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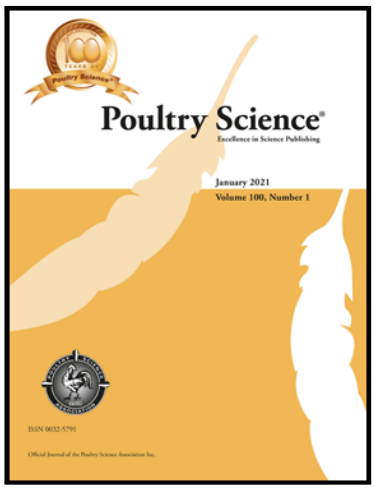

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Running head: EGG YOLK IGY AGAINST NECROTIC ENTERITIS

Hyperimmune egg yolk antibodies developed against *Clostridium perfringens* antigens protect against necrotic enteritis

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ABSTRACT

Necrotic enteritis (NE) is a widespread infectious disease caused by *Clostridium perfringens* that inflicts major economic losses on the global poultry industry. Due to regulations on antibiotic use in poultry production, there is an urgent need for alternative strategies to mitigate the negative effects of NE. This paper presents a passive immunization technology that utilizes hyperimmune egg yolk immunoglobulin Y (IgY) specific to the major immunodominant antigens of *C. perfringens*. Egg yolk IgYs were generated by immunizing hens with four different recombinant *C. perfringens* antigens, and their protective effects against NE were evaluated in commercial broilers. Six different spray-dried egg powders were produced using recombinant *C. perfringens* antigens: α-toxin, NE B-like toxin (NetB; EB), elongation factor-Tu (ET), pyruvate:ferredoxin oxidoreductase, a mixture of four antigens (EM-1), and a non-immunized control (EC). The challenged groups were either provided with different egg powders at a 1% level or no egg powders (EN). The NE challenge model based on *Eimeria maxima* and *C. perfringens* dual infection was used. In Experiments 1 and 2, the EB and ET groups exhibited increased body weight gain (BWG; $P < 0.01$), decreased NE lesion scores ($P < 0.001$), and reduced serum NetB levels ($P < 0.01$) compared to the EN and EC groups. IgY against NetB significantly reduced Leghorn Male Hepatocellular cytotoxicity in an *in vitro* test ($P < 0.01$). In Experiment 3, the protective effect of the IgYs mixture (EM-2) against *C. perfringens* antigens (NetB and EFTu) and *Eimeria* antigens (elongation factor-1-alpha: EF1α and *Eimeria* profilin: 3-1E) was tested. The EM-2 group showed similar body weight, BWG, and feed intake from d 7 to 22 compared to the NC group ($P < 0.05$). On d 20, the EM-2 group showed comparable intestinal permeability, NE lesion scores, and jejunal NetB and collagen adhesion protein levels to the NC group ($P < 0.05$). In conclusion, dietary IgY mixture containing NetB and EFTu provides

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protection against experimental NE in chickens through passive immunization.

Key words: Broiler, *Clostridium perfringens*, Egg yolk immunoglobulin Y, Necrotic enteritis, Necrotic enteritis B-like toxin

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INTRODUCTION

Necrotic enteritis (NE), caused by *Clostridium perfringens*, is a widespread infectious disease inflicting great economic losses of more than $6 billion globally on the poultry industry worldwide (Van der Sluis, 2000; Wade and Keyburn, 2015). NE usually occurs in broiler chickens at 2-6 weeks of age and may present as an acute clinical disease or subclinical infection. Acute infection is characterized by a sudden onset of mortality with few clinical signs, while subclinical NE causes a decrease in growth performance by about 12% compared to healthy chickens, accounting for a major portion of the economic loss caused by NE (Skinner et al., 2010). In the past few decades, prophylactic supplementation of in-feed antibiotics has been used as a major strategy to mitigate the impact of NE. However, with the ban on the use of antibiotics for growth promotion in the European Union and the increasing regulatory restrictions on the use of antibiotics in the United States, the incidence and severity of NE outbreaks have increased in recent years (Casewell et al., 2003; Gaucher et al., 2015). Therefore, there is a timely need to develop antibiotic-alternative strategies to mitigate NE (Seal et al., 2013).

One potential alternative strategy in the prevention of NE is passive immunization using antigen-specific hyperimmune egg yolk antibodies, also known as immunoglobulin Y (IgY). IgY from egg yolks collected after repeated immunization of laying hens with specific antigens has previously been shown to be effective in the prevention and treatment of intestinal infectious diseases (Gadde et al., 2015). One of the advantages of using IgY as an antibiotic alternative in the control of NE is the high stability of egg yolk IgY (Gadde et al., 2015). Spray-dried egg yolk IgY can be stored at room temperature for approximately 6 months and for considerably longer periods when stored under refrigeration or freezing conditions (Fu et al., 2006; Nilsson et al.,

2012). Importantly, IgY is also known to be stable when processed under high heat and pressure as a feed additive (Shimizu et al., 1992, 1994).

The mechanism of action of IgY is mainly through an antigen-antibody reaction, resulting from antigen-specific immunoglobulins binding to the pathogen to induce various anti-bacterial effects (Rahman et al., 2013). For example, IgY binding to bacterial structures such as flagella and pili inhibits bacterial adhesion to the intestinal wall, thereby reducing bacterial growth and colonization in the intestine (Jin et al., 1998). In addition, IgY can interfere with bacterial growth and toxin production in a variety of ways including bacterial aggregation, toxin neutralization, inhibition of enzyme activity, and reduction of bacterial signaling cascades (Wang et al., 2011; Xu et al., 2011; Rahman et al., 2013). Another important characteristic of IgY-mediated passive immunization is its immediate effects compared to active immunization which can take several days or longer to induce an antigen-specific immune response (Rahman et al., 2014). Additionally, egg yolk IgY antibodies that target *C. perfringens* can more effectively defend against enteric bacterial diseases through passive immunization. Some studies have shown no significant effects of dietary IgY antibodies against *C. perfringens*. Wilkie et al. (2006) reported that egg yolk IgY did not affect the level of colonization of *C. perfringens* whereas Tamilzarasan et al. (2009) reported that the mortality rate of chickens infected with *C. perfringens* was reduced by egg yolk IgY. These varying observations could be due to many factors including the specificity and dose of IgY antibodies as well as the type of infection model.

For example, pathogenic and toxin-producing *C. perfringens* strains can induce NE, however, in most field NE cases, coccidiosis has been shown to be an important predisposing factor for NE infection. This is because intracellular development of *Eimeria* parasites in the gut damages the intestinal epithelium and facilitates the colonization and proliferation of *C.*

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perfringens (Van Immerseel et al., 2009). Physically damaged epithelial cells by *Eimeria* can result in the leakage of plasma proteins, promoting *C. perfringens* growth (Van Immerseel et al., 2004). In addition, damaged epithelial cells expose certain types of collagens within the extracellular matrix (ECM) to the lumen. As a result, *C. perfringens* with collagen adhesin protein (CNA) efficiently binds to collagen, promoting colonization (Lepp et al., 2021; Goo et al., 2023). Previous studies have reported that *Eimeria*-specific IgYs are likely to mitigate the effects of coccidiosis (Lee et al., 2009a, b). Therefore, the combination of *Eimeria*-specific and *C. perfringens*-specific IgY antibodies can effectively synergize to mitigate NE infection. The objective of the current study was to develop egg yolk IgY antibodies against the major immunodominant antigens of *C. perfringens* and *Eimeria* to investigate their combined protective effect against experimental NE through passive immunization.

MATERIALS AND METHODS

Cloning, Expression, and Purification of Recombinant C. perfringens and Eimeria Proteins

The method for the production of recombinant proteins for immunization of hens was previously described (Lee et al., 2010, 2011; Jang et al., 2012; Lin et al., 2017). Briefly, full-length coding sequences of *C. perfringens* α-toxin, NE B-like toxin (NetB), *C. perfringens* elongation factor Tu (EFTu), and a partial sequence of pyruvate: ferredoxin oxidoreductase (PFO), as well as full-length coding sequences of *Eimeria* elongation factor 1 alpha (EF1α) and 3-1E (*Eimeria* recombinant profilin protein), were cloned into the pET32a (+) vector with an NH_2-terminal polyhistidine tag and transformed into *Escherichia coli*. Transformed *E. coli* DH5α bacteria were cultured for 16 h at 37℃ and induced with 1.0 mM of isopropyl-β-d-

thiogalactopyranoside (Amresco, Cleveland, OH) for 5 h at 37℃. The bacteria were then harvested by centrifugation and disrupted by sonication on ice (Misonix, Farmingdale, NY). The supernatant was incubated with Ni-NTA agarose (Qiagen, Valencia, CA) for 1 h at room temperature, and the resin was washed with phosphate-buffered saline (PBS). Purified proteins were eluted, and their purity was confirmed on Coomassie blue-stained SDS-acrylamide gels.

Production of C. perfringens and Eimeria-specific Egg Yolk IgY

Laying hens (25 – 30 weeks of age, Brown Leghorn, Slonaker Farms, Harrisonburg, VA) were immunized with 50 – 100 µg of the purified recombinant *C. perfringens* or *Eimeria* antigens: (1) AgA (α-toxin antigen); (2) AgB (NetB antigen); (3) AgT (EFTu antigen); (4) AgP (PFO antigen); (5) AgM-1 (a mixture of AgA, AgB, AgT, and AgP); (6) AgM-2 (a mixture of AgB, AgT, EF1α antigen, and 3-1E antigen) by an intramuscular injection into the breast muscle. Freund's Complete Adjuvant (FCA) was used for the first injection, and Freund's Incomplete Adjuvant (FIA) was used for the boost injection. For the primary immunization, 0.5 ml was injected into each breast muscle (total of 1.0 ml injected), and for the boost immunization, 0.5 ml was injected into one breast muscle (total of 0.5 ml injected). The second immunization was administered 4 weeks after the first immunization, with subsequent boosts given every 4 weeks. Egg collection began 1 week after the first boost, and the antibody titers were monitored by enzyme-linked immunosorbent assay (ELISA) at regular intervals. After demonstrating of increasing specific antibody titers in the egg yolks, the eggs were collected, homogenized, and then spray-dried. The resultant egg powders were used as a source of protective antibodies and control egg powder was obtained from the non-immunized hens. The different egg powders produced include (1) EA (antibody against AgA); (2) EB (antibody against AgB); (3) ET

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(antibody against AgT); (4) EP (antibody against AgP); (5) EM-1 (antibody against AgM-1); (6) EM-2 (antibody against AgM-2); (7) EC (non-immunized control hens).

