Veterinary Immunology and Immunopathology 210 (2019) 28–37

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

# Heat stress, *Eimeria* spp. and *C. perfringens* infections alone or in combination modify gut Th1/Th2 cytokine balance and avian necrotic enteritis pathogenesis

Atilio Sersun Calefi[1,*], Wanderley Moreno Quinteiro-Filho[1], Adriana de Siqueira[1], Ana Paula Nascimento Lima[1], Daniel Sanzio Gimenes Cruz[1], Nicolle Queiroz Hazarbassanov[1], Fernanda Auciello Salvagni[1], Anderlise Borsoi[1], Cristina de Oliveira Massoco Salles Gomes[1], Paulo César Maiorka[1], Antonio José Piantino Ferreira[1], João Palermo-Neto[1]

*Department of Pathology, School of Veterinary Medicine and Animal Science, University of São Paulo, SP, Brazil*

ARTICLE INFO

*Keywords:*
Gut-brain axis
Necrotic enteritis
Broiler chickens
Neuroimmunomodulation

ABSTRACT

Information on the dynamics of the chicken immune system during bacterial or parasite challenge in the presence or absence of stressful situations may provide a better understanding of the complex mechanisms behind these diseases. Necrotic enteritis (NE) had been controlled previously by the proper use of antimicrobial agents; however, more recently, NE has reemerged in many countries. The imposed restrictions on antimicrobial use and/or the intensive productive programs implemented by producers are challenges the birds, leading to large host adaptive responses that in many instances are like those elicited by stressors. This study analyses the effects of heat stress on Th1/Th2 cytokine balance, pathological features, and Toll-like receptor expression in the small intestine of broiler chickens infected with *Clostridium perfringens* type A in the presence or absence of *Eimeria* spp. co-infection. This co-infection model was experimentally used because it reproduces the findings commonly observed in the field during avian NE. For this purpose, broiler chickens infected with *C. perfringens* and/or *Eimeria* spp. were reared in isolator chambers subjected or not to heat stress intermittently. It was observed that heat stress directs the expression of Th2-type cytokines, increases Toll-like receptor 4 expression in the intestine and reduces the disease severity induced by *Eimeria* spp. and *C. perfringens* infections alone or in combination, most likely as a consequence of stress-induced changes in brain-gut axis activity.

## 1. Introduction

Necrotic enteritis (NE) is a reemerging disease that annually produces large losses in the poultry industry (Shane, 2005). Several models have been proposed to experimentally reproduce this disease in chickens as it occurs in the field (Lee et al., 2011). However, due to the difficulties in reproducing the NE using *C. perfringens* infection alone, some authors have suggested an association between the bacterial load and factors known to predispose poultry to NE. Thus, the addition of thioglycolate broth culture medium (Cooper and Songer, 2010) and/or fish meal into the feed supplied to the poultry (Truscott and Al-Sheikhly, 1977) as well as co-infection with other pathogens, such as *Eimeria* spp. (McReynolds et al., 2004) be useful tools to predispose NE development. Environmental challenges have also been identified as an NE predisposing factor (Calefi et al., 2014). Stress is known to reduce food intake (Quinteiro-Filho et al., 2012), to induce intestinal damage (Burkholder et al., 2008; Lambert, 2009) and to modify the immune response of poultry (Honda et al., 2015). Within this context, the type, intensity and duration of the stressors applied to birds are known to produce different stress responses that range from an increase in the animal's immune response (Star et al., 2007) to a reduction in their immunity due to stress-induced antigen challenge (Zulkifli et al., 2000).

Information on the dynamics of the immune system during infectious challenges and/or stressful situations may provide a better understanding of the mechanisms underlying the development of NE. This fact is relevant because it would allow the direct application of rational and suitable measures to control NE development.

Previous reports from our group have shown that heat stress reduces

* Corresponding author.
*E-mail address:* ascalefi@gmail.com (A. Sersun Calefi).
[1] Address: Av. Prof. Dr. Orlando Marques de Paiva, 87, 05508 270, São Paulo, SP, Brazil.

https://doi.org/10.1016/j.vetimm.2019.03.001
Received 24 June 2018; Received in revised form 24 February 2019; Accepted 3 March 2019

intestinal inflammation and decreases *C. perfringens* adhesion to the intestinal epithelium of infected poultry (Calefi et al., 2014); the most crucial finding described in this work was the reduction in the migration of heterophilic cells. It was suggested that the thioglycolate broth medium (MFT) employed and/or the *C. perfringens*-induced infection modified the brain-gut axis activity in poultry leading to the development of the disease (Calefi et al., 2016a,b). Using an experimental model of NE that employed co-infection with *Eimeria* spp., Tsiouris et al. (2015a,b) demonstrated that stressors, such as cold or high population density are predisposing factors for the development of *C. perfringens* infection.

Cytokines released into the gut are known to assist the suppression of *Eimeria* spp. during infection in poultry (Min et al., 2001). Accordingly, the addition of some cytokines (IL-8, lymphotactin, IFN-γ, IL-15, TGF-β4, IL-1β) during *Eimeria* spp. vaccination was reported to modify and even prevent the pathogenesis of coccidiosis (Min et al., 2001). Understanding the role of cytokines during *C. perfringens* infection could lead to the development of an effective tool for NE control. Although there have been studies on the effects of stressors in NE models, none of them have analyzed cytokine modulation during NE development in the presence of stressors. Thus, the current study was designed to analyze the expression of cytokines and toll-like receptors (TLRs) in the small intestine and cecal tonsils of broilers during an experimental NE model with and without heat stress. The serum levels of corticosterone and the scores of macro and microscopic intestinal injuries were also analyzed in the broiler chickens.

## 2. Materials and methods

### 2.1. Animals

One-day-old broiler chickens (Cobb®, São Paulo, Brazil) were acquired from a breeder hatchery free of *Salmonella* spp. and *Mycoplasma* spp. The animals were housed in isolation chambers (Alesco, São Paulo, Brazil) containing air filters with high-efficiency separation of particulate (HEPA) from the first experimental day (ED1) to ED23. Water and food (hanging feeders) were provided *ad libitum* to the chickens, and their health status and behavior were observed continuously. The relative environmental humidity was monitored and controlled (maintained to not fall below 45%).

