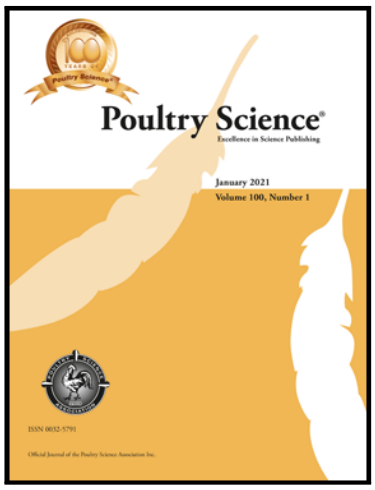

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Intestinal changes and immune responses during *Clostridium perfringens*-induced necrotic enteritis in broiler chickens

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ABSTRACT

Clostridium perfringens-induced necrotic enteritis (NE) is an economically important disease of broiler chickens. The present study evaluated the effect of *C. perfringens* on the intestinal histomorphometry, enteric microbial colonization and host immune responses using three experimental NE reproduction methods. The experimental groups consisted of 1) unchallenged Control diet (corn-soybean meal), 2) Control diet + *Eimera* inoculation at d 11 followed by *C. perfringens* challenge at d 15 (ECp), 3) Wheat-based diet + *C. perfringens* challenge (WCp), and 4) Wheat-based diet + *Eimeria* inoculation followed by *C. perfringens* challenge (WECp). The results showed that chickens receiving ECp and WECp had reduced ($P< 0.05$) bird performance coupled with enteric gross lesions and epithelial damage at d 17 and 24 of age compared to unchallenged control birds. These ECp and WECp administered birds also had increased ($P< 0.05$) ileal colonization by clostridia and *E. coli* at d 17 and 24, while the resident *Lactobacillus* counts were reduced ($P<0.05$) at d 24 of age. Furthermore, at d 24, jejunal transcription of IL-6, IL-10, annexin-A1 and IL-2 genes was upregulated ($P< 0.05$) in the ECp group, whereas the transcription of TNF receptor associated factor (TRAF)-3 gene was increased ($P<0.05$) in WECp treated birds when compared to unchallenged control group. Additionally, stimulation of chicken splenocytes and cecal tonsilocytes with virulent *C. perfringens* bacilli or their secretory proteins resulted in a higher ($P< 0.05$) frequency of T cells and their upregulation of MHC-II molecule, as determined by flow cytometry. These findings suggest that *C. perfringens*, while inducing epithelial damage and changes in microbiota, can also trigger host immune responses. Furthermore, NE reproduction methods using coccidia with or without the wheat-based dietary predisposition seem to facilitate an optimal NE reproduction in broiler chickens and thus, may provide better avenues for future *C. perfringens* research.

Key words: Necrotic enteritis, *Clostridium perfringens*, Broiler chicken, Histomorphometry, Immune response

INTRODUCTION

Necrotic enteritis (NE), caused by *Clostridium perfringens* type G bacteria, is one of the most economically important diseases of chickens affecting poultry worldwide. The global annual losses due to NE are estimated around $6 billion (Wade, 2015). *C. perfringens* uses many virulence strategies including metabolic enzymes or adhesion molecules and importantly, tissue-degrading toxins such as NetB and possibly, TpeL (Prescott et al., 2016a). We have previously shown that antibodies to toxins and certain metabolic enzymes and proteins are important in NE immunity and hence, these proteins may also have a role in NE pathogenesis (Kulkarni et al., 2006; Kulkarni et al., 2007). Our recent work has shown that some of the *netB+ C. perfringens* isolates carrying *tpeL* possess enhanced virulence ability in chickens (unpublished data), which is agreement with other reports (Coursodon et al., 2012; Gu et al., 2019). The pathogenesis of NE is complex and NetB has been shown to be critical virulence factor. Recent reports also suggest that there are NE-causing unique strains that possess certain signature NE-associated virulence gene(s) that are absent in commensal avirulent non-NE isolates of *C. perfringens* (Lepp et al., 2010; Prescott et al., 2016a).

Development of NE is often predisposed by factors such as high dietary protein, soluble non-starch polysaccharides (NSPs) or wheat-based diets that induce dysbiosis or enteric infections, primarily coccidiosis (Moore, 2016). These predisposing factors have been effectively utilized over the years in the experimental reproduction of NE to investigate NE pathogenesis, immunity or prevention (Lee et al., 2011; Shojadoost et al., 2012). In this context, many studies have utilized various experimental NE models involving intestinal predisposition induced by coccidial infection and /or use of wheat-based or fish meal containing diets to study the effects of *C. perfringens* on the intestinal damage, gut microbial colonization or host immunity (Lee et al., 2016; Wu et al., 2014). However, the results from these studies have depended on the type of model used, specifically, the method of induction of intestinal predisposition and the virulence of

the *C. perfringens* strain used in the challenge. Additionally, immunological understanding of intestinal tissue responses or immune cell responses against virulent *C. perfringens* are not fully understood.

Here, we used *netB*+ and *tpeL*+ virulent *C. perfringens* strains to infect broilers in three NE models employing different predisposing factors that included birds fed with wheat-based diet inoculated with *C. perfringens* (WCp), a conventional diet co-inoculated with *Eimeria* and *C. perfringens* (ECp), or those fed with a wheat-based diet and co-inoculated with *Eimeria* and *C. perfringens* (WECp). Using these models, we evaluated the effects of *C. perfringens* on the intestinal histomorphology, colonization of resident *Lactobacillus* commensals and important enteric pathogens and immune responses against *C. perfringens*.