Experiment 1

Determination of IgY Levels in Egg Yolk and Egg Powder Egg samples collected from immunized and non-immunized hens at regular intervals were used to monitor the specific antibody levels. Total IgY was extracted from egg yolks using the Pierce™ Chicken IgY Purification Kit (Thermo Fisher Scientific, Waltham, MA). Briefly, 2 ml of egg yolk contents was mixed with 10 ml of delipidation reagent, and IgY was purified following the manufacturer's instructions. Spray-dried egg powder samples were reconstituted in sterile PBS at a concentration of 1 mg/ml and filtered through a 0.22 µm membrane filter. Specific IgY levels in the egg yolk or egg powder samples were measured by indirect ELISA. Flat-bottom, 96-well microplates (Corning® Costar®, Corning, NY) were coated with 10 µg/ml purified recombinant proteins in carbonate buffer (BupH Carbonate-Bicarbonate buffer packs, Thermo Scientific, Rockford, IL) and incubated overnight at 4°C. The plates were washed twice with PBS containing 0.05% Tween 20 (PBS-T) (Sigma-Aldrich, St. Louis, MO) and blocked with 100 µl of PBS containing 1% Bovine Serum Albumin (BSA), incubating for 1 h at room temperature. Diluted (in PBS with 0.1% BSA) 100 µl of IgY samples from either egg yolk or egg powder were then added to the plates with triplicate wells and incubated for 2 h at room temperature with constant shaking. As a blank control, PBS with 0.1% BSA was used. The plates were then washed with PBS-T and treated with peroxidase-conjugated rabbit anti-chicken IgY (IgG) (1:500; Sigma-Aldrich, St. Louis, MO), incubated for 30 min, followed by color development for 10 minutes with 0.01% tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO) in

0.05 M pH 5.0 Phosphate-Citrate buffer. Bound antibodies were detected by measuring the optical density at 450 nm (OD_{450}) using a microplate reader (Bio-Rad, Richmond, CA).

Chickens and Experiment Design Experiment 1 was approved by the Beltsville Agriculture Research Center Small Animal Care and Use Committee and the husbandry followed guidelines for the care and use of animals in agriculture research (FASS, 1999). A total of 120 one-day (d) old broiler chickens (Ross 708, Longenecker's Hatchery, Elizabethtown, PA) were obtained and housed in brooder units in an *Eimeria*-free facility for 2 weeks. The chickens were then transferred to finisher cages where they were infected and kept until the end of the experimental period. Feed and water were provided *ad libitum*. At 17 d of age, 120 chickens were randomly assigned to one of the 8 treatments (n = 15). Chickens in the control (NC) group were non-infected and given a non-supplemented basal diet. Chickens in the other treatments were experimentally coinfected with *Eimeria maxima* + *C. perfringens* to induce NE. The treatments consisted of a non-supplemented egg powder diet (EN), diet supplemented with EC, diets supplemented with five different immunized egg powders (EA, EB, ET, EP, and EM-1) at a 1% level. The experimental model used for NE induction included oral inoculation of chickens at 17 d of age with *E. maxima* strain 41A (1×10^4 oocysts/chicken) followed by oral administration with *C. perfringens* strain Del-1 (1×10^9 colony forming unit (cfu)/chicken) 4 days after *E. maxima* infection (d 21) (Park et al., 2008; Jang et al., 2013; Lee et al., 2013). To facilitate the development of NE, all the chickens were given an antibiotic-free starter diet containing a low level (18%) of crude protein from d 1 to 20 and then switched to a standard grower diet with high crude protein levels (24%) from d 21 to 28 (Table 1). All chickens were weighed individually on d 17 (inoculation day of *E. maxima*) and on d 28 (7 days post inoculation (dpi) of *C. perfringens* inoculation and 11 dpi of *E. maxima* inoculation) to calculate body weight gain

(BWG).

Jejunal Necrotic Enteritis Lesion Scores Three chickens per treatment group were randomly selected, euthanized, and approximately 20 cm intestinal segments extending 10 cm anterior and posterior from the Meckel's diverticulum were obtained on d 23 (2 dpi of *C. perfringens* inoculation). Intestinal sections were scored for NE lesions on a scale of 0 (none) to 4 (high) by three independent observers (Shojadoost et al., 2012).

Experiment 2

Chickens and Experiment Design Experiment 2 was approved by the Beltsville Agriculture Research Center Small Animal Care and Use Committee, and the husbandry followed guidelines for the care and use of animals in agriculture research (FASS, 1999). A total of 50 broiler chickens were randomly assigned to one of the five treatments (n = 10) at d 17. The treatments consisted of NC, EN, EC, EB, and ET. The procedures for the induction of NE and the experimental diets were the same as those described for Experiment 1. All chickens were weighed individually on d 17 (inoculation day of *E. maxima*) and d 28 (7 dpi of *C. perfringens* inoculation and 11 dpi of *E. maxima* inoculation) to calculate BWG.

Sandwich ELISAs for Determination of Serum α-toxin and NetB Levels On d 21, three blood samples per treatment were collected from the wing vein 6 h after *C. perfringens* inoculation. The sera were separated by centrifugation at 1,000 × *g* for 20 min to determine the levels of α-toxin and NetB by sandwich ELISA as previously described (Lee et al., 2013). Briefly, α-toxin and NetB monoclonal antibodies (mAbs) were coated onto 96-well microplates at a concentration of 5 μg/ml using carbonate buffer (BupH Carbonate-Bicarbonate buffer packs, Thermo Scientific, Rockford, IL) and incubated overnight at 4°C. The plates were washed and

blocked as described previously. Serum samples (100 µl) were added to the microplates, and the plates were incubated at 4℃ by overnight. Following incubation, the plates were washed and treated with 2 µg/ml unconjugated rabbit polyclonal antibody to α-toxin and NetB, incubated at room temperature for 30 min. After washing the plates for five times with PBS-T, 1 ml of a 1:10,000 dilution of anti-rabbit IgG horseradish peroxidase (HRP)-conjugated second detection antibody was added and incubated for 30 minutes. After incubation, the plates were washed and developed with 100 µl of TMB substrate (Sigma-Aldrich, St. Louis, MO) for 10 min, and followed by the addition of 2 N H_2SO_4 stop solution. The plates were read at OD_{450} using a microplate reader (Bio-Rad, Richmond, CA).

IgY-NetB Neutralization Assay The Leghorn male hepatocellular (LMH) cell cytotoxicity assay, as outlined by Keyburn et al. (2008), was used to assess the neutralizing activity of anti-NetB IgY against recombinant NetB protein. LMH cells (LMH, CRL-2117, ATCC, Manassas, VA) were added onto 96-well tissue culture plates (Corning) at a density of 5×10^3 cells in Waymouth's medium. The cells were pre-incubated for 24 h at 37℃ and 5% CO_2. IgY extracted from the egg yolks of control unimmunized hens (AgC) and IgY from hyperimmunized hens with AgB were incubated with recombinant NetB protein at a ratio of NetB:IgY = 1:20 for 1 h at room temperature. The pre-incubated IgY-NetB mixtures and NetB (390 pg) were added to the LMH cells in triplicate wells and incubated for 4 h at 37℃. The dehydrogenase activity in the viable cells was measured using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) and used to calculate LMH cell cytotoxicity.

C. perfringens Growth Inhibition Assay The efficacy of IgY from hens hyperimmunized with AgT in inhibiting the growth of *C. perfringens* in culture was investigated and the results were compared to those of the AgC group. The *C. perfringens* Del-1 strain was cultured anaerobically

in Brain Heart Infusion (BHI, Becton Dickinson, NJ) broth overnight at 37°C. Specific and non-specific egg yolk IgY solutions were sterilized by filtering through a 0.22 μm membrane filter. Five milliliters of each IgY solution were then added to an equal volume of *C. perfringens* culture (2.4×10^7 cfu/ml) and incubated in anaerobic conditions at 37°C. The final concentration of the IgY tested was 1 mg/ml. Samples (1 ml) were collected at 0, 2, 4, 6 and 24 h, and serial dilutions were plated on Perfringens agar plates (Thermo Scientific, Lenexa, KS) in triplicate. The inoculated plates were incubated at 37°C for 24 h and the colonies were counted to determine the cfu.

Experiment 3

Chickens and Experiment Design Experiment 3 was conducted at the Poultry Research Center, University of Georgia, following the approved protocol by the Institutional Animal Care and Use Committee (A2020 01-018). The animal husbandry followed the Cobb 2018 nutritional and management guidelines (Cobb, 2018). A total of 200 0-d-old Cobb 500 broiler chickens were obtained and raised in battery cages, with feed and water provided *ad libitum*. On d 7, the chickens were randomly assigned to 4 treatments with 5 replicates, and each replicate consisted of 10 chickens. The four treatments included NC, EN, EC, and EM-2, with EC and EM-2 were provided at the 1% level of diet. The experimental NE infection model used oral inoculation of chickens on d 14 with *E. maxima* strain 41A (7.5×10^3 oocysts/chicken) followed by oral administration of *C. perfringens* strain Del-1 (1×10^9 cfu/chicken) on d 18 (4 dpi). To facilitate the development of NE, all the chickens were fed with a starter diet containing 21% crude protein diet from d 0 to 17, and then switched to a 24% high crude protein diet from d 18 to 22 (Table 2). Individual body weight (BW), BWG, feed intake (FI), and feed conversion ratio (FCR)

were recorded for all chickens on d 7 and 22.

Intestinal Permeability Intestinal permeability was assessed on d 20 (6 dpi) using fluorescein isothiocyanate-dextran (FITC-d; Molecular weight 4,000; Sigma-Aldrich, Canada) following a modified version of previous experiments (Teng et al., 2020; Choi et al., 2022). In brief, a solution of FITC-d with a concentration of 2.2 mg/ml was prepared in PBS under dark condition. One chicken per cage was orally administered the FITC-d solution. Two hours after administration, the chickens were euthanized by CO_2 asphyxiation, and blood samples were collected. The collected blood samples were stored in a completely dark room for two hours and then centrifuged at 2,000 × *g* for 12 min to obtain serum. To determine the FITC-d level, a standard curve was generated by serial dilution of serum samples extracted from five non-experimental chickens. Subsequently, 100 µl of the serum samples were transferred to 96-well dark plates, and the fluorescence was measured at $OD_{485/525}$ using a Spectra Max 5 microplate reader (Molecular Devices, Sunnyvale, CA).