The animals were kept and used by the rules settled by the Ethics Committee on Animal Use of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo, Brazil (Approved protocol number 3071/2013).

### 2.2. Experimental groups

After their arrival into the experimental facilities, the chickens were randomly separated into eight groups of seven animals each, as follows: control group (C); a group of animals infected by *Eimeria* spp. (Ei); a group of animals infected with *C. perfringens* (Cp); a group of animals infected with *Eimeria* spp. plus *C. perfringens* (Cp + Ei); control group heat stressed (C/HS35); a group of animals infected by *Eimeria* spp. and heat stressed (Ei/HS35); a group of animals infected with *C. perfringens* and heat stressed (Cp/HS35); a group of animals infected with *Eimeria spp* plus *C. perfringens* and heat stressed (Cp + Ei/HS35).

### 2.3. Heat stress protocol

Chickens in the heat-stressed groups (C/HS35, Ei/HS35, Cp/HS35, Cp + Ei/HS35) were kept at room temperature (34 ± 1 °C) from ED17 to ED23. The heat stress was performed employing a 12-h cycle, i.e., 12 h of heat stress followed by 12 h of thermal comfort. During the thermal comfort period, the stressed birds were maintained under a normothermic environmental temperature specified by the producer for this broiler strain.

### 2.4. Eimeria spp. Infection protocol

On ED18, animals in the Ei, Cp + Ei, Ei/HS35, Cp + Ei/HS35 groups received the BioCoccivet-R vaccine (Biovet, SP, Brazil) via gavage at a concentration 30 times higher (≈12000 oocysts per bird) than that recommended by the manufacturer. The vaccine contains live oocysts of *Eimeria acervulina*, *E. brunetti, E. hagani, E. tenella, E. necatrix, E. mivati, E. maxima* and *E. praecox*.

### 2.5. Preparation of the inoculum and the infection protocol for C. Perfringens

This protocol was adapted from Calefi et al. (2014). A pathogenic strain of *C. perfringens* type A with a genotype to produce Tpel toxin (netB negative) was used. This bacterial strain was maintained in glycerol at −80 °C until the beginning of cultivation. Two alternative cultivations produced the inoculum: cooked meat medium (CMM Becton, Dickinson, and Company) and Brain Heart Infusion (BHI, Becton, Dickinson and Company, MD, USA) medium containing 2% yeast extract (Becton, Dickinson and Company). The inoculum ($1 \times 10^8$ cfu per mL) was given once daily via gavage (5 mL) to every bird in groups Cp, Cp/HS35, Cp + Ei and Cp + Ei/HS35 on ED19 through ED23. From ED1 to ED24, the animals received feed formulated for broilers with 24% crude protein, without antibiotics, organic acids or other feed additives. The birds in the control groups (C and C/HS35) received only the feed throughout the experiment.

### 2.6. Quantification of free and total corticosterone

Blood samples from the brachial veins were collected from all groups on ED24; sera were obtained for hormone quantification. The corticosterone levels were determined using a commercial ELISA kit (Test Arbor, Michigan, USA) according to the manufacturer's instructions, preceded by standard dilutions. The concentration of free corticosterone was determined from the concentration of corticosterone that was not bound to plasma proteins; the total corticosterone was determined before the dissociation of corticosterone from plasma proteins. Corticosterone concentration was determined via graphical interpolation on a standard curve and was expressed in picograms per milliliter of plasma corticosterone (pg/ml). The results were multiplied by the dilution factor used in the test.

### 2.7. Quantification of C. Perfringens in cecal contents

On ED24, the intestinal contents taken from the left cecum of each animal was diluted 1:2 in 2% peptone water (Becton, Dickinson, and Company), followed by 10-fold dilutions; the contents were seeded in petri dishes containing BHI agar with 0.5% yeast extract and 5% of citrated bovine blood. The material was incubated under anaerobic conditions at 37 °C for 48 h. After incubation, each bacterial colony that presents double-zone hemolysis was counted to determine the cfu.

### 2.8. Intestinal gross evaluation

After the animals were euthanized (ED24) the intestines were cut for macroscopic evaluation of the entire length of the intestine. We evaluated the intestinal macroscopic appearance of animals infected by C. *perfringens* and the uninfected control animals (groups C, C/HS35, Cp, Cp/HS35, Ei + Cp, Ei + Cp/HS35) by employing a lesion scoring system proposed by Calefi et al. (2014). The lesion scores of 0 to +4 proposed by Johnson and Reid (Johnson and Reid, 1970) for *E. acervulina*, *E. maxima* and *E. brunette* in induced intestinal lesions was employed in each bird to evaluate the data from birds in groups Ei and Ei/HS35. Because both scores use the same scale, the values were compared between the groups. The definition of separate scores became necessary due to the differences in the pathogenesis of infectious

challenges.

### 2.9. Intestinal microscopic evaluation

Two and a half cm sections of the lower duodenal flexure, jejunum (the region between the end of the duodenum and Meckel's diverticulum) and ileum (the middle region between Meckel's diverticulum and the ileocecal junction) were collected on ED24 for histopathology. These intestinal sections were taken from each animal, regardless of the presence or absence of pathological changes. The tissues were fixed in 10% formalin for 48 h. Subsequently, the materials were embedded in paraffin according to standard procedures used in our laboratories. Five μm cross-sections were stained with HE. Histopathological examination was performed using optical microscopy under 40×, 200× and 400× magnifications.

For the diagnosis of intestinal injuries and the comparison of data across the groups, the areas of three transverse intestinal sections in terms of intestinal portion (duodenum, jejunum, and ileum) were evaluated for the presence of necrosis, inflammation (infiltration of polymorphonuclear cells in the villi and crypt and mononuclear infiltrate), fusion of villi, edema, crypt abscess and transmural lesions. Each type of injury was classified as either mild (score 1), moderate (score 2) or marked to severe (score 3) for necrosis and inflammation. Villi fusion was scored as (1): less than 3 mergers per histological section; (2): between 3–6 fused villi per histological section; and (3): more than 6 fused villi per histologic section. The occurrence of crypt abscesses and transmural lesions was recorded as being present (score 1) or absent (score 0). The total lesion score per animal was taken as the total sum of the observed lesion scores.