MATERIALS AND METHODS

All animal experiments were approved by the Institutional Animal Care and Use Committee (Ferdowsi University of Mashhad, Protocol# 3/42449; North Carolina State University, Protocol# 19-077-A) and performed in accordance with the guidelines and regulations.

Birds, Diets and Management

A total of 360 one-d-old male Cobb 500 chicks were obtained from a local commercial hatchery, randomly housed in disinfected fresh floor pens covered with wood shavings and reared until 10 d of age based on Cobb 500 guideline and specifications. On d 11, birds were weighed and randomly assigned to 4 experimental groups of 6 replicates with 15 birds per replica. The experimental groups were as follows: 1) a corn-soybean meal-based diet as control (Ctrl); 2) Ctrl + orally challenged birds with *Eimeria* and *C. perfringens* (ECp); 3) Wheat-based diet (W) + orally challenged birds with *C. perfringens* (WCp); 4) W + orally challenged birds with *Eimeria* and *C. perfringens* (WECp). All diets contained no antibiotic growth promotants, were in mash form, and formulated to be isonitrogenous and isoenergetic meeting the requirements of Cobb

500 (Table 1). Birds were monitored daily for any mortality as well as clinical signs during NE challenge period.

C. perfringens strains and experimental Challenge

Birds were challenged with *Eimeria spp.* as described previously (Wu et al., 2014) with some modification. Briefly, on d 11, chickens in the ECp and WECp groups were gavaged with 0.2 mL of coccidial vaccine solution (1 mL LIVACOX®T = 100 doses; Biopharm Co., Prague, Czech Republic), while birds in Ctrl group received 0.2 mL oral dose of sterile PBS. As per the manufacturer's claim, one dose of the vaccine (0.01 mL) contained 300 sporulated oocysts each of *E. acervulina*, *E. maxima* and *E. tenella*. On d 15, birds in WCp and WECp groups were inoculated via oral gavage with 2×10^8 cfu in 0.5 mL of *C. perfringens* type G isolate JRTK44, a *netB*+ *tpeL*+ clinical isolate recovered from a broiler chicken presented with a case of necrotic enteritis. While the NE challenge experiments were carried out at the Ferdowsi University of Mashhad, Iran, the *in vitro* immunology experiments were performed at the North Carolina State University, United States. Hence, in the *in vitro* work, a different C. perfringens isolate (CP26) that also carried *netB* and *tpeL* genes and was tested to be virulent in chickens (unpublished data) was used to assess T cell responses. The CP26 isolate was kindly provided by Dr. John F Prescott, University of Guelph, Canada for use in this study.

Growth Performance, Sampling and NE lesion scoring

Body weight and feed intake of birds in each of the pens were recorded on d 17 and 24 to calculate the average daily gain (ADG), ADFI and FCR. Mortality per pen, if any, was recorded daily and FCR was adjusted accordingly. On d 17 and 24, two birds from each pen (12 birds/treatment) on each sampling day were randomly selected and a cut of 5cm mid-jejunum was collected for histomorphometry analysis. The ileum was gently squeezed to aseptically collect ileal content into the sterile tubes for microbiological analysis. On d 24, a section of 2cm mid-jejunum was sampled, rinsed in cold PBS, put in RNAlater (Qiagen, Germantown, MD),

and stored at −80ºC for subsequent analysis of immune gene expression. For gross pathology examination, NE lesions of duodenum, jejunum and ileum were scored on a scale of 0 (none) to 4 (high) as previously described (Kulkarni et al., 2007; Thompson et al., 2006).

Intestinal Histomorphometry

A previously described method to prepare samples for histomorphometry analysis was used (Kermanshahi et al., 2017a). Briefly, the jejunal content was flushed out with physiological saline and a section of 2 cm of the tissue (midway between the Meckel's diverticulum and the entrance of the bile ducts) was stored in 10% formalin for 48 h followed by paraffin embedding cut to a thickness of 5 µm using a microtome. Sections were stained with hematoxylin and eosin, and examined microscopically. A total of 9 slides were obtained from each jejunal section per bird and 10 villi were measured per slide (90 villi/bird). Villus width (VW: the base of each villus), villus height (VH: from the top of the villus to the villus-crypt junction), crypt depth (CD: from the base of the adjacent villus to the sub-mucosa), and the ratio of VH to CD was measured on the total of 90 villi per bird with their average representing the values for each bird.

Bacterial Enumeration

The collected ileal content was used to enumerate the population of *E. coli*, *C. perfringens*, *Lactobacillus spp.*, and the total anaerobic bacteria based on the method previously described (Kermanshahi et al., 2017b). Briefly, the ileal contents of a sample were mixed and a 10-fold serial dilution (10^{-1} to 10^{-7}) was performed, followed by plating for enumeration. *Lactobacillus spp.* were anaerobically grown using MRS agar (Difco, Laboratories, Detroit, MI) and. *E. coli* were assayed using MacConkey agar (Difco Laboratories, Detroit, MI) aerobically. The Shahidi-Ferguson Perfringens (SFP) agar and blood agar plates (Oxoid, Basingstoke, UK) were used to anaerobically cultivate *C. perfringens*. All the microbiological analyses were performed in triplicate, and average values were used for statistical analyses and results were expressed in colony forming units ($\log_{10}$ cfu/g of ileal content).