Jejunal Necrotic Enteritis Lesion Scores On d 20 (6 dpi), three chickens per cage were randomly selected and euthanized to collect approximately 30 cm intestinal segments extending 15 cm anterior and posterior from the Meckel's diverticulum. The intestinal segments were then examined for NE lesions using a blind method by two independent observers. The severity of the lesions was assessed on a scale ranging from 0 (no lesions) to 4 (severe lesions) as described in the previous study (Shojadoost et al., 2012).

Fecal Oocyst Counting To perform *E. maxima* oocyst counting, clean trays were placed under the cages on d 19 (1 d before sample collection). On d 20, approximately 100 g of fresh fecal samples were collected, homogenized, and stored at 4°C for further analysis. The oocyst counting was carried out following a previously described method (Choi et al., 2022) with slight

modifications. In brief, 5 g of feces was mixed with 30 ml of tap water and vigorously vortexed. After vortexing, 1 ml of the fecal sample was mixed with 10 ml of a saturated salt solution and vortexed again. Then, 650 µl of the feces mixture with the saturated salt solution was added to a McMaster chamber (Vetlab Supply, Palmetto Bay, FL). The oocysts were counted by three different individuals. Total number of *E. maxima* oocysts per gram of feces was expressed as $\log_{10}$.

Sandwich ELISAs for Determination of NetB and CNA in Jejunal Digesta On d 20, two jejunal digesta samples per treatment were collected and diluted with sterile PBS at a ratio of 1:10. The diluted digesta samples were then centrifuged at 2,000 × *g* for 10 min, and the supernatants were collected to determine the levels of NetB and CNA using sandwich ELISA. The sandwich ELISAs were performed following a method previously described by Goo et al. (2023) with slight modifications. In summary, NetB and CNA capture mAbs were coated onto 96-well microplates at a concentration of 5 µg/ml using carbonate buffer (BupH Carbonate-Bicarbonate buffer packs, Thermo Scientific, Rockford, IL) and incubated overnight at 4°C. The plates were washed twice with PBS-T and then blocked with blocking buffer (Superblock™ Blocking Buffer, Thermo Scientific, Rockford, IL). Next, diluted digesta samples (100 µl) were added to the microplates and incubated for 2 h. After the incubation, the plates were washed 6 times with PBS-T, and HRP-conjugated NetB and CNA detection mAbs, at a concentration of 0.33 µg/ml, were added and incubated at room temperature for 1 h. After washing the plates again for 6 times with PBS-T, 100 µl of TMB substrate (Sigma-Aldrich, St. Louis, MO) was added to the each well and incubated at room temperature for 5 min. The color development reaction was stopped by adding 50 µl of 2 M H_2SO_4 stop solution. The fluorescence values were then measured at OD_{450} using a microplate reader (Bio-Rad, Richmond, CA).

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Statistical analysis

The statistical analysis was conducted using SAS software (version 9.4, SAS Institute Inc., Cary, NC). The data were expressed as mean ± standard error of the mean (SEM) for each treatment. All experiments involving ELISA, cell neutralization, and *C. perfringens* growth inhibition assays were performed in triplicate. For data analysis, a one-way analysis of variance (ANOVA) was applied, and if the *P*-value was less than 0.05 ($P < 0.05$), indicating a significant difference, Tukey's honestly significant difference (HSD) test was used to determine the differences among treatments.

RESULTS

Experiment 1

Antibody Levels of Egg Yolk Antibodies and Spray-Dried Egg Powder from Hyperimmunized Hens The average antibody levels in the egg yolks of hyperimmunized hens are shown in Figure 1. The chicken egg yolks exhibited significantly higher antibody levels to the respective immunizing antigens compared to those from the unimmunized hens. The specific antibody levels of the spray-dried egg powder as determined by indirect ELISA, are shown in Figure 2. All the tested egg powders, including EA, EB, ET, and EP, demonstrated significantly higher antibody levels compared to that of the EC.

Body Weight Gain The result of dietary egg powder supplementation on BWG from d 17 to 28 is shown in Figure 3. The EN, EC, EA, and EP groups showed significantly decreased BWG compared to the NC group ($P < 0.001$). Dietary supplementation with EB, ET, and EM-1

significantly increased BWG compared to the EN and EC groups. The BWG of the EB, ET, and EM-1 groups did not show any statistical difference compared to the NC group.

Jejunal Necrotic Enteritis Lesion Score The result of dietary EP supplementation on NE lesion score on d 23 (6 dpi) is presented in Figure 4. All groups showed significantly increased NE lesion scores compared to the NC group ($P < 0.001$). The EB and ET groups showed significantly decreased NE lesion scores compared to the EN group. No significant differences were observed in NE lesion scores of EC, EA, EP, and EM-1 groups compared to the EN group.

Experiment 2

Body Weight Gain The result of BWG in Experiment 2 is shown in Figure 5. The BWG of chickens in the EB and ET groups was significantly higher compared to that of the EN and EC groups ($P < 0.01$). There were no significant differences in BWG between the EN and EC groups. Both the EN and EC groups showed significantly decreased BWG compared to the NC group.

Serum α-toxin and NetB Levels The results of serum α-toxin and NetB levels are shown in Figure 6. No significant levels of α-toxin and NetB were detected in the serum of the NC group. The levels of both α-toxin and NetB in the serum of chickens from the EB and ET groups were significantly lower compared to those of the EN group ($P < 0.01$). However, α-toxin and NetB were also shown to be significantly decreased in the EC group compared to the EN group.

In vitro NetB Neutralization and C. perfringens Inhibition Assay The result of *in vitro* NetB neutralization assay of egg yolk IgY against AgB is shown in Figure 7. NetB-specific hyperimmune IgY significantly neutralized the cytotoxic effect of NetB on LMH cells, reducing it from 66% (control group without IgY) to 12% ($P < 0.01$). The NC group did not exhibit any

neutralizing effect on NetB. The result of in vitro *C. perfringens* growth inhibition assay is shown in Fig. 8. Neither the NC group nor the egg yolk IgY against AgT demonstrated any inhibitory effect on the growth of *C. perfringens*.

Experiment 3

Growth Performance The growth performance results from d 7 to 22 are shown in Figure 9. The chickens in the EN and EC groups showed significantly decreased BW, BWG, and FI compared to the NC group ($P < 0.05$). The BW, BWG, and FI of chickens in the EM-2 group did not show differences compared to the NC group ($P < 0.05$). No statistical differences were observed in FCR throughout the experimental period.

Intestinal Permeability The result of intestinal permeability on d 20 (6 dpi) is shown in Figure 10. The chickens in the EN and EC groups showed significantly increased intestinal permeability compared to the NC group ($P < 0.05$). No significant differences in intestinal permeability were observed between the EN and EC groups. The chickens in EM-2 group did not exhibit differences in intestinal permeability compared to the NC group ($P < 0.05$).

Jejunal Necrotic Enteritis Lesion Scores The result of the NE lesion score on d 20 (6 dpi) is presented in Figure 11. The EN and EC groups exhibited a significant increase in NE lesion score compared to the NC group ($P < 0.01$). There were no significant differences in the NE lesion score between the EN and EC groups. The chickens in the EM-2 group showed a similar NE lesion score compared to the NC group ($P < 0.05$).

Fecal E. maxima Oocyst Counting The result of *E. maxima* oocyst counting on d 20 (6 dpi) is presented in Figure 12. No *E. maxima* oocysts were detected in the NC group, while all the NE-infected groups (EN, EC, and EM-2) showed a significant increase in *E. maxima* counts

compared to the NC group ($P < 0.001$). There were no significant differences observed among the NE-infected groups.

NetB and CNA Levels in Jejunal Digesta The results of the levels of NetB and CNA in jejunal digesta are presented in Figure 13. NetB and CNA were detected in very small amounts in all samples from the NC group. The levels of NetB in jejunal digesta on d 20 and 22 (6 and 8 dpi) were significantly higher in the EN group compared to the NC group, while no differences were found between the EM-2 and NC groups on d 20 and 22 ($P < 0.001$). The EN and EC groups showed significantly increased CNA levels in jejunal digesta on d 20 compared to the NC group ($P < 0.05$). The EM-2 group showed similar CNA levels compared to the NC group ($P < 0.05$). No significant difference in CNA levels was found in jejunal digesta on d 22.

DISCUSSION

Six different egg powders containing specific IgYs detecting immunodominant *C. perfringens* antigens were produced by hyperimmunizing layers with immunodominant antigens of pathogenic *C. perfringens* and/or *Eimeria* (AgA, AgB, AgT, AgP, AgM-1, and AgM-2). *C. perfringens* strains can be grouped into seven toxin types (A to G) based on the type of toxins (α-toxin, β-toxin, ε-toxin, ι-toxin, enterotoxin, and NetB) (Lee and Lillehoj, 2022). A zinc metalloenzyme phospholipase C sphingomyelinase, α-toxin, has been considered a major virulence factor in the pathogenesis of NE in chickens for more than 20 years (Van Immerseel et al., 2009), and α-toxin plays a role in host cell membrane damage. NetB, a pore-forming toxin, is a 33 kDa beta-barrel toxin that forms small or large pores in the cell membrane (Lee and Lillehoj, 2022). Keyburn et al. (2006) demonstrated that α-toxin is not essential for generating

NE pathogenesis and provided strong evidence to show that a novel pore-forming NetB protein is the major cause of NE pathogenesis (Keyburn et al., 2010). EFTu is a component of the prokaryotic mRNA translation apparatus that has a role in the elongation cycle of protein synthesis (Schirmer et al., 2002). PFO is a metabolic enzyme that catalyzes the conversion of pyruvate to acetyl-CoA and has been associated with most anaerobic bacteria including *C. perfringens* (Kulkarni et al., 2007; Lee et al., 2011).