### 2.10. Total RNA extraction and cDNA synthesis

The total RNA samples (50 mg of tissue) taken from the small intestine (duodenum, jejunum, and ileum) and the left cecal tonsils of all animals were extracted with the aid of Trizol reagent (Invitrogen Canada Inc. Burlington, Ont., Canada) as described in Abdul-Careem et al. (2006). Then, the total RNA was processed for cDNA synthesis using a commercial kit according to the manufacturer's instructions (iScript, Bio-Rad, USA).

### 2.11. Quantification of cytokines using real-time PCR

Real-time PCR was employed to quantify the relative expression of cytokines in the processed cDNA samples (1:10 in DEPC-treated water). The sequences of the genes and primers used are described in Table 1. The PCR reactions were prepared in a total volume of 20 μl, using SYBR Green Select (Thermo Fisher Scientific, Waltham, MA, USA) as a marker. The reactions were processed using StepOnePlus equipment (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, the applied amplification conditions consisted of pre-incubation for 10 min at 94 °C followed by 60 cycles of 95 °C for 10 s; 54–64 °C for annealing for 5 s, and acquisition and elongation at 72 °C for 10 s. The melting curve analysis was performed in three steps: 95 °C for 10 s, cooling to 55 °C for 1 min and heating to 97 °C. The geometric mean of two endogenous control genes (genes β-actin and glyceraldehyde 3-phosphate dehydrogenase, GAPDH) was used to analyze the relative gene expression. We used a relative threshold cycle (CT) to determine the expression of the target gene compared to control tissue without (calibrator). For each sample, the mean CT of the target gene was normalized using the formula ΔCt = Ct target gene − Ct mean of GAPDH and β-actin. To determine the relative expression levels, the following formula was used: ΔΔCt = ΔCt (sample) − ΔCt (calibrator); this value was used to calculate the relative expression by using the $2^{-\Delta\Delta Ct}$ expression. The Ct values were centered in the C group. The cytokines that presented negative result for the amplification were assigned as 0 (zero). For the understanding of the Th1/Th2 balance, the cytokines that plays a significant role in the Th1 balance are IFN-γ, TNFSF, IL-2, and the Th2 cytokines are IL-4, IL-6, IL-10 and IL-13. The other cytokines deppends the context of these cytokines to interpret and can play a regulatory function such as TGF and IL-12.

### 2.12. Statistical analysis

Quantifications of corticosterone and the cfu of C. *perfringens* were analyzed using the Shapiro-Wilk test for normality after were performed the one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test (Tukey's HSD). The results of the macroscopic and microscopic lesion scores were analyzed using the Kruskal-Wallis test followed by the *post-hoc* multiple comparisons test among groups using the *kruskalmc* function from the *pgirmess* package in the R statistical software package version 3.2.3. $P \leq 0.05$ was considered significant.

To generate the heat maps the *heatmap. 2* function from the *gplot* package (version 2.12.1) in the R statistical software package version 3.2.3 was used. To assess the similarity of the cytokine relative expression between two groups and relative to the overall cytokine expression matrix a score was computed (z-score) and the data were centered to normalize the values. The heat maps were reorganized in their rows and columns to identify similarities between cytokine relative expression and were based on the Euclidean distance and

**Table 1**
Sequence of primers used to evaluate the expression of cytokines and chemokines.

| Target gene | Sense | Antisense | Accession Number |
|---|---|---|---|
| β-actin | 5'-CAACACAGTGCTGTCTGGTGG-3' | 5'-ATCGTACTCCTGCTTGCTGAT-3' | X00162 |
| GAPH | 5'-GGTGGTGCTAAGCGTGTTAT-3' | 5'-ACCTCTGTCATCTCTCCACA | K01458 |
| IFN-α | 5'-GACATCCTTCAGCATCTCTTCA-3' | 5'-AGGCGCTGTAATCGTTGTCT-3' | AB021154 |
| IFN-γ | 5'-AGCTGACGGTGGACCTATTATT-3' | 5'-GGCTTTGCGCTGGATTC-3' | Y07922 |
| IL-1β | 5'-TGGGCATCAAGGGCTACA-3' | 5'-TCGGGTTGGTTGGTGATG-3' | Y15006 |
| IL-2 | 5'-TCTGGGACCACTGTATGCTCT-3' | 5'-ACACCAGTGGGAAACAGTATCA-3' | AF000631 |
| IL-6 | 5'-CAAGGTGACGGAGGAGGAC-3' | 5'-TGGCGAGGAGGGATTTCT-3' | AJ309540 |
| IL-10 | 5'-CGGGAGCTGAGGGTGAA-3' | 5'-GTGAAGAAGCGGTGACAGC-3' | AJ621614 |
| IL-12 | 5'-AGACTCCAATGGGCAAATGA-3' | 5'-CTCTTCGGCAAATGGACAGT-3' | NM_213571 |
| IL-13 | 5'-CCAGGGCATCCAGAAGC-3' | 5'-CAGTGCCGGCAAGAAGTT-3' | AJ621735 |
| IL-17 | 5'-CTCCGATCCCTTATTCTCCTC-3' | 5'-AAGCGGTTGTGGTCCTCAT-3' | AJ493595 |
| LITAF | 5'-TGTGTATGTGCAGCAACCCGTAGT -3' | 5'-GGCATTGCAATTTGGACAGAAGT-3' | AY765397 |
| TGF-β4 | 5'-CGGGACGGATGAGAAGAAC-3' | 5'-CGGCCCACGTAGTAAATGAT -3' | M31160 |
| TNFSF15 | 5'-CCTGAGTATTCCAGCAACGCA-3' | 5'-ATCCACCAGCTTGATGTCACTAAC-3' | NM_01024578 |
| LITAF | 5′-TGTGTATGTGCAGCAACCCGTAGT-3′ | 5′-GGCATTGCAATTTGGACAGAAGT-3′ | AY765397 |
| MIP3-α | 5'-CTCGGAAGGTCATTAAGG-3' | 5'-CTTAGGATTTACGCAGGC-3' | NM_204438 |

Note: IL – interleukin; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; IFN – interferon; LITAF – LPS-induced TNF-alpha factor; TGF – transforming growth factor; TNFSF – tumor necrosis factor superfamily; MIP – macrophage inflammatory proteins.

complete agglomeration method, i.e. rows and columns were sorted based on the hierarchical clustering result. The colors were assigned to represent the cytokine relative expression in each group and each figure represents an intestinal portion. A colour key relative to the z-score with a histogram with the overall score distribution in the matrix were placed in the top-left corner in the figures. The distances between cytokine relative expression by group are represented by dendrograms in the x- and y-axes of the graphic. This approach was preferred to replace conventional presentations to demonstrate similarities and dissimilarities of neurotransmitters between all analytes and experimental groups. Thus, subtle variations and clusters are easily depicted in one figure, a fact that is not evident in graphic presentations in bars and boxplots.