Intestinal Immune Gene Expression

Jejunal samples were thawed from −80°C and homogenized using 3 mm glass beads using the Bioprep-24 homogenizer. Total RNA was extracted, cDNA was synthetized from 1µg of total RNA using the Easy cDNA synthesis kit (Pars Tous, Iran) following the manufacturer's protocol, and stored at −20°C. Transcript abundance of two reference genes (GAPDH and β-actin) and five target genes (tumor necrosis factor receptor associated factor 3, annexin A1 (ANXA1), interleukin (IL)-1, IL-6, IL-10 were determined by quantitative real-time PCR, as described previously (Daneshmand et al., 2020) using ABI 7300 system (Applied Biosystems, Foster City, CA) and 2× SYBR Green RealTime-PCR master mix (Pars Tous, Iran) reagent. Thermal condition for all transcripts set as an initial denaturation phase at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 20 sec, annealing and extension at 63°C for 15 sec. Primer details are shown in Table 2. Relative expression of target genes were normalized by geometric means of two reference genes and the standard curve method used to calculate the efficiency of reactions. All efficiencies were within 90 and 110%, and calculated R^2 was 0.99 for all reactions.

Cellular Stimulation Assay and Flow Cytometry

In order to assess the cellular immune responses of splenocytes and cecal tonsilocytes against *C. perfringens* bacilli and their secretory proteins, spleen and cecal tonsil tissues from six clinically healthy 21 d old male broiler chickens were collected. Single cell suspensions were prepared and counted and 100 µL of each cell suspension was seeded in 96 well plates at a density of 1 $\times 10^6$/ml in RPMI medium (Laursen et al., 2018). Cells were stimulated with 10^3 *C. perfringens* (CP26) bacilli per well for 24 h. For collecting *C. perfringens* supernatant proteins, a previously described method was followed (Kulkarni et al., 2006). Briefly, the bacteria were grown in a fluid thioglycolate medium for 14-16 h and the culture supernatants were dialyzed and concentrated by use of 10-kDa cut-off Amicon centrifugal filters (Millipore Inc., Billerica, MA)

to obtain secreted proteins. The splenocytes and tonisillocytes were stimulated for 24 h with concentrated secretory supernatant at 1:5 ratio in RPMI dilutions per well.

Cells post-stimulation were collected and washed twice in FACS buffer (PBS containing 1% BSA) and stained for 30 minutes on ice with fluorescent mouse monoclonal antibodies directed to bind chicken CD3-PacBlue (clone CT-3), CD4-PE Cy7 (CT-4), CD8-APC (CT-8) and MHC-II-PE (2G11) obtained from Southern Biotech Inc., Birmingham, AL. The Invitrogen Live/Dead fixable near-IR staining was also used to exclude dead cells during data acquisition and subsequent analysis. The cells were washed twice in FACS buffer, fixed in 2% paraformaldehyde (PFA) before immunophenotyping analysis. Flow cytometry was performed using a LSR-II flow-cytometer (BD Bioscience, San Jose, CA) and data were analyzed using FlowJo Software v10 (Tree Star, Ashland, OR). The gating strategy included removal of doublet and dead cells followed by gating on CD3+ cells as the backbone for T cell subset analysis (Figure 2A).

Statistical Analysis

All data were statistically analyzed using the GLM model procedure of SAS program (The SAS system for Windows) in a completely randomized design by ANOVA using the General Linear Model (GLM) procedure of SAS software. Tukey's test was used to compare differences among means of treatments and P values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Due to the growing consumer demand for raising poultry without antibiotics, there has been a renewed research interest to focus on studying *C. perfringens* pathogenesis, immunity, and vaccines (Van Immerseel et al., 2016). These studies have used different NE reproduction models to study *C. perfringens* infection *in vivo* (Prescott et al., 2016b). Here, we employed three methods to reproduce NE in broiler chickens to study the effects of *C. perfringens* on the

intestinal pathology, enteric colonization of commensal and pathogenic bacteria, and host immune responses during NE. Our findings showed that while the inclusion of *Eimeria* as part of the challenge (ECp) could significantly increase NE pathology and reduce bird performance compared to controls, the addition of wheat-based diet to this challenge (WECp) can exacerbate the disease severity. The ECp or WECp challenged birds showed increased immune genes expression *in vivo* and an augmented T cell response *in vitro* as well as promoted enteric colonization of pathogens while reducing the resident *Lactobacillus* population.

Growth Performance, Intestinal histomorphometry, and Gross pathology

The effects of *C. perfringens* administered under different NE reproduction conditions on the growth performance of birds receiving ECp and WECp showed a reduction ($P < 0.05$) in the group mean values of BW (874 for ECp; 841 for WECp), ADG (41.6 for ECp; 39.1 for WECp), and ADFI (60.3 for ECp; 59.2 for WECp) at d 24 of age when compared to unchallenged control birds (BW: 1105; ADG: 58.2; ADFI: 76.6), as given in Table 3. There was also an increase ($P < 0.05$) in the FCR in these birds (1.45 for ECp; 1.51 for WECp) at d 24 when compared to control (1.32). Similarly, the % mortality in ECp (0.7 and 1.1 on d 17 and 24, respectively) and WECp (0.6 and 1.0 on d 17 and 24, respectively) groups was higher ($P < 0.05$) compared to no mortality in the control group (Table 3). These results indicated that coccidial infection can exacerbate *C. perfringens*-induced negative effects on the bird performance.