The selection of these four proteins (α-toxin, NetB, EFTu, and PFO) of *C. perfringens* as immunizing antigens for hyperimmunization in this study is based on our previous finding that showed strong immunogenicity of these antigens in experimentally induced NE infection (Lee et al., 2011). Indeed, hyperimmunized IgY serum and spray-dried egg powders from hens injected with selected *C. perfringens* antigens showed high antibody levels in indirect ELISA. Therefore, we conducted a series of experiments to investigate the protective effects of passive immunization against an experimental NE model using these hyperimmunized IgY antibodies (Lee et al., 2011) in commercial broiler chickens.

In Experiment 1, dietary supplementation of young chickens with EB, ET, and EM-1 significantly increased BWG compared to the control groups (EN and EC). However, supplementation with EA and EP showed no significant differences compared to the groups treated with EN and EC. NE lesion scores were significantly reduced in both the EB and ET groups compared to the EN group, whereas the EM-1 group showed no difference in NE lesion scores. Several studies have been published on the effectiveness of recombinant vaccination with native or recombinant α-toxin in protecting chickens from NE challenge (Kulkarni et al., 2007; Zekarias et al., 2008; Valipouri et al., 2022). In our study, α-toxin in egg powder failed to protect chickens from NE challenge. Effective protection against NE following immunization with the

NetB protein has been well documented previously. Keyburn et al. (2013a) reported that vaccination with subcutaneous injection of recombinant NetB partially protected broiler chickens from a mild challenge with a virulent *C. perfringens* isolate. Similar results were reported by Fernandes et al. (2013), who showed that immunization with NetB toxoid increased serum antibody levels and provided partial protection against NE. Jang et al. (2012) showed that chickens vaccinated with recombinant NetB emulsified in ISA 71 VG adjuvant induced a significant level of protection against NE challenge, as demonstrated by increased BWG and reduced gut lesion scores. Furthermore, maternal immunization with NetB toxoid vaccine induced a strong serum IgY response and protected the progeny from subclinical NE (Keyburn et al., 2013b). Our results are consistent with these previously published studies that demonstrated the protective effects of NetB-induced protective immunity against NE. Our studies clearly showed that dietary treatment of young chickens with egg yolk IgYs detecting immunodominant antigens of *C. perfringens* protects from NE. The protective effect of EFTu and PFO immunization in conferring protection against NE was shown in our previous work (Jang et al., 2012), which demonstrated the effective intramuscular vaccination against NE using recombinant EFTu or PFO in ISA 71 VG adjuvant. Both EFTu and PFO vaccination reduced NE lesion scores in chickens following NE infection, but only PFO resulted in increased BWG. In the current study, however, dietary supplementation of EFTu IgY (ET group) increased BWG and decreased NE lesion scores compared to the EN group, but PFO IgY (EP group) showed no difference in both BWG and NE lesion scores compared to the control groups (EN and EC).

In Experiment 2, we confirmed the protective effects of EB and ET IgY antibodies. The experimental results showed that dietary supplementation with EB and ET IgYs significantly increased BWG compared to the EN and EC groups following NE challenge. Reduced serum

levels of both α-toxin and NetB were found in the NE-challenged chickens following dietary treatment with EB and ET IgYs in feed. Since EFTu is expressed intracellularly and appears on the bacterial cell surface, treatment with IgY against EFTu may reduce the adhesion of bacteria to intestinal epithelial cells (Severin et al., 2007; Lee et al., 2011). To understand the mechanism of action of *C. perfringens*-specific IgYs in protection against NE, we performed an *in vitro* toxin neutralization assay using anti-NetB IgY and *C. perfringens* growth inhibition assays using anti-EFTu IgY. As shown by the results of the toxin neutralization assay, the protective effect mediated by anti-NetB IgY can be demonstrated by its neutralization of NetB to reduce cytotoxicity in LMH cells. In the current study, we used the Del-1 strain, which expresses both α-toxin and NetB (Gu et al., 2019), and we speculate that anti-NetB IgY neutralized the biological activity of NetB, limiting its biological function (Gadde et al., 2015). This may also account for the reduced levels of NetB in the serum of chickens fed the diet supplemented with NetB IgY (EB group) following NE challenge. The reason for the reduced α-toxin level with NetB in the serum following EB IgY treatment is not clear, but EB IgY may be the reason for the reduced activity of *C. perfringens* by neutralizing the NetB antigen. An *In vitro* bacterial growth inhibition assay was also performed to investigate whether anti-EFTu IgY reduces *C. perfringens* growth; however, in the current experiment, the anti-*C. perfringens* activity of EFTu IgY was not demonstrable.

In Experiment 3, *C. perfringens*-specific NetB and EFTu IgYs and *Eimeria*-specific EF1α and 3-1E IgYs were combined and tested. EF1α, an evolutionarily conserved protein, commonly found in eukaryotic cells (Sasikumar et al., 2012), plays a key role in protein synthesis by mediating aminoacyl-tRNA loads in the A site of the 80S ribosome (Lin et al., 2017). In addition, EF-1α is an essential component of parasitic invasion, as it is associated with

the cytoskeleton of the apical region (Matsubayashi et al., 2013) and regulates assembly, cross-linking, and binding to actin filaments (Doyle et al., 2011). Another *Eimeria* immunodominant antigen, 3-1E, is expressed in the posterior cytoplasm of merozoites and sporozoites by *Eimeria* profilin, which has previously been used to induce protective immunity against coccidiosis through vaccination (Lillehoj et al., 2005; Lee et al., 2007). Therefore, the combination of these *C. perfringens* and *Eimeria*-specific recombinant antigens is expected to induce strong protective immunity against the NE infection model based on dual infection with *E. maxima* and *C. perfringens*. As a result, both control groups (EN and EC) exhibited a decrease in BWG and FI. However, the EM-2 group, which received treatment with a mixture of NetB, EFTu, EF1α, and 3-1E, did not show any reduction in BWG or FI compared to the NC group. This result was consistent throughout Experiments 1, 2, and 3 and indicates that chickens treated with egg powder containing NetB and EFTu IgY were not statistically different from the NC group. Additionally, both intestinal permeability and NE lesion scores showed that the EM-2 group was statistically similar to the NC group. The recurring results with reduced NE lesion scores in groups including EB are similar to those of several previous studies that showed recombinant NetB immunization reduced NE lesion scores in chickens infected with NE (Jang et al., 2012; Keyburn et al., 2013a, b; Shamshirgaran et al., 2022). To date, there are no reports that show dietary effects of IgY antibodies affecting intestinal permeability. However, several toxins in *C. perfringens* are known to increase intestinal permeability, especially α-toxin or enterotoxin, which damages the intestinal barrier and reduces the expression of claudin or occludin (Awad et al., 2017). In this study, the *C. perfringens*-specific IgY of the EM-2 group neutralized the activity of NetB, resulting in decreased intestinal permeability. Despite the dietary supplementation with anti-*Eimeria* IgY antibodies (EF1α and 3-1E) in the EM-2 group, the

number of *E. maxima* oocysts did not decrease in the NE-challenged group.

Similar to Experiment 2, the level of NetB in the jejunal digesta decreased in the EM-2-treated group, and CNA also decreased in Experiment 3. CNA is a bacterial cell wall-anchored protein and has the key ability to attach to the host cell wall (Arora et al., 2021). Collagen is one of the essential components of the extracellular matrix molecules, and for most pathogenic Gram-positive bacteria, attachment to the host cell wall with their specific bacterial adhesin is the key step for colonization (Krogfelt, 1991; Klemm et al., 2007; Martin and Smyth, 2010). Recently, CNA has been reported in some *C. perfringens* strains that were implicated in NE in chickens (Wade et al., 2015). In addition, it has been reported that a CNA-deleted *C. perfringens* strain does not cause NE lesions (Wade et al., 2016). In the current study, there was no significant difference in the levels of CNA and NetB, and the NE lesion scores of the EM-2 group compared to the NC group. These results support the protective effects of *C. perfringens*-specific IgY of the EM-2 group, which binds to NetB and/or EFTu antigens of *C. perfringens* in chicken intestines, and decreases the CNA level as we have previously shown a close correlation between CNA and NetB levels (Goo et al., 2023).

Unlike active immunity achieved by vaccination or exposure to pathogens, passive immunization relies on the transfer of humoral immunity in the form of preformed antibodies from one individual to another (Baxter, 2007). However, the protection given is short-lived and lasts only for a few weeks. Passive immunization with dietary hyperimmune IgY offers several advantages over passive immunity through maternal immunity since IgY levels will be maintained as long as chickens are fed with egg yolk IgYs. Maternally derived antibodies (from hen to chicken through embryonic circulation) protect chickens in the early stages of life, but their levels decrease within 1 to 2 weeks after hatching (Szabó, 2012). In contrast, high levels of

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protective antibodies can be maintained in the intestine with continuous feeding of hyperimmune egg yolk IgY in the diet by passive immunization (Lee et al., 2009b; Gadde et al., 2015). The main functions of egg yolk IgY, including inhibition of bacterial enzymes, blocking the attachment of pathogenic microorganisms, and toxin neutralization (Müller et al., 2015), can all be performed effectively in the intestinal environment. To enhance the stability of egg yolk IgYs in the intestine (Rahman et al., 2013; Mitragotri et al., 2014), encapsulation methods can be used to maximize IgY stability, which can increase the activity of IgY in the intestinal tract, further enhancing passive immunization (Xia et al., 2022). Pathogen-specific egg yolk IgYs have been successfully employed in the prevention and treatment of various enteric infections in swine (Marquardt et al., 1999; Kweon et al., 2000; Zuo et al., 2009) and cattle (Ikemori et al., 1997; Vega et al., 2011). However, studies on the development and use of hyperimmune egg yolk IgY to prevent NE in chickens are still insufficient.

In conclusion, dietary feeding with spray-dried egg powders from hens hyperimmunized with various *C. perfringens*-specific antigens significantly protected naïve chickens against NE based on enhanced growth performance, decreased NE lesion scores, and reduced intestinal damage. Based on these results, we conclude that passive immunization with hyperimmune egg yolk IgY shows great potential as an antibiotic-alternative strategy against NE infection in broiler chickens. Passive immunization using hyperimmune IgYs is an antibiotic-alternative strategy that can be effectively applied to mitigate NE infection in broiler chickens. Further studies are necessary to develop effective oral delivery strategies to maintain the stability of egg yolk IgYs in commercial applications.

Declaration of Competing Interest

There is no conflict of interest.