To visualize the correlations between cytokines, the technique proposed by Hurley (2004) for multidimensional data visualization was used. For each correlation between cytokines, a score was given to rank the absolute correlation values. After permutation of the variables and the proximity of the merits, the graphics were rearranged near the diagonal axis and grouped at their higher level of correlation. Colors were assigned based on the level of merit: red for the top third of merit ($> 0.7$; high correlation), blue for the middle third of merit (0.3– 0.7) and yellow for the bottom third of merit ($< 0.3$). The transition pattern of correlation matrices was identified by the scatter distribution of the red graphics along the diagonal axis without forming defined clusters of highly correlated cytokines. Thus, it was possible to visualize whether the correlations were positive or negative following the direction of dispersion of the points in each graph. A summary table was created for each set of matrices indicating the highest cytokine cluster correlations. The correlation matrix graphics used to produce the summary tables are provided as supplementary data. These data evaluation and presentation was chosen to produce a global view of the data with the interaction between parameters and to reduce the P-value inflation due to multiple tests. The statistical analyses and graphs were generated with the *gclus* package in the R statistical software package version 3.2.3.

## 3. Ethics statement

Animals were used and maintained with the approval of the Ethics Committee on Animal Use from the School of Veterinary Medicine and Animal Science (CEUA/FMVZ), Brazil (permit number 3071/2013); the guidelines are similar to the guidelines of the National Institutes of Health (NIH), USA (for Laboratory Animal Research (US) and (US), 2010).

## 4. Results

### 4.1. Quantification of total and free corticosterone serum levels

The animals infected with *Eimeria* (Ei/HS35 group) and those in the Cp + Ei co-infected and heat-stressed group (Ei + Cp/HS35 group) had decreased concentrations of free and total corticosterone when subjected to heat stress relative to their respective unstressed groups (Ei and Ei + Cp groups) (Fig. 1). Due to the variability and sample size only the group infected with *Eimeria* spp. and heat-stressed (Ei/HS35) presented a significant reduction of free corticosterone concentrations when compared to the respective group infected (Ei; $P < 0.05$; Fig. 1B).

### 4.2. Counts of Clostridium perfringens in the cecal contents of broiler chickens

Heat stress increased the number of colony-forming units (cfu) measured in the cecum of animals in the Cp/HS35 and Ei + Cp/HS35 groups relative to the Cp group ($P < 0.05$; Fig. 2). Birds in the Ei + Cp group presented intermediate cfu counts ($P > 0.05$; Fig. 2).

### 4.3. Gross intestinal evaluation

The animals in the C group did not show macroscopic lesions in the duodenum, jejunum or ileum (Fig. 3). Co-infection with *Eimeria* + *C. perfringens* (Ei + Cp group) significantly increased the median lesion scores to +2 in the duodenum and jejunum when compared to the birds in the C group ($P < 0.05$). Interestingly, birds in the Ei + Cp/HS35 group presented a decrease in the macroscopic lesion scores compared to those of group Ei + Cp (Fig. 3; $P > 0.05$). Similarly, birds in the Ei/HS35 group had decreased lesion scores in the jejunum relative to those in the Ei group ($P > 0.05$). Finally, animals in the Cp/HS35 group exhibited higher gross lesion scores than those in the Cp group (Fig. 3; $P > 0.05$).

### 4.4. Intestinal microscopic evaluation

The microscopic scores evaluated in the different groups are shown in Fig. 4, as well as in Supplementary Tables 1 and 2. The histopathological evaluation confirmed the lesions previously observed and described during the gross evaluation given above. Birds in the Ei + Cp group exhibited increased median lesion scores ($P < 0.05$ in all intestinal portions) relative to those in group C. In turn, it was shown that the Ei group exhibited increased ($P < 0.05$ in duodenum and jejunum) intestinal lesion scores relative to those of the C group. Furthermore, decreased lesion scores were observed in the Ei/HS35 group relative to those in the Ei group ($P > 0.05$). In support of the gross lesion scores described above (Fig. 3), increased microscopic median lesion scores in the ileum were observed in the Cp/HS35 group relative to those of the Cp group (Fig. 4; $P < 0.05$).

### 4.5. Relative expression of cytokines in the small intestine and cecal tonsils of broilers

To facilitate the presentation of the data on the relative expression of cytokines due the distinct pattherns in each intestinal portion of the experimental animals by group, the duodenal, jejunal, ileal and cecal tonsils results are presented separately. This was accomplished for the representation of cytokines IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, IL-13, IL-16, IL-17, LITAF, MIP1α, MIP3α, IFN-α, IFN-γ, TNF and TGF-β. The results were subsequently divided into subtopics for MyD88, TLR2, and TLR4 expression data analysis.

#### 4.5.1. Duodenum

Fig. 5 details the relative expression of cytokines regarding the challenges imposed on the animals in this study. The Ei + Cp group exhibited increased cytokine expression after the infectious challenges relative to the other groups, except IL-6, that produces a cytokine profile similar to stressed animals. The Cp and Ei groups presented similar cytokine expression patterns opposed to the stressed animals that present a similar pattern (C/HS35, Cp/HS35, and Ei + Cp/HS35 groups). The IL-6, IL-17, MIP3α, and MIP1α expression were excluded from the multivariate regression analysis due the absence of correlation with other cytokines that were not observed in the other cytokines (IFN-α, IFN-γ, IL-1, IL-2, IL-4, IL-10, IL-12, IL-13, IL-16, LITAF, TGF-β, TNFSF15) analyzed in all of the intestinal regions. The experimental groups that exhibited high correlations between cytokines were specified in Table 2. The heat stressed animals from groups Ei and Cp (Ei/HS35 and CP/HS35 groups) presented a Th2 cytokine shift when compared to non-stressed animals (Ei and Cp groups; Table 2). Otherwise the Ei + Cp/HS35 group presented a Th1 shift when compared to the Ei + Cp group. Supplementary Fig. 3 shows the entire correlation matrix graphs. Supplementary Table 3 shows the means and standard errors of the relative duodenal cytokine expressions.