Evaluation of intestinal gross lesions and histomorphometry in birds in response to different treatments are given in Table 4 and Table 5, respectively. The gross lesion scores in the ECp and WECp groups were higher ($P < 0.05$) in the jejunum (1.97 for ECp; 2.01 for WECp) and ileum (0.98 for ECp; 1.04 for WECp) segments in comparison to WCp (jejunum: 0.57; ileum: 0.16) and control (score 0) groups at d 24 (Table 4). Similar increase ($P < 0.05$) in the lesion scores was also observed at d 17 in the ECp and WECp groups compared to WCp and controls (Table 4). Histomorphometry analysis showed lower ($P < 0.05$) mean values, in the ECp and WECp groups,

for jejunal VH (1177.1 for ECp; 1148.4 for WECp), thinner VW (154.4 for ECp; 137.9 for WECp), deeper CD (213.8 for ECp; 229.9 for WECp) and lower VH/CD ratio (5.5 for ECp; 4.99 for WECp) compared to birds fed control diet (VH: 1343.8; VW: 202.6; CD: 179.2; VH/CD ratio: 7.49) at d 24 (Table 5). Similar decrease ($P< 0.05$) in the values for VH, VW, CD and VH/CD ratio was also observed at d 17 in the ECp and WECp groups compared to controls (Table 5). While the observation of ECp-induced effects is in agreement with previous reports (Belote et al., 2018; Collier et al., 2008), the present study also found that coupling dietary predisposition such as feeding a wheat-containing diet with *Eimeria* inoculation (WECp) can slightly enhance, although not statistically significant, the NE severity compared to ECp, as determined by bird performance and intestinal pathology. This may suggest that the soluble NSPs in wheat could increase the viscosity of digesta and facilitate the initial growth of *Eimeria* and subsequent colonization of *C. perfringens* (Annett et al., 2002). However, *C. perfringens* inoculation of birds receiving wheat-based diet with no *Eimeria* inoculation in the present study could not induce a productive NE compared to ECp or WECp. It is likely that the NSP content provided by wheat-based diet was relatively low (25%) compared to previous reports (Annett et al., 2002; Kermanshahi et al., 2018). In cases of '*C. perfringens* only' NE model, where coccidial predisposition is excluded, NE reproduction likely requires wheat and fish meal-based diet coupled with a heavy challenge consisting of twice-daily *C. perfringens* inoculations for 3-5 d (Kulkarni et al., 2007; Thompson et al., 2006). However, based on the present study findings, it is logical to suggest that coccidia-induced intestinal predisposition plays a key role in NE reproduction in experimental models that rely on the use of single oral administration of *C. perfringens* (Lee et al., 2011). Another critical factor affecting the severity of experimental NE also seems to depend, at least in part, on the carriage of an additional *tpeL* toxin gene by the *netB*+ *C. perfringens* isolates (Coursodon et al., 2012; Gu et al., 2019). This notion is also in agreement with our recent NE investigation (unpublished work). It is noteworthy that the *C.*

perfringens strains used in the present study were *netB+tpel+*, and that may also have been a contributing factor in the reproduction of NE.

Intestinal Microbial Colonization

Enteric infections are known to cause an imbalance in the resident commensal population while promoting the colonization by the pathogenic bacteria (Broom and Kogut, 2018b). To this end, the effects of different NE induction models on the ileal bacterial counts (Log_{10} CFU g^{-1}) were evaluated (Table 6). Broilers receiving ECp and WECp had a higher ($P< 0.05$) number of total anaerobes (4.96 for ECp; 5.25 for WECp), *E. coli* (4.12 for ECp; 4.37 for WECp), and *C. perfringens* (3.91 for ECp; 4.16 for WECp) and a lower ($P< 0.05$) counts of *Lactobacillus* species (3.84 for ECp; 3.76 for WECp) in their ileal digesta compared to unchallenged birds (total anaerobes: 4.35; *E. coli*: 3.7; *C. perfringens*: 3.46; *Lactobacillus spp*.: 4.86) at d 24 of age. Similar increase ($P< 0.05$) in the total anaerobe, *E. coli* and *C. perfringens* counts was also observed at d 17 in the ECp and WECp groups compared to controls (Table 6). At d 24, the *Lactobacillus* species CFU count in the WCp group (4.13) was also lower ($P< 0.05$) than the control (4.86). However, no statistically significant changes between the treatment groups in the numbers of *Lactobacillus* species were observed at d 17 (Table 6). In agreement with the alternation in the gut microbial populations, a previous study reported that *Eimeria* and *C. perfringens* inoculation negatively affected the intestinal microflora in broilers and that the NE infection can impair the epithelial structure and immune function, thus perturbing the intestinal microflora, including the displacement of Lactobacilli via bacteriocins (Wu et al., 2014).

Immune response evaluation

The gut-associated lymphoid tissue consisting of various cells releasing inflammatory and anti-inflammatory molecules in maintaining gut homeostasis serves as an immunological barrier to pathogen invasion (Broom and Kogut, 2018a). The present investigation sought to measure the intestinal expression of certain immune genes that predominantly belong to pro-inflammatory