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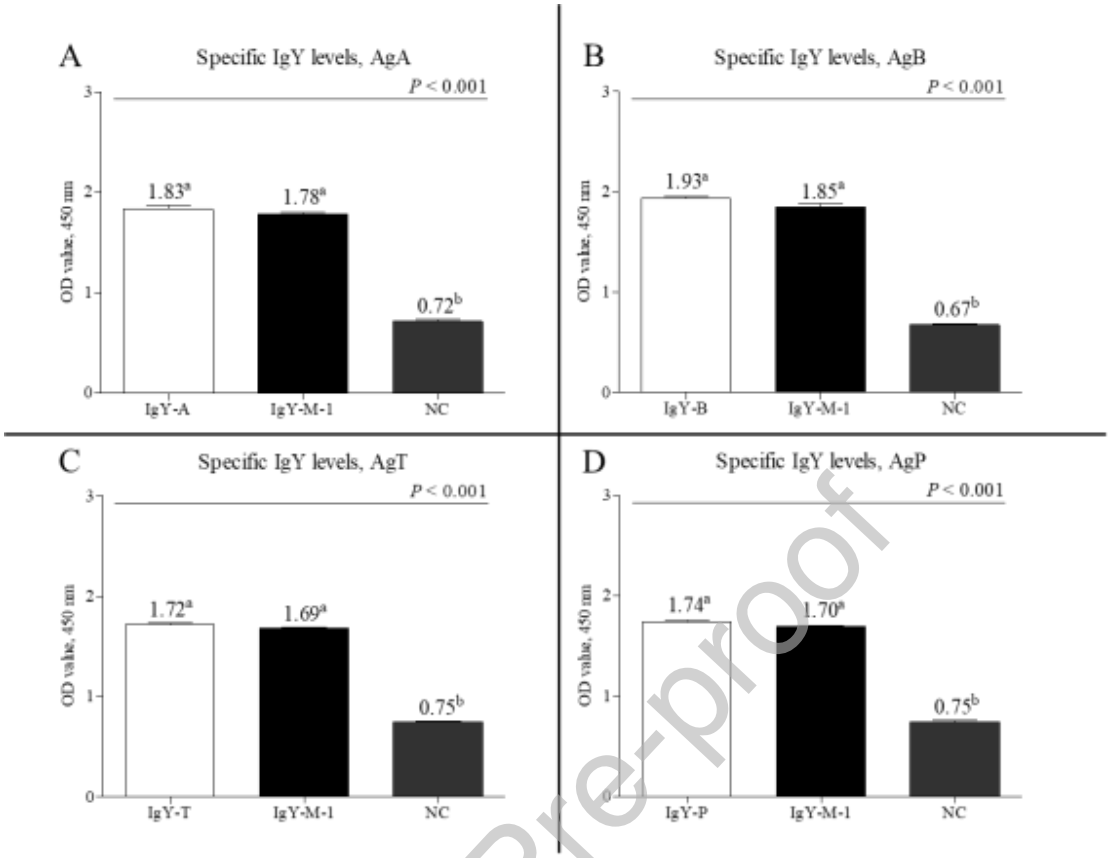

Figure 1. Specific IgY levels in the egg yolks collected from hens hyperimmunized with immunodominant antigens of *C. perfringens* in Experiment 1. Abbreviations: Ag, recombinant *C. perfringens* antigen; AgA, α-toxin antigen; AgB, necrotic enteritis B-like toxin (NetB) antigen; AgT, elongation factor Tu (EFTu) antigen; AgP, pyruvate : ferredoxin oxidoreductase (PFO) antigen; IgY-A, egg yolk IgY of AgA; IgY-B; egg yolk IgY of AgB; IgY-T, egg yolk IgY of AgT; IgY-P, egg yolk IgY of AgP; IgY-M-1, egg yolk IgY of the four Ag mixture; NC, unimmunized control egg yolk. A) IgY specificity test to AgA. B) IgY specificity test to AgB. C) IgY specificity test to AgT. D) IgY specificity test to AgP. Purified egg yolk mixtures were diluted to 10 μg/ml in carbonate buffer. [a-b] Treatment means with different letters are statistically different if $P < 0.05$. Standard error of means is represented by vertical bars (n = 3).

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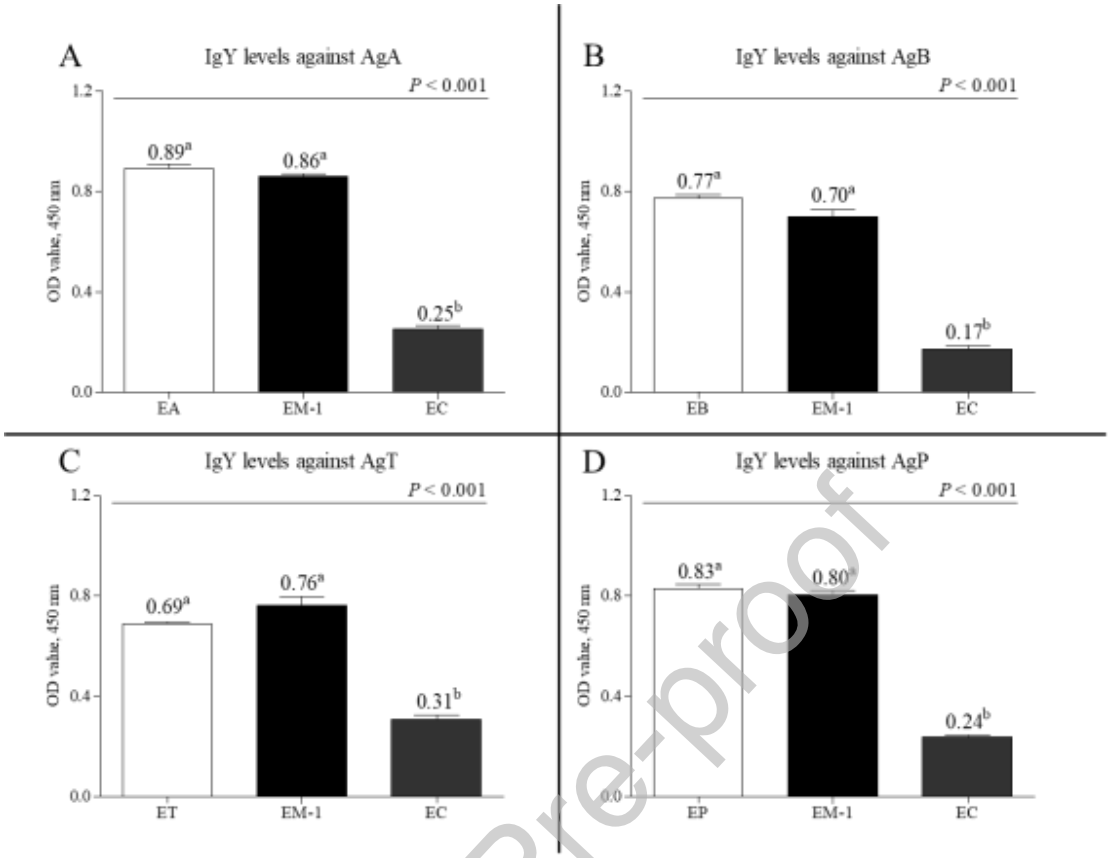

Figure 2. IgY levels in the spray-dried egg powders tested against immunodominant antigens of *C. perfringens* in Experiment 1. Abbreviations: Ag, recombinant *C. perfringens* antigen; AgA, α-toxin antigen; AgB, necrotic enteritis B-like toxin antigen; AgT, elongation factor Tu antigen; AgP, pyruvate : ferredoxin oxidoreductase antigen; EA, egg powder with antibody against AgA; EB, egg powder with antibody against AgB; ET, egg powder with antibody against AgT; EP, egg powder with antibody against AgP; EM-1, egg powder with antibody against AgM-1; EC, unimmunized control egg powder. A) IgY specificity test to AgA. B) IgY specificity test to AgB. C) IgY specificity test to AgT. D) IgY specificity test to AgP. Spray-dried egg powders were reconstituted in sterile PBS and diluted to 10 μg/ml in carbonate buffer. [a-b] Treatment means with different letters are statistically different if $P < 0.05$. Standard error of means is represented by vertical bars (n = 3).

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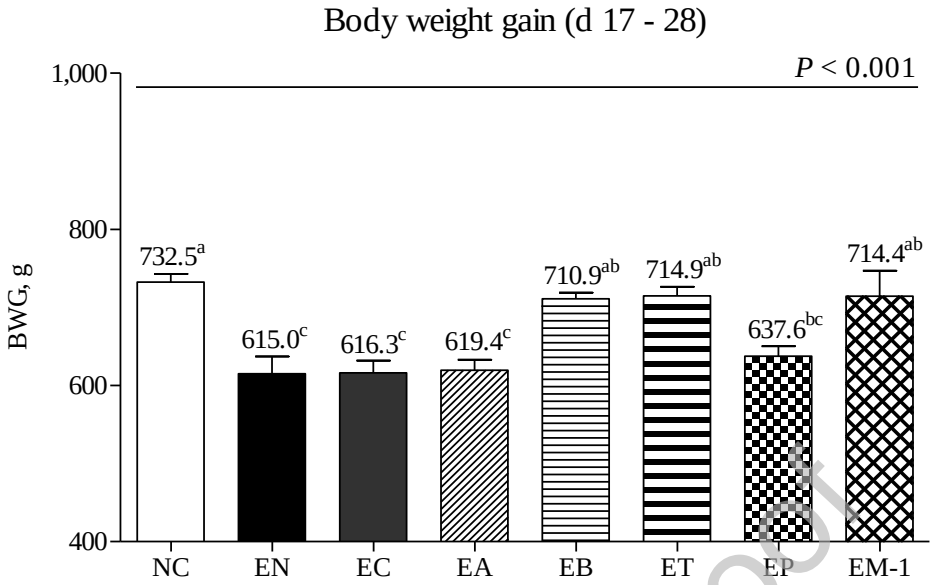

Figure 3. The effect of dietary supplementation of spray-dried egg powder IgYs on body weight gain (BWG) in necrotic enteritis (NE)-afflicted broiler chickens in Experiment 1. Abbreviations: NC, non-challenged control; EN, NE challenged control; EC, unimmunized control egg powder with NE challenge; EA, egg powder with antibody against α-toxin with NE challenge; EB, egg powder with antibody against NE B-like toxin (NetB) with NE challenge; ET, egg powder with antibody against elongation factor Tu (EFTu) with NE challenge; EP, egg powder with antibody against pyruvate : ferredoxin oxidoreductase (PFO) with NE challenge; EM-1, egg powder with antibody against mixed 4 antigens (α-toxin, NetB, EFTu, and PFO) with NE challenge. The feed contained 1% egg powder supplementation. At d 17, chickens in the NE challenged groups were orally inoculated with 1×10^4 sporulated oocysts of *E. maxima* followed by oral inoculation with 1×10^9 cfu of *C. perfringens* at d 21. [a-c] Treatment means with different letters are statistically different if $P < 0.05$. Standard error of means is represented by vertical bars (n = 12).