#### 4.5.2. Jejunum

Fig. 6 details the occurrence of small variations in expression among

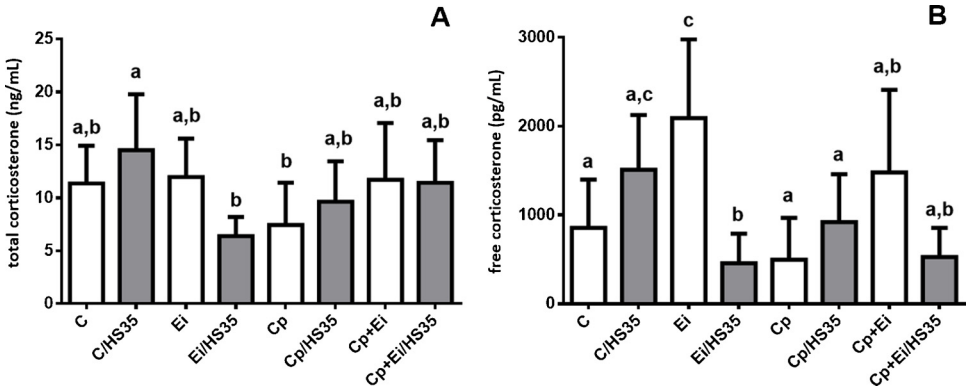


**Fig. 1.** Serum levels of free and total corticosterone in broilers. The bars represent the average serum corticosterone levels per group ± standard deviation. Control group (C); Group infected with *Eimeria* spp. (Ei); group infected with *Clostridium perfringens* (Cp); group infected with *Clostridium perfringens* + *Eimeria* spp. (Ei + Cp). '/HS35' beside the group name indicates the presence of heat stress. Different letters on the averages indicate significant differences with $P < 0.05$ (Tukey HSD test).

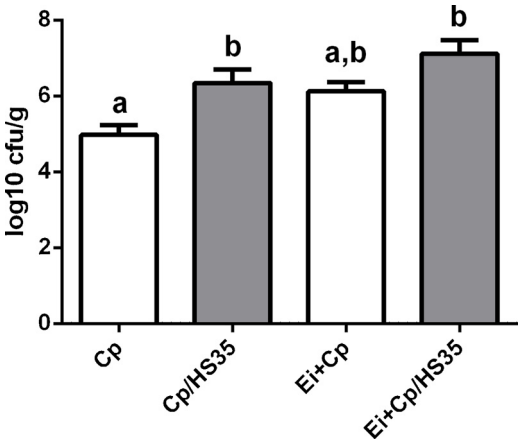


**Fig. 2.** Effects of heat stress on *Clostridium perfringens* counts in the cecum of broiler chickens. Average ± standard deviation cfu/g of *C. perfringens* within the cecal content. The letters above the bars indicate significant differences at $P < 0.05$ (Tukey's HSD test). Group infected with *Eimeria* spp. (Ei); group infected with *Clostridium perfringens* (Cp); group infected with *Clostridium perfringens* + *Eimeria* spp. (Ei + Cp). '/HS35' beside the group name indicates the presence of heat stress. Different letters on the averages indicate significant differences with $P < 0.05$ (Tukey HSD test).

cytokines in the C and Cp groups. The application of heat stress increased cytokine expression in the Ei + Cp/HS35 group when compared to the Ei + Cp group (Fig. 6). Multivariate regression analysis revealed high positive correlations between some cytokines (such as IFN-α, IFN-γ, IL-4, IL-13, IL-16, LITAF, TGF-β4, TNFSF15) in the different experimental groups (Table 3). The heat stressed animals from groups Ei + Cp (Ei + Cp/HS35) presented a Th2 cytokine shift when compared to non-stressed animals (Ei + Cp; Table 3). Supplementary Fig. 4 shows the entire correlation matrix graphs. The means and standard errors of the relative expression of the jejunal cytokines are presented in Supplementary Table 4.

#### 4.5.3. Ileum

Fig. 7 details the data on cytokine expression in the ileum of chickens. It is feasible to highlight the data of the Ei + Cp/HS35 group because cytokines were highly expressed in this group and also the distance between this group and the other groups was maximal, except the MIP1α levels (Fig. 7). Furthermore, we observed proximity between the data from the C, Cp and Ei groups suggesting that these groups exhibit similar cytokine expression. Multivariate regression analysis revealed cytokine correlation patterns in the different experimental groups (Table 4). The heat stressed animals from groups C, Ei and Cp (C/HS35, Ei/HS35 and CP/HS35 groups) presented a Th2 cytokine shift when compared to non-stressed animals (C, Ei and Cp groups; Table 2).