(IL-6 and TRAF3) and anti-inflammatory or immunomodulatory (IL-10 and ANXA1) categories, as well as T cell cytokine, IL-2. Birds receiving an inoculation of coccidia followed by *C. perfringens* had an elevated (P< 0.05) jejunal transcription of IL-2, IL-6, IL-10, and ANXA1 genes on d 24 (Figure 1). Increased IL-6 transcription during NE indicating an infection-induced inflammation has previously been reported (Park et al., 2008). ANXA1, a member of the annexin proteins family secreted by the antigen-presenting cells, has been shown to migrate to the infected sites to help reduce inflammation via mechanisms that include IL-10 production by resident regulatory T cells (Perretti and D'Acquisto, 2009). To this end, our observation of elevated expression of ANXA1 and IL-10 genes suggest that these effects are likely elicited in response to infection resolution. It is also of note here that IL-10 production during coccidial and NE infections may work to the advantage of the pathogens as part of their immune-evasive properties (Lee et al., 2011). Furthermore, *Eimeria* and *C. perfringens* co-inoculated birds fed on wheat-based diet had increased (P< 0.05) jejunal expression of TRAF3 gene, which was in agreement with a previous report (Kim et al., 2015), suggesting that TRAF3, an immune signalling molecule, could promote inflammatory response against co-infection of *Eimeria* and *C. perfringens* during NE in broilers (Broom and Kogut, 2019; Yang et al., 2015). The present study also observed an elevated (P< 0.05) intestinal transcription of IL-2, a T cell cytokine, in co-infected birds fed the diet without wheat (Figure 1) indicating that *C. perfringens* infection could induce host T cell responses in affected birds. To this end, chicken primary cells from two lymphoid tissues representing local (cecal tonsils) and systemic (spleen) responses were stimulated with *C. perfringens* bacilli as well as their secretory component. The findings showed that T cell (CD3+) frequencies as well as their surface upregulation of MHC-II in the splenocyte and cecal tonsilocytes stimulated with *netB*+ *tpeL*+ virulent *C. perfringens* bacilli were significantly increased (P< 0.05) compared to unstimulated controls (Figure 2B and 2D). Additionally, analysis of T cell subsets showed that stimulation of splenocytes and tonsilocytes

with *C. perfringens* bacilli led to an increase (P< 0.05) in the CD4+ and CD8+ T cell populations compared to controls (Figure 2B and 2D). Furthermore, analysis of effects of *C. perfringens* culture supernatants on T cell responses showed that while the splenocytes stimulated with secretory proteins had higher (P< 0.05) frequencies of CD4+ and CD8+ T cell subsets, as well as T cell upregulation (P< 0.05) of MHC-II (Figure 2C), the cecal tonsilocyte stimulation also led to increased CD4+ T cell frequencies (P< 0.05) compared to unstimulated controls (Figure 2E). However, no statistical differences were found in the treatments related to frequencies of CD8+ T cells or T cell expression of MHC-II in cecal tonsils. Taken together, these observations suggested that the *C. perfringens* bacilli and their secretory proteins can induce augmented T cell responses, including the CD4+ and CD8+ T cell subsets, which are the source of IL-2 and anti-bacterial effector functions (Shepherd and McLaren, 2020). Additionally, the significant increase in the expression of MHC-II antigen-presenting molecules on T cells indicated their elevated cellular activation status of T cells during *C. perfringens* infection (Holling et al., 2004).

CONCLUSION

The present study used three methods of NE reproduction to evaluate the effects of virulent *C. perfringens* on the intestinal pathology, enteric bacterial colonization and host immune responses in broilers. The current findings showed that co-inoculation of broiler chickens with *Eimeria* and *C. perfringens* fed on a conventional diet or a wheat-based diet can lead to a significantly reduced bird performance coupled with severe epithelial damage and a gut microbial imbalance in favour of enteric pathogens in comparison to the resident lactobacilli. The intestinal immune response was associated with an increased transcription of pro- and anti-inflammatory immune genes, in addition, virulent *C. perfringens* stimulation of chicken splenocytes and cecal tonsilocytes showed augmented T cell responses. These findings provide useful information on the suitability of different NE reproduction methods that use *Eimeria* and/or dietary predisposition in studying *C. perfringens* pathogenesis, immunity and vaccine development.

Furthermore, these results provide additional data on the intestinal as well as cellular immune responses against *C. perfringens*.

CONFLICT OF INTEREST

The authors claim that there is no conflict of interest for this manuscript and the results.