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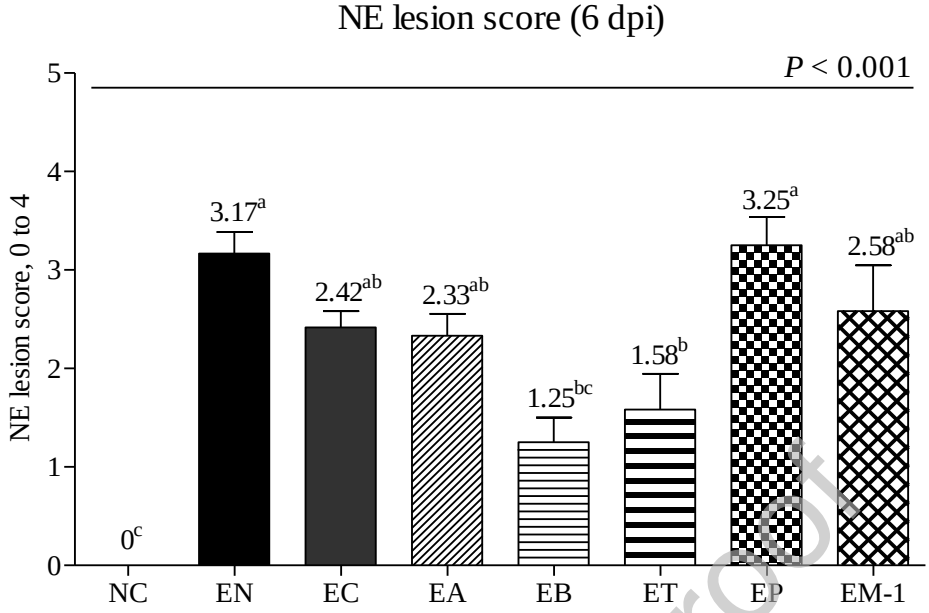

Figure 4. The effect of dietary supplementation with spray-dried egg powder IgYs on the intestinal lesion score of necrotic enteritis (NE)-afflicted broiler chickens in Experiment 1. Abbreviations: NC, non-challenged control; EN, NE challenged control; EC, unimmunized control egg powder with NE challenge; EA, egg powder with antibody against α-toxin with NE challenge; EB, egg powder with antibody against NE B-like toxin (NetB) with NE challenge; ET, egg powder with antibody against elongation factor Tu (EFTu) with NE challenge; EP, egg powder with antibody against pyruvate : ferredoxin oxidoreductase (PFO) with NE challenge; EM-1, egg powder with antibodies against four immunodominant antigens (α-toxin, NetB, EFTu, and PFO) with NE challenge. The feed contained 1% of egg powder antibodies against 4 different antigens. Jejunal sections were collected on d 23 (6 dpi of *E. maxima* inoculation and 2 dpi of *C. perfringens* inoculation) and scored for NE lesions on a scale of 0 (none) to 4 (high). [a-c] Treatment means with different letters are statistically different if $P < 0.05$. Standard error of means is represented by vertical bars (n = 3).

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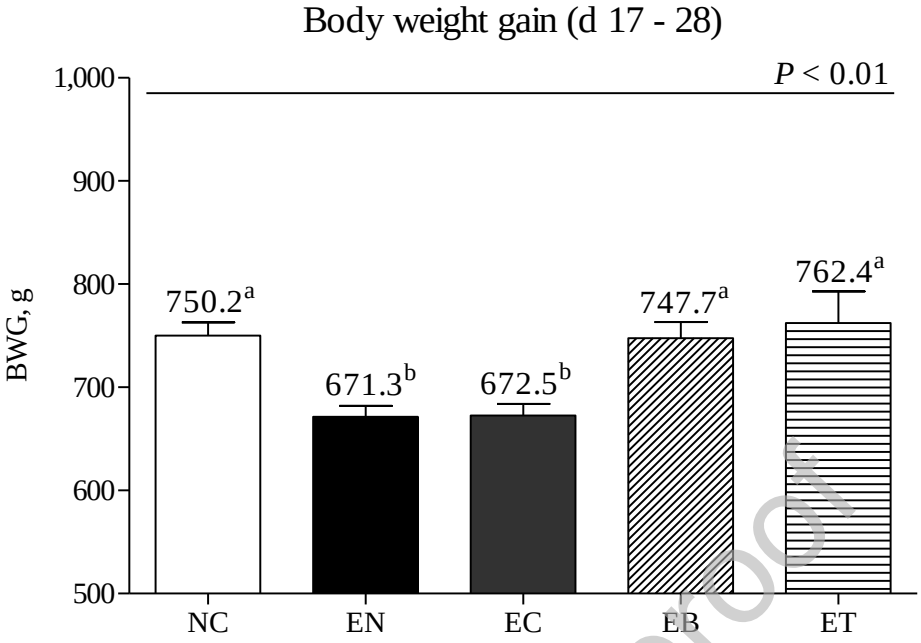

Figure 5. The effect of dietary supplementation with spray-dried egg powder IgYs on the body weight gain (BWG) of necrotic enteritis (NE)-afflicted broiler chickens in Experiment 2. Abbreviations: NC, non-challenged control; EN, NE challenged control; EC, unimmunized control egg powder with NE challenge; EB, egg powder with antibody against NE B-like toxin (NetB) with NE challenge; ET, egg powder with antibody against elongation factor Tu (EFTu) with NE challenge; Each egg powder was supplemented to the feed by 1 %. On d 17, chickens in the NE challenged groups were orally inoculated with 1×10^4 sporulated oocysts of *E. maxima* followed by oral inoculation with 1×10^9 cfu of *C. perfringens* on d 21. [a-b] Treatment means with different letters are statistically different if $P < 0.05$. Standard error of means is represented by vertical bars (n = 10).

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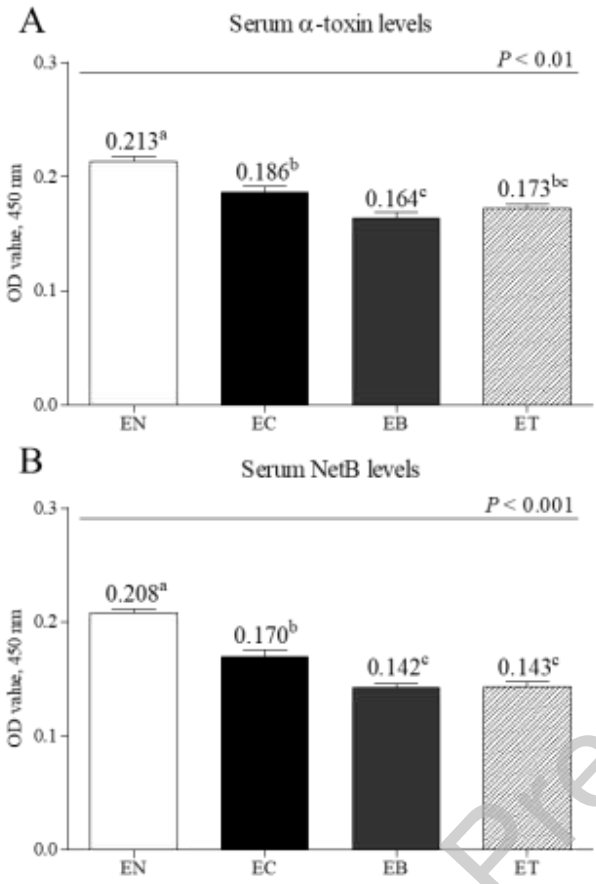

Figure 6. The effect of dietary supplementation with spray-dried egg powder IgY on serum α-toxin and necrotic enteritis B-like toxin (NetB) levels in Experiment 2. Abbreviations: EN, NE challenged control; EC, unimmunized control egg powder with NE challenge; EB, egg powder with antibody against NE B-like toxin (NetB) with NE challenge; ET, egg powder with antibody against elongation factor Tu (EFTu) with NE challenge; The feed contained 1% egg powder. Serum samples were collected at 6 h after *C. perfringens* infection on d 21 and used to measure toxin levels by sandwich ELISA. α-toxin and NetB were not detected in the non-challenged group (data not shown). A) serum α-toxin level test by sandwich ELISA. B) serum NetB level test by sandwich ELISA. $^{A-C}$ Treatment means with different letters are statistically different if $P < 0.05$. Standard error of means is represented by vertical bars (n = 3).

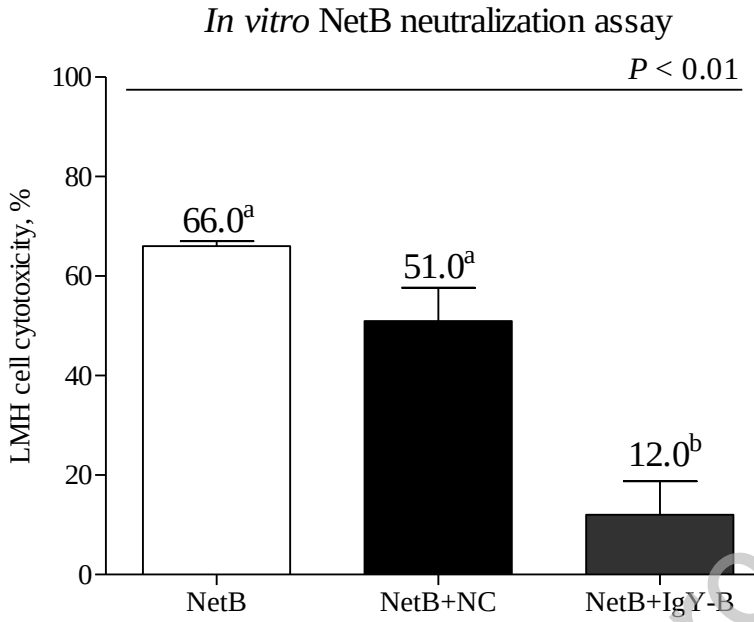

Figure 7. *In vitro* necrotic enteritis B-like toxin (NetB) neutralization assay of egg yolk IgY in Experiment 2. Abbreviations: NC, unimmunized control egg yolk; IgY-B, egg yolk IgY of NetB antigen. NC and IgY-B samples were incubated with recombinant NetB (NetB + NC or IgY-B) for 1 h at room temperature. The IgY mixtures were then added to the Leghorn male hepatocellular (LMH) cells in triplicates in a 96-well plate and incubated for 4 h at 37℃ and 5% CO_2. The LMH cell cytotoxicity (%) was measured using the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Rockville, MD). [a-b] Treatment means with different letters are statistically different if $P < 0.05$. Standard error of means is represented by vertical bars (n = 3).

