal injury of broiler chickens with NE.

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