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Figure legends

Figure 1

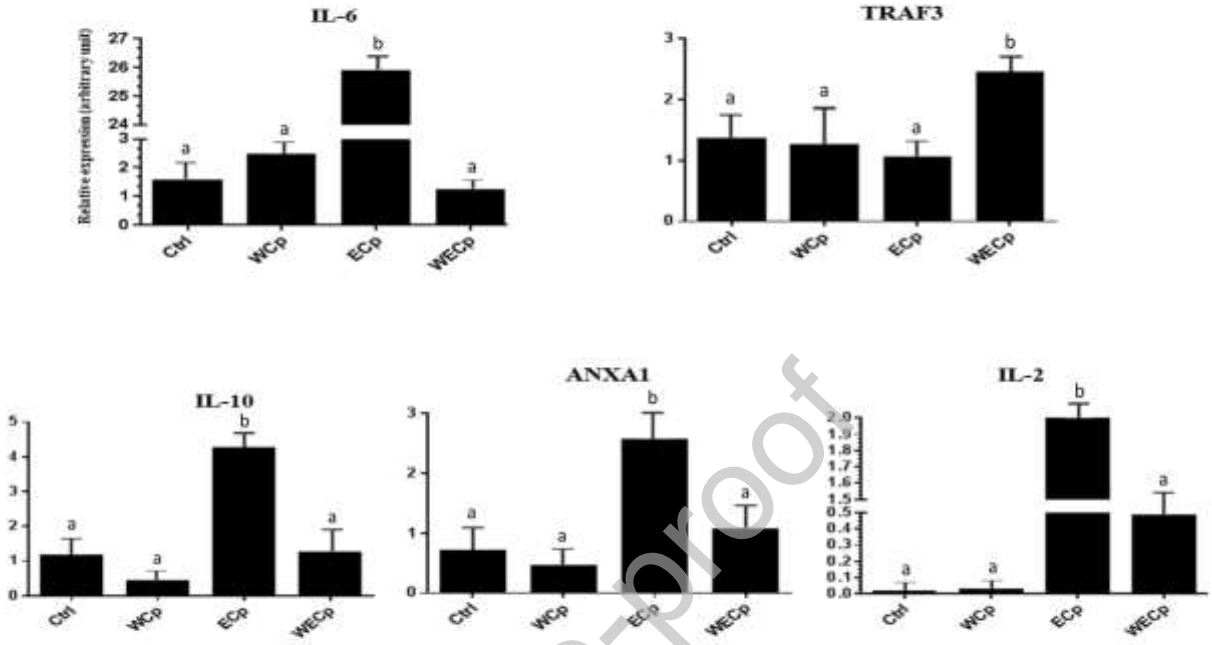

Figure 1. Immune genes expression induced by *C. perfringens* infection. Jejunal tissues collected at 24 d of age from broiler chickens infected with *C. perfringens* in three NE models were collected and processed for gene expression analysis using qRT-PCR. The genes, GAPDH and β-actin, were used as the reference. **Ctrl**, control, corn soybean meal-based diet; **ECp**, Control + *Eimeria* + *Clostridium perfringens* co-inoculation; **WCp**, Control + 25% corn replaced by wheat (W) + *Clostridium perfringens* inoculation; **WECp**, Control + 25% corn replaced by wheat (W) + *Eimeria* and *Clostridium perfringens* co-inoculation; **ANXA1**, annexin A1; **TRAF3**, tumor necrosis factor receptor-associated factor 3. Different letters above the bars within each graph indicate the differences were statistically significant ($P < 0.05$).

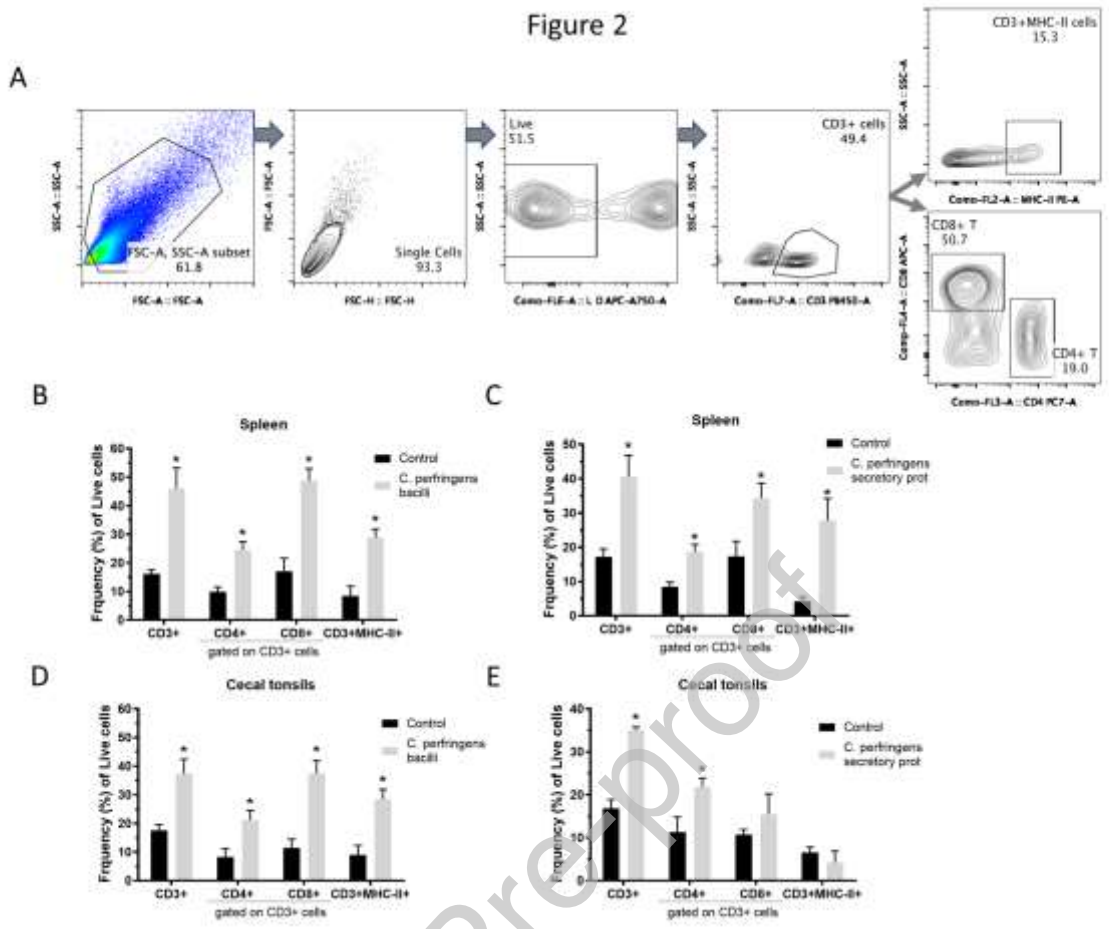

Figure 2. **Stimulation of splenocytes and cecal tonsilocytes with *C. perfringens* bacilli and secretory proteins**. Spleens and cecal tonsils from broiler chickens were collected and mononuclear cell suspension was prepared. Splenocytes and tonsilocytes were stimulated with virulent *C. perfringens* bacilli as well as their cell-free secretory component for 24 h followed by staining with antibodies against chicken CD3, CD4, CD8 and MHC-II molecules for flow cytometry analysis. Gating strategy for cellular analysis is shown in panel A. The frequencies of splenocytes stimulated with *C. perfringens* bacteria (panel B) and their secretory proteins (panel C) and those of cecal tonsilocytes stimulated with *C. perfringens* (panel D) and their secretory proteins (panel D) are shown as bar graphs. Different letters above the bars within each cell set population indicate the differences were statistically significant ($P < 0.05$).