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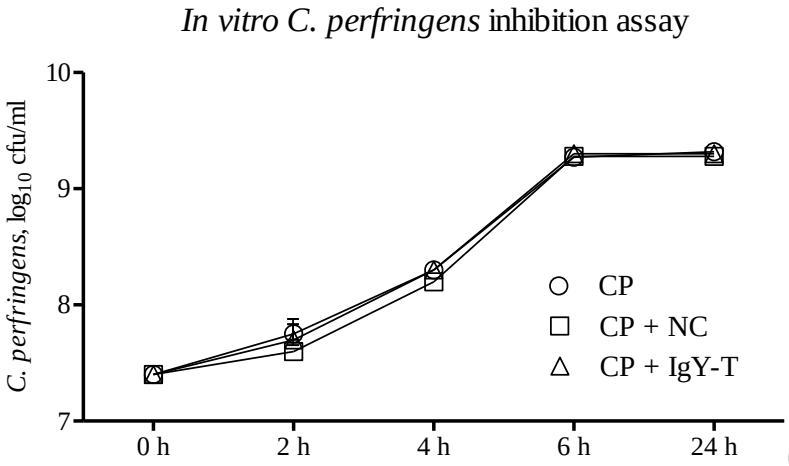

Figure 8. The growth curve of the *in vitro C. perfringens* inhibition assay of egg yolk IgY in Experiment 2. Abbreviations: CP, *C. perfringens*; NC, unimmunized control egg yolk; IgY-T, egg yolk IgY of immunized with elongation factor Tu (EFTu) antigen. Five ml of each NC and IgY-T sample were added to an equal volume of *C. perfringens* culture media (2.4×10^7 cfu/ml) and incubated under anaerobic conditions at 37°C. Samples (1 ml) were taken at 0, 2, 4, 6 and 24 h and dilutions were plated on *Perfringens* agar plates (Thermo Scientific, Lenexa, KS) in duplicates. The plates were then incubated at 37°C for 24 h and the colonies were counted to determine the cfu. No significant difference was observed between the treatments throughout the entire *C. perfringens* inhibition assay. Standard error of means is represented by vertical bars (n = 3).

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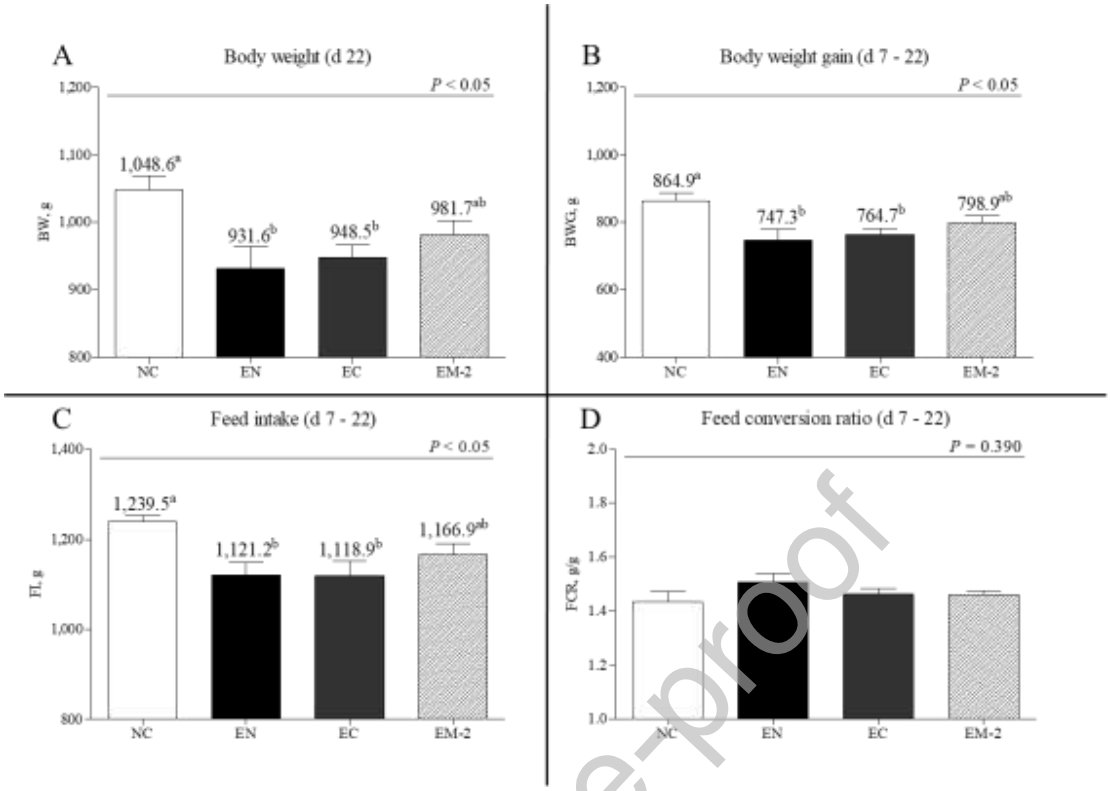

Figure 9. Effects of dietary supplementation with spray-dried egg powder IgY on the growth performance of necrotic enteritis (NE)-afflicted broiler chickens from d 7 to 22 in Experiment 3. Abbreviations: NC, non-challenged control; EN, NE challenged control; EC, unimmunized control egg powder with NE challenge; EM-2 egg powder with antibody against four combined *C. perfringens* and *Eimeria* antigens (NE B-like toxin, elongation factor Tu, elongation factor 1 alpha, and 3-1E) with NE challenge. Each egg powder was supplemented to the feed by 1%. At d 14, chickens in the NE challenged groups were orally inoculated with 7.5×10^3 sporulated oocysts of *E. maxima* followed by oral administration of 1×10^9 cfu of *C. perfringens* at d 18. A) final body weight at d 22 in Experiment 3. B) body weight gain from d 7 to 22 in Experiment 3. C) feed intake from d 7 to 22 in Experiment 3. D) feed conversion ratio from d 7 to 22 in Experiment 3. [a-b] Treatment means with different letters are statistically different if $P < 0.05$. Standard error of means is represented by vertical bars (n = 5).

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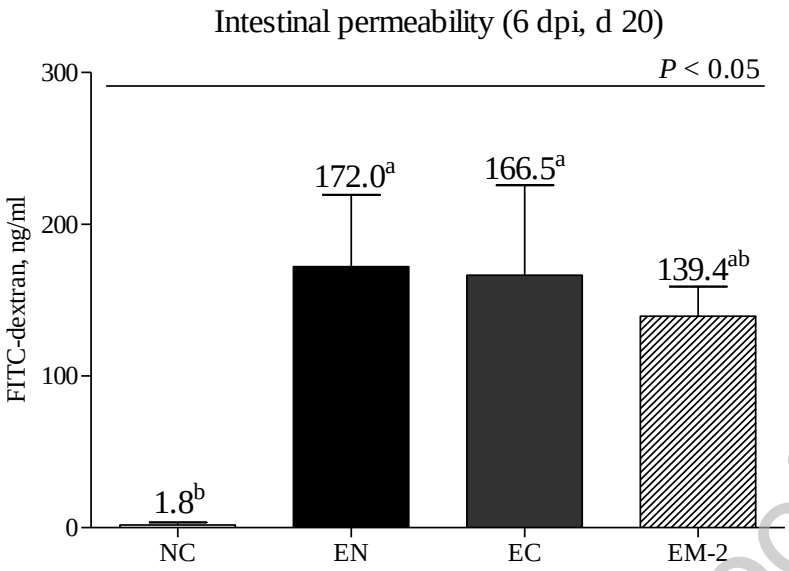

Figure 10. Effects of dietary supplementation with spray-dried egg powder IgY on the intestinal permeability of necrotic enteritis (NE)-afflicted broiler chickens on d 20 (6 dpi) in Experiment 3. Abbreviations: NC, non-challenged control; EN, NE challenged control; EC, unimmunized control egg powder with NE challenge; EM-2 egg powder with antibody against four combined 4 *C. perfringens* and *Eimeria* antigens (NE B-like toxin, elongation factor Tu, elongation factor 1 alpha, and 3-1E) with NE challenge. Each egg powder was supplemented to the feed by 1%. On d 20, two hours after the inoculation of fluorescein isothiocyanate-dextran solution, serum samples were collected, and fluorescence was measured at $OD_{485/525}$ using a Spectra Max 5 microplate reader (Molecular Devices, Sunnyvale, CA). [a-b] Treatment means with different letters are statistically different if $P < 0.05$. Standard error of means is represented by vertical bars (n = 5).