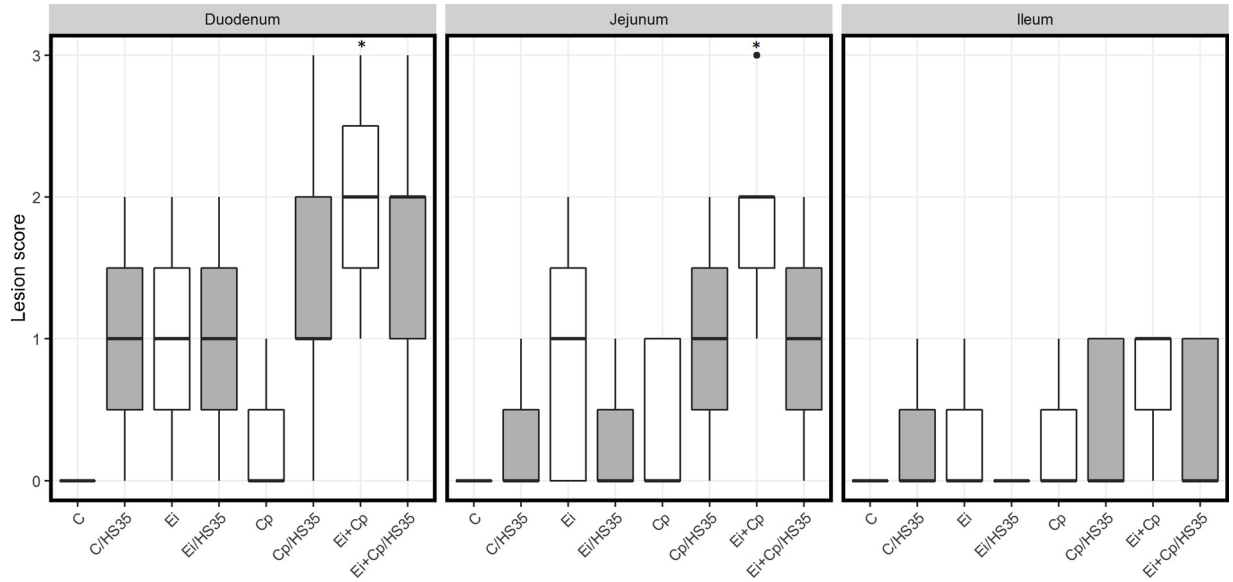


**Fig. 3.** Gross lesion scores in the duodenum, jejunum and ileum of broilers. The box plots represent the median plus the maximum and minimum lesion scores per group. * represent statistically significant differences relative to the C group data (Kruskal-Wallis test). Control group (C); group infected with *Eimeria* spp. (Ei); group infected with *Clostridium perfringens* (Cp); group infected with *Clostridium perfringens* + *Eimeria* spp. (Ei + Cp). '/HS35' beside the group name indicates the presence of heat stress. ● represents outliers.

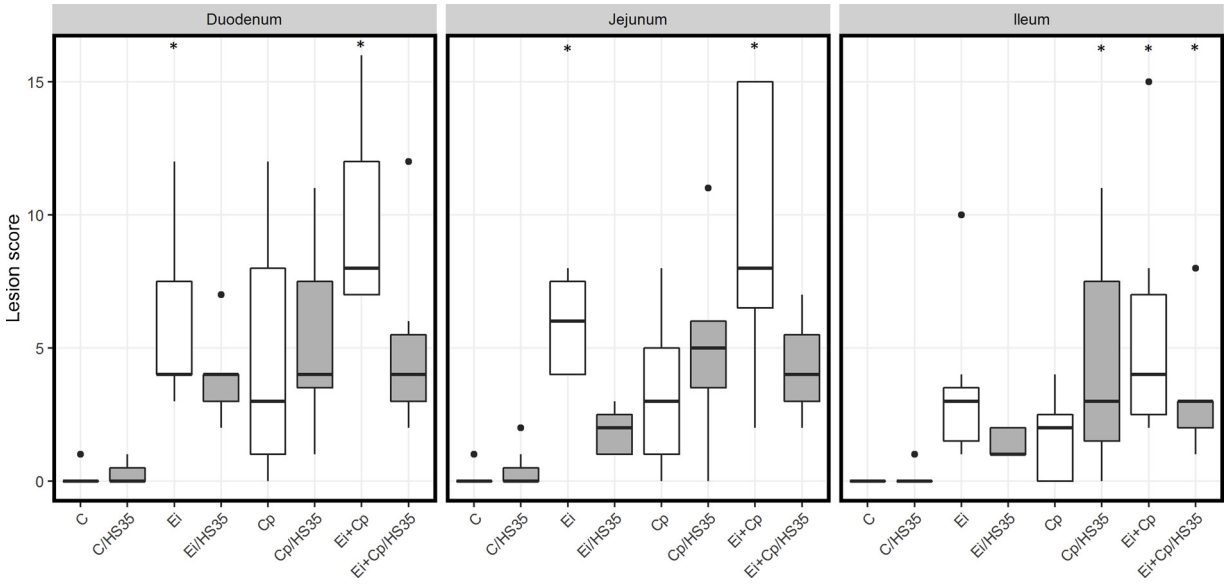


**Fig. 4.** Scores of microscopic lesions in the duodenum, jejunum and ileum of broilers. The box plots represent the median plus the maximum and minimum scores by group. * represents a statistically significant difference relative to the C group (Krukal-Wallis test). Control group (C); group infected with *Eimeria* spp. (Ei); group infected with *Clostridium perfringens* (Cp); group infected with *Clostridium perfringens* + *Eimeria* spp. (Ei + Cp). '/HS35' beside the group name indicates the presence of heat stress. ● represents outliers.

Supplementary Fig. 5 shows the entire correlation matrix graphs. The data on cytokine expression in the ileum of chickens are presented in Supplementary Table 5.

#### 4.5.4. Cecal tonsil

The heat map of cytokine expression in the cecal tonsils did not indicate a characteristic pattern (Fig. 8). However, multivariate regression analysis revealed statistically significant correlations between some cytokines, such as IL-4, IL-10, LITAF, TNFSF15 in the Ei + Cp group (Table 5). Supplementary Fig. 6 shows the entire correlation matrix graphs. Data on the relative expressions of cytokines in the cecal tonsils are presented in Supplementary Table 6.

## 5. Discussion

Glucocorticoids are considered classical markers of stress. However, significant differences in the total and free corticosterone levels between controlled stressed and non-stressed birds (C and C/HS35

**Table 2**
Groups of highly positively correlated cytokines in the duodenum.

| Group | High correlation cytokine cluster |
|---|---|
| C | Transition pattern |
| C/HS35 | Transition pattern |
| Ei | LITAF, IFN-γ, IL-12, IL-16 |
| Ei/HS35 | IL-1, TNFSF15, LITAF, IL-10, IL-12, IL-13, IFN-γ |
| Cp | IFN-α, IFN-γ, IL-2, IL-10, IL-12 |
| Cp/HS35 | IFN-γ, IL-2, IL-10, IL-13, TNFSF15 |
| Ei + Cp | IL-2, IL-10, IL-13, IFN-α, TGF-β4 |
| Ei + Cp/HS35 | IFN-α, IFN-γ, LITAF, IL-2, IL-10, IL-12, IL-13, TNFSF15 |

Note: data extracted from the correlation matrix from Supplementary Fig. 1. Control group (C); group infected with *Eimeria* spp. (Ei); group infected with *Clostridium perfringens* (Cp); group infected with *Clostridium perfringens* + *Eimeria* spp. (Ei + Cp). '/HS35' beside the group name indicates the presence of heat stress.