Table 1. Composition of experimental diets (11-24 d of age).

Ingredient (%)	Control	Control with wheat
Corn	58.16	34.81
Wheat	-	25.00
Soybean meal (44.0 %)	34.85	32.58
Soybean oil	3.35	3.97
Dicalcium phosphate	1.65	1.68
Limestone	0.92	0.85
Salt	0.30	0.35
Mineral-vitamin premix[1]	0.50	0.50
DL-Methionine	0.15	0.17
L-Lysine HCl	0.12	0.09
Calculated nutrients		
AME (kcal/kg)	3025	3025
Crude protein (%)	19.0	19.0
Calcium (%)	0.84	0.84
Available phosphorus (%)	0.42	0.42
Sodium (%)	0.16	0.17
Methionine (%)	0.47	0.48
Methionine + cysteine (%)	0.86	0.87
Lysine (%)	1.18	1.19
Threonine (%)	0.77	0.78
Analyzed nutrients		
Crude protein (%)	18.8	18.7
Calcium (%)	0.80	0.79

[1] Added per kg of feed: vitamin A, 7,500 UI; vitamin D3 2100 UI; vitamin E, 280 UI; vitamin K3, 2 mg; thiamine, 2 mg; riboflavin, 6 mg; pyridoxine, 2.5 mg; cyanocobalamin, 0.012 mg, pantothenic acid, 15 mg; niacin, 35 mg; folic acid, 1 mg; biotin, 0.08 mg; iron, 40 mg; zinc, 80 mg; manganese, 80 mg; copper, 10 mg; iodine, 0.7 mg; selenium, 0.3 mg.

Table 2. Sequences of primer pairs used for amplification of target and reference genes.[1]

Gene	Strand	Sequence (5′→ 3′)	Ta	Product size (bp)	GenBank Accession No.
ANXA1[2]	Forward	CTGCCTGACTGCCCTTGTGA	63	98	NM_206906.1
	Reverse	GTTTGTGTCGTGTTCCACTCCC			
TRAF3	Forward	CTGAGAAAAGATTTGCCAGACCA	63	101	XM_421378
	Reverse	CATGAAACCATGACACACGGG			
IL-2	Forward	TTATGGAGCATCTCTATCATCAGCA	63	122	XM_01576098.1
	Reverse	CCTGGGTCTCAGTTGGTGTGTAG			
IL-6	Forward	CTGTTCGCCTTTCAGACCTACC	63	141	NM_204628.1
	Reverse	GACCACTTCATCGGGATTTATCA			
IL-10	Forward	GGACTATTTTCAATCCAGGGACG	63	136	NM_001004414.2
	Reverse	GGGCAGGACCTCATCTGTGTAG			
GAPDH	Forward	TTGTCTCCTGTGACTTCAATGGTG	63	128	NM_204305
	Reverse	ACGGTTGCTGTATCCAAACTCAT			
β-Actin	Forward	CCTGGCACCTAGCACAATGAA	63	175	NM_205518.1
	Reverse	GGTTTAGAAGCATTTGCGGTG			

[1]For each gene the primer sequence for forward and reverse (5′→ 3′), the product size (bp), and the annealing temperature (Ta) in °C are shown; [2]ANXA1, annexin A1; TRAF3, tumor necrosis factor receptor associated factor 3; IL-, interleukin-; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

Table 3. Effects of different NE reproduction methods on growth performance of broiler chickens from 11 to 24 d of age.

	BW[1] (g)			ADG (g)			ADFI (g)			FCR (g/g)			Mortality[2] (%)	
Groups[3]	11	17	24	11-17	17-24	11-24	11-17	17-24	11-24	11-17	17-24	11-24	17	24
Control	293	597^{a}	1105^{a}	43.4^{a}	72.6^{a}	58.2^{a}	51.5^{a}	101.7^{a}	76.6^{a}	1.18^{b}	1.40^{b}	1.32^{b}	0.0^{b}	0.0^{b}
ECp	292	492^{ab}	874^{b}	28.6^{b}	54.6^{b}	41.6^{b}	38.4^{b}	82.2^{b}	60.3^{b}	1.34^{a}	1.51^{a}	1.45^{a}	0.7^{a}	1.1^{a}
WCp	297	533^{a}	962^{ab}	33.7^{ab}	61.3^{ab}	47.5^{ab}	42.7^{ab}	90.3^{ab}	66.5^{ab}	1.26^{ab}	1.47^{ab}	1.40^{ab}	0.3^{ab}	0.6^{ab}
WECp	293	440^{b}	841^{b}	21.6^{b}	57.3^{b}	39.1^{b}	29.2^{b}	89.3^{b}	59.2^{b}	1.39^{a}	1.56^{a}	1.51^{a}	0.6^{a}	1.0^{a}
SEM[4]	2.6	5.3	9.8	3.01	6.90	4.96	3.52	9.11	6.04	0.012	0.019	0.031	0.29	0.72
P-value	0.428	0.022	0.036	0.028	0.042	0.039	0.012	0.026	0.031	0.013	0.039	0.018	0.043	0.018

$^{a-b}$ Group mean values within a column with different letters differ significantly (P< 0.05).