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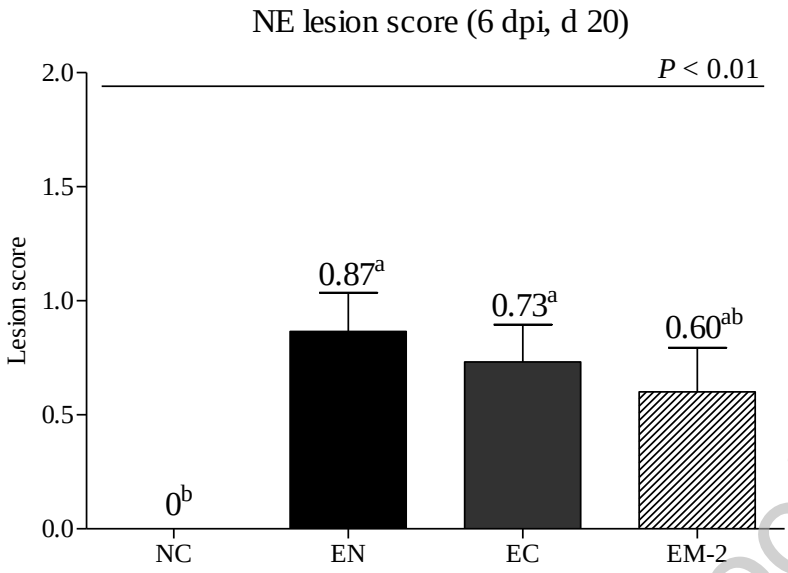

Figure 11. Effects of dietary supplementation with spray-dried egg powder IgY on the jejunal necrotic enteritis (NE) lesion score of NE-afflicted broiler chickens on d 20 (6 dpi) in Experiment 3. Abbreviations: NC, non-challenged control; EN, NE challenged control; EC, unimmunized control egg powder with NE challenge; EM-2 egg powder with antibody against four combined *C. perfringens* and *Eimeria* antigens (NE B-like toxin, elongation factor Tu, elongation factor 1 alpha, and 3-1E) with NE challenge. Each egg powder was supplemented to the feed by 1%. Jejunal sections were collected on d 20 (6 dpi of *E. maxima* inoculation and 2 dpi of *C. perfringens* inoculation) and scored for NE lesions on a scale of 0 (none) to 4 (high) using a blind method by 2 independent observers. [a-b] Treatment means with different letters are statistically different if $P < 0.05$. Standard error of means is represented by vertical bars (n = 5).

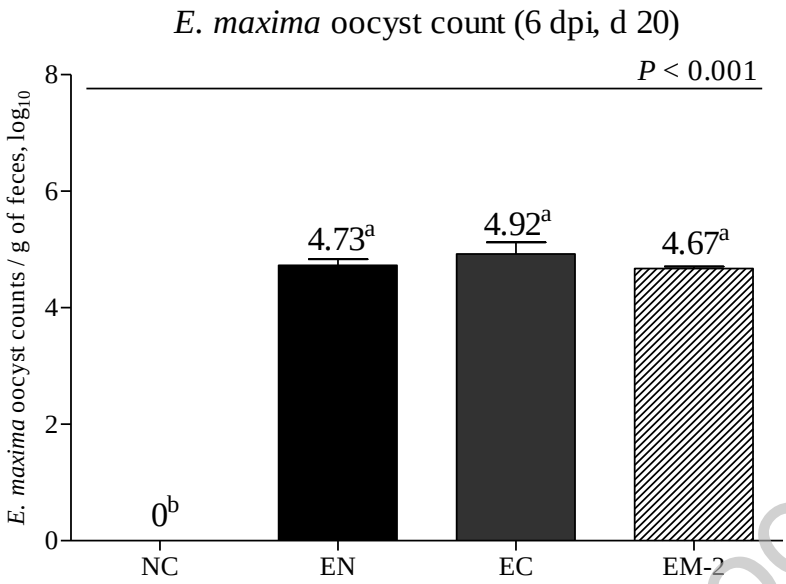

Figure 12. Effects of dietary supplementation with spray-dried egg powder IgY on the *E. maxima* oocyst count of necrotic enteritis (NE)-afflicted broiler chickens on d 20 (6 dpi) in Experiment 3. Abbreviations: NC, non-challenged control; EN, NE challenged control; EC, unimmunized control egg powder with NE challenge; EM-2 egg powder with IgY antibodies against four combined *C. perfringens* and *Eimeria* antigens (NE B-like toxin, elongation factor Tu, elongation factor 1 alpha, and 3-1E) with NE challenge. Each egg powder was supplemented to the feed by 1%. On d 20, approximately 100 g of fecal samples were collected from mixed fresh feces and the live *E. maxima* oocysts were counted using a McMaster chamber (Vetlab Supply, Palmetto Bay, FL). The total *E. maxima* oocysts per gram of feces were expressed as $\log_{10}$. [a-b] Treatment means with different letters are statistically different if $P < 0.05$. Standard error of means is represented by vertical bars (n = 5).

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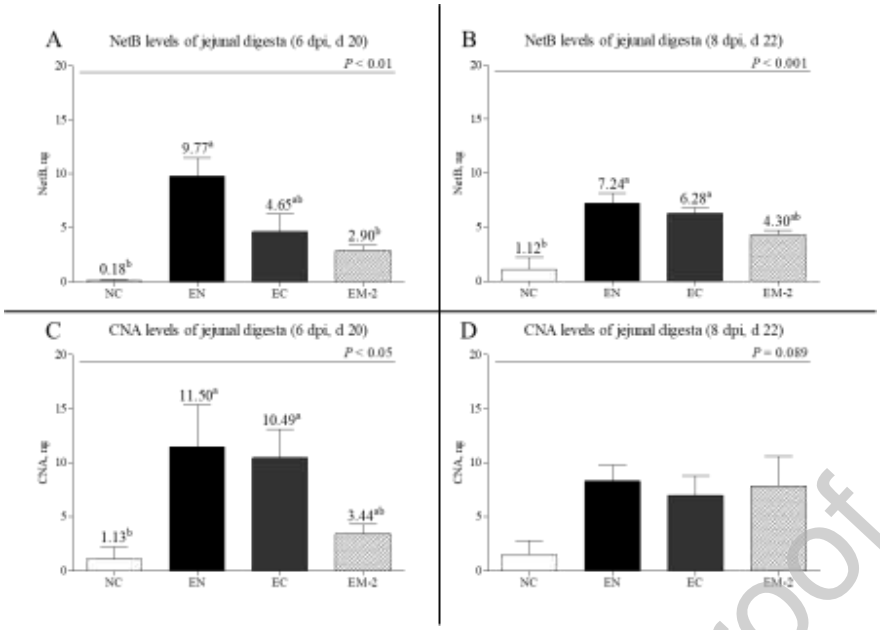

Figure 13. Effects of dietary supplementation with spray-dried egg powder IgY on necrotic enteritis B-like toxin (NetB) and collagen adhesin protein (CNA) levels in jejunal digesta of necrotic enteritis (NE)-afflicted broiler chickens on d 20 and 22 (6 and 8 dpi) by sandwich ELISA in Experiment 3. Abbreviations: NC, non-challenged control; EN, NE challenged control; EC, unimmunized control egg powder with NE challenge; EM-2 egg powder with antibodies against four combined *C. perfringens* and *Eimeria* antigens (NetB, elongation factor Tu, elongation factor 1 alpha, and 3-1E) with NE challenge. Each egg powder was supplemented to the feed by 1%. Jejunal digesta samples were collected on d 20 and 22 (6 and 8 dpi) and used to measure NetB and CNA levels by sandwich ELISA. A) NetB levels of jejunal digesta at 6 dpi by sandwich ELISA. B) NetB levels of jejunal digesta at 8 dpi by sandwich ELISA. C) CNA levels of jejunal digesta at 6 dpi by sandwich ELISA. B) CNA levels of jejunal digesta at 8 dpi by sandwich ELISA. $^{a-b}$ Treatment means with different letters are statistically different if $P < 0.05$. Standard error of means is represented by vertical bars (n = 5).

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Table 1. Ingredient compositions of basal diets of Experiments 1 and 2 (as-fed basis, %)

Ingredients, %	Low protein diet, d 1 to 20	High protein diet, d 21 to 28
Corn	69.01	55.78
Soybean meal	23.99	37.03
Soybean oil	2.75	2.97
Dicalcium phosphate	2.00	1.80
Calcium carbonate	1.40	1.51
Common salt	0.35	0.38
Vitamin mixture[1]	0.20	0.22
Mineral mixture[2]	0.15	0.15
DL-Met	0.10	0.10
60% Choline chloride	0.05	0.06
Total	100.0	100.0
Calculated values, %		
Crude protein	18.00	24.00
Calcium	1.19	1.20
Available phosphorus	0.54	0.51
Lys	1.00	1.40
TSAA	0.65	0.80
ME, Mcal/kg	3.6	3.5

[1]Vitamin mixture provided the following nutrients in kg of diet: vitamin A, 2,000 IU; vitamin D_3, 22 IU; vitamin E, 16 mg; vitamin K, 100 μg; vitamin B_1, 3.4 mg; vitamin B_2, 1.8 mg; vitamin B_3, 23.8 mg; vitamin B_5, 8.7 mg; vitamin B_6, 6.4 mg; vitamin B_7, 170 μg; vitamin B_9, 800 μg; vitamin B_{12}, 13 μg.

[2]Mineral mixture provided the following nutrients in kg of diet: Fe, 400 mg; Zn, 220 mg; Mn, 180 mg; Cu, 21 mg; Co, 1.3 mg; Se, 0.2 mg.

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Table 2. Ingredient compositions of basal diets of Experiment 3 (as-fed basis, %)

Ingredients, %	Low protein diet, d 0 to 17	High protein diet, d 18 to 22
Corn, grain	58.58	53.61
Soybean meal – 46%	31.72	39.61
Soybean oil	2.76	3.69
Sand	2.00	0.00
Dicalcium phosphate	1.69	1.24
Salt	1.38	0.35
Limestone	1.16	0.99
L-Lys HCl	0.28	0.00
DL-Met	0.15	0.08
Thr	0.15	0.00
Mineral premix[1]	0.08	0.08
Vitamin premix[2]	0.05	0.05
Titanium dioxide	0.00	0.30
Total	100.0	100.0
Calculated values, %		
Crude protein	21.00	24.00
Calcium	0.90	0.76
Available phosphorus	0.45	0.38
Lys	1.20	1.20
TSAA	0.85	0.85
ME, Mcal/kg	3.0	3.1

[1] Mineral premix provided the following per kg of diet: Mn, 100.5 mg; Zn, 80.3 mg; Ca, 24 mg; Mg, 20.1 mg; Fe, 19.7 mg; Cu, 3 mg; I, 0.75 mg; Se, 0.30 mg.

[2] Vitamin premix provided the following per kg of diet: vitamin A, 3,527 IU; vitamin D_3, 1,400 IU; vitamin E, 19.4 IU; niacin, 20.28 mg; D-pantothenic acid, 5.47 mg; riboflavin, 3.53 mg; vitamin B_6, 1.46 mg; menadione, 1.10 mg; thiamin, 0.97 mg; folic acid, 0.57 mg; biotin, 0.08 mg; vitamin B_{12}, 0.01 mg.

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