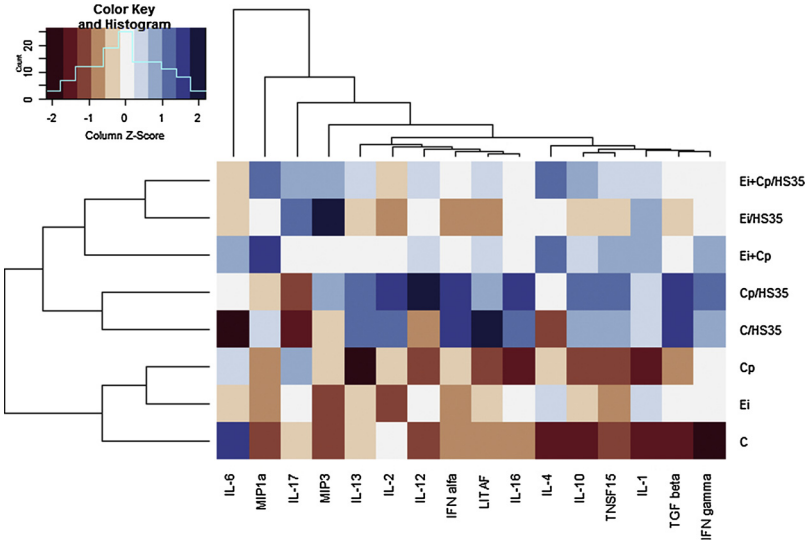


**Fig. 5.** Heat map of Z-score hierarchical clustering based on the cytokines relative expression in the duodenum of chickens. The data were normalized to the mean of the control group (C). Groups within the rows and columns were made to identify the similarities between cytokines and were based on the Euclidean distance and complete agglomeration method. The distances were represented by dendrograms depicted in the x- and y-axes. Control group (C); group infected with *Eimeria* spp. (Ei); group infected with *Clostridium perfringens* (Cp); group infected with *Clostridium perfringens* + *Eimeria* spp. (Ei + Cp). '/HS35' beside the group name indicates the presence of heat stress.

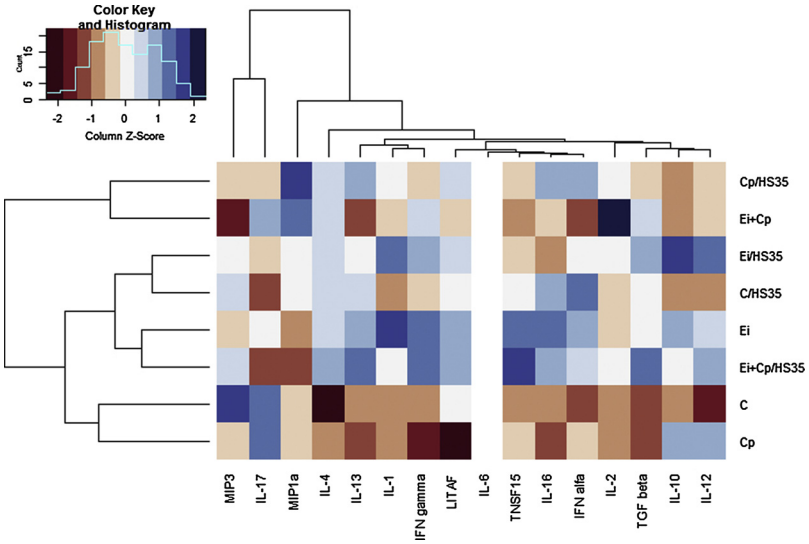


**Fig. 6.** Heat map of Z-score hierarchical clustering based on the cytokines relative expression in the jejunum of chickens. The data were normalized to the mean of the control group (C). Groups within the rows and columns were made to identify similarities between cytokines and were based on the Euclidean distance and complete agglomeration method. The distances were represented by dendrograms depicted in the x- and y-axes. Control group (C); group infected with *Eimeria* spp. The (Ei); group infected with *Clostridium perfringens* (Cp); group infected with *Clostridium perfringens* + *Eimeria* spp. (Ei + Cp). '/HS35' beside the group name indicates the presence of heat stress.

**Table 3**
Groups of highly positively correlated cytokines in the jejunum.

| Group | High correlation cytokine cluster |
|---|---|
| C | Transition pattern |
| C/HS35 | IFN-α, IL-2, IL-10, TGF-β4 / IFN-γ, IL-4, IL-13, TNFSF15 |
| Ei | IFN-α, IL-2, IL-4, IL-10, LITAF, TGF-β4 |
| Ei/HS35 | IFN-α, IFN-γ, IL-2, IL-10, TGF-β4 / IFN-α, IL-1, IL-4, TNFSF15 |
| Cp | IL-2, IL-10, IL-16, LITAF, TGF-β4 |
| Cp/HS35 | Transition pattern |
| Ei + Cp | IFN-α, IFN-γ, IL-4, IL-13, IL-16, LITAF, TGF-β4, TNFSF15 |
| Ei + Cp/HS35 | IFN-α, IL-13, IL-16, LITAF, TGF-β4 |

Note: data extracted from the correlation matrix from Supplementary Fig. 2. Control group (C); group infected with *Eimeria* spp. (Ei); group infected with *Clostridium perfringens* (Cp); group infected with *Clostridium perfringens* + *Eimeria* spp. (Ei + Cp). '/HS35' indicates the groups stressed by heat. "/" between cytokines delimit highly correlated groups.