[1]BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio

[2] Mortality data are the records of dead birds due to NE

[3] Experimental groups were, Control: Corn-soybean based conventional diet; ECp: Control diet + oral co-inoculation with *Eimeria spp.* and *C. perfringens*; WCp, Control diet + 25% corn replaced by wheat + *C. perfringens* inoculation*;* WECp, Control diet + 25% corn replaced by wheat + oral co-inoculation with *Eimeria spp.* and *C. perfringens*.

[4] SEM: results are given as means of 6 pens of 15 birds/treatment).

Table 4. Effects of different NE reproduction methods on intestinal lesion scores in broilers at 17 and 24 d of age.

	d 17		d 24	
Groups[1]	Jejunum	Ileum	Jejunum	Ileum
Control	0[b]	0[b]	0[b]	0[b]
ECp	1.88[a]	0.82[a]	1.97[a]	0.98[a]
WCp	0.49[b]	0.10[b]	0.57[b]	0.16[b]
WECp	1.84[a]	0.88[a]	2.01[a]	1.04[a]
P-value	0.001	0.001	0.004	0.011
SEM[2]	0.17	0.09	0.27	0.13

[a-b] Group mean values within a column with different letters differ significantly ($P < 0.05$).

[1] Experimental groups were, Control: Corn-soybean based conventional diet; ECp: Control diet + oral co-inoculation with *Eimeria spp.* and *C. perfringens*; WCp, Control diet + 25% corn replaced by wheat + *C. perfringens* inoculation; WECp, Control diet + 25% corn replaced by wheat + oral co-inoculation with *Eimeria spp.* and *C. perfringens*.

[2] SEM: results are given as means (n = 12) for each treatment.

Table 5. Effects of different NE reproduction methods on intestinal histomorphology (μm) of broiler chickens at 17 and 24 d of age.

	d 17				d 24			
Groups[2]	VH[1]	VW	CD	VH/CD	VH	VW	CD	VH/CD
Control	958.7[a]	271.2[a]	203.3 [b]	4.72[a]	1343.8[a]	202.6[a]	179.2[b]	7.49[a]
ECp	799.8[b]	197.1[b]	226.4[a]	3.53[b]	1177.1[b]	154.4[b]	213.8[a]	5.50[b]
WCp	849.9[ab]	239.1[ab]	214.7[ab]	3.95[ab]	1295.6[ab]	186. 2[ab]	199.4[ab]	6.49[ab]
WECp	741.2[b]	174.3[b]	238.2[a]	3.11[b]	1148.4[b]	137.9[b]	229.9[a]	4.99[b]
SEM[3]	9.56	9.02	8.94	0.184	30.01	9.03	8.99	0.294
P-value	0.032	0.011	0.012	0.038	0.021	0.013	0.029	0.034

[a-b] Group mean values within a column with different letters differ significantly (P< 0.05).

[1]VH: villus height; VW: villus width; CD: crypt depth; VH/CD: the ratio of VH to CD.

[2] Experimental groups were, Control: Corn-soybean based conventional diet; ECp: Control diet + oral co-inoculation with *Eimeria spp.* and *C. perfringens*; WCp, Control diet + 25% corn replaced by wheat + *C. perfringens* inoculation*;* WECp, Control diet + 25% corn replaced by wheat + oral co-inoculation with *Eimeria spp.* and *C. perfringens*.

[3]SEM (results are given as means (n = 12) for each treatment).

Table 6. Effects of different NE reproduction methods on ileal microflora (log_{10} CFU g^{-1}) in broilers at 17 and 24 d of age.

	d 17				d 24			
Groups[1]	Total anaerobes	*E. coli*	*C. perfringens*	*Lactobacillus spp.*	Total anaerobes	*E. coli*	*C. perfringens*	*Lactobacillus spp.*
Control	4.60[b]	3.74[b]	2.89[b]	4.26	4.35[b]	3.70[b]	3.46[b]	4.86[a]
ECp	5.01[a]	4.34[a]	3.66[a]	3.73	4.96[a]	4.12[a]	3.91[a]	3.84[b]
WCp	4.83[ab]	3.98[ab]	3.11[b]	3.96	4.83[ab]	3.89[ab]	3.70[ab]	4.13[b]
WECp	5.30[a]	4.51[a]	3.73[a]	3.83	5.25[a]	4.37[a]	4.16[a]	3.76[b]
P-value	0.009	0.022	0.037	0.215	0.040	0.031	0.017	0.041
SEM[2]	0.241	0.379	0.421	0.386	0.251	0.314	0.263	0.413

[a-b] Group mean values within a column with different letters differ significantly (P< 0.05).

[1] Experimental groups were, Control: Corn-soybean based conventional diet; ECp: Control diet + oral co-inoculation with *Eimeria spp.* and *C. perfringens*; WCp, Control diet + 25% corn replaced by wheat + *C. perfringens* inoculation*;* WECp, Control diet + 25% corn replaced by wheat + oral co-inoculation with *Eimeria spp.* and *C. perfringens*.

[2] SEM: results are given as means (n = 12) for each treatment.