**Table 4**
Groups of highly positively correlated cytokines in the ileum.

| Group | High correlation cytokine cluster |
|---|---|
| C | Transition pattern |
| C/HS35 | IL-2, IL-4, IL-10, IL-12, IFN-α |
| Ei | Transition pattern |
| Ei/HS35 | IFN-α, IL-2, IL-10, IL-12, TGF-β4, LITAF |
| Cp | Transition pattern |
| Cp/HS35 | IFN-α, TGF-β4, IL-2, IL-4, IL-10, IL-12, LITAF |
| Ei + Cp | IFN-α, IFN-γ, IL-10, IL-13, IL-16, TGF-β4, TNFSF15 |
| Ei + Cp/HS35 | IFN-α, IL-1, IL-10, IL-12, IL-16, LITAF |

Note: data extracted from the correlation matrix from Supplementary Fig. 3. Control group (C); group infected with *Eimeria* spp. (Ei); group infected with *Clostridium perfringens* (Cp); group infected with *Clostridium perfringens* + *Eimeria* spp. (Ei + Cp)./HS35 next to group names indicates the presence of heat stress."/" between cytokines delimit highly correlated groups.

groups) were not detected in the current study. The lack of corticosterone-related changes after the application of multiple or intense stressors have been reported in birds previously (McFarlane and Curtis, 1989) and was likely due to the activation of negative HPA-axis feedback loops (Vandenborne et al., 2005). However, the current data demonstrated that the *Eimeria*-induced infections acted as a stressor *per se* as it increased the levels of free corticosterone in the Ei group relative to controls. Their intensity might explain differences in corticosterone responses to stressors among the different experimental groups. Calefi et al. (2014; 2016a,b) reported decreased inflammatory intestinal responses and decreased intestinal injuries in chickens, appearing in the presence of small variations in serum corticosterone levels. Similar

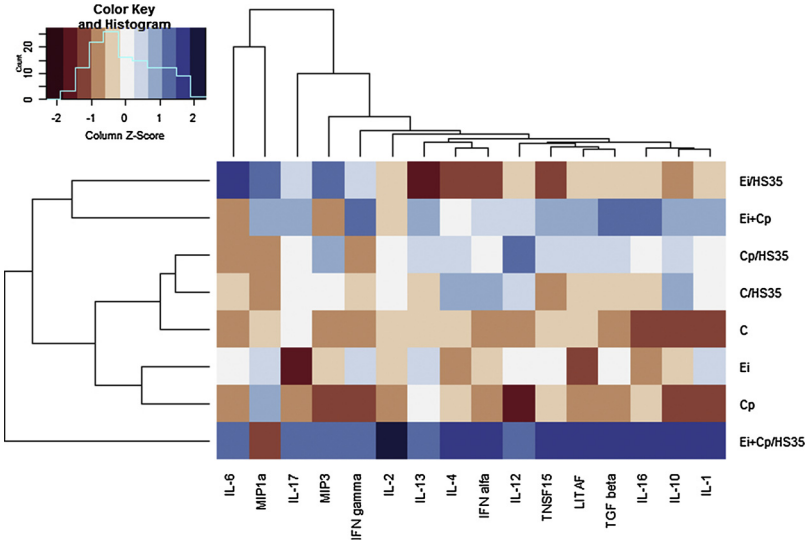


**Fig. 7.** Heat map of Z-score hierarchical clustering based on the cytokines relative expression in the ileum of chickens. The data were normalized to the mean of the control group (C). Groups within the rows and columns were made to identify similarities between cytokines and were based on the Euclidean distance and complete agglomeration method. The distances were represented by dendrograms depicted in the x- and y-axes. Control group (C); group infected with *Eimeria* spp. (Ei); group infected with *Clostridium perfringens* (Cp); group infected with *Clostridium perfringens* + *Eimeria* spp. (Ei + Cp). '/HS35' beside the group name indicates the presence of heat stress.

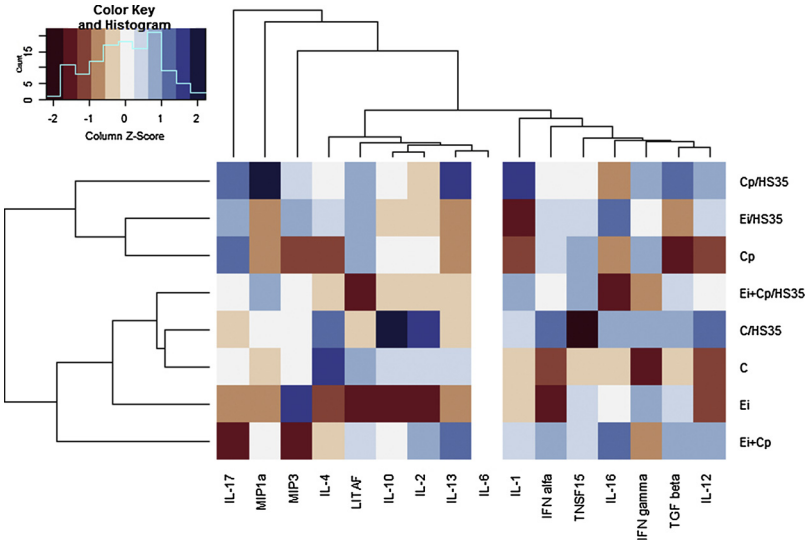


**Fig. 8.** Heat map of Z-score hierarchical clustering based on the cytokines relative expression in the cecal tonsils of chickens. The data were normalized to the mean of the control group (C). Groups within the rows and columns were created to identify similarities between the phenotypes based on the Euclidean distance and the complete agglomeration method. The distances were presented by dendograms depicted in the x- and y-axis. Control group (C); group infected with *Eimeria* spp. (Ei); group infected with *Clostridium perfringens* (Cp); group infected with *Clostridium perfringens* + *Eimeria* spp. (Ei + Cp). '/HS35' beside the group name indicates the presence of heat stress.

**Table 5**
Groups of highly positively correlated cytokines in the cecal tonsil.

| Group | High correlation cytokine cluster |
|---|---|
| C | Transition pattern |
| C/HS35 | IL-2, IL-10, IL-12 / IL-13, IL-16, TNFSF15, IFN-α |
| Ei | IFN-α, IFN-γ, IL-4, TNFSF15 |
| Ei/HS35 | IL-4, IL-12, IL-13, TGF-β4 / IL-1, IL-16, TNFSF15, |
| Cp | IFN-α, IL-4, IL-13, IL-16, TGF-β4 |
| Cp/HS35 | Transition pattern |
| Ei + Cp | IL-4, IL-10, LITAF, TNFSF15 |
| Ei + Cp/HS35 | IFN-γ, IL-4, IL-10, IL-12, TNFSF15 |

Note: data extracted from the correlation matrix from Supplementary Fig. 4. Control group (C); group infected with *Eimeria* spp. (Ei); group infected with *Clostridium perfringens* (Cp); group infected with *Clostridium perfringens* + *Eimeria* spp. (Ei + Cp). '/HS35' beside the group name indicates the presence of heat stress. "/" between cytokines delimit highly correlated groups.

findings were observed in the current study in the Ei/HS35 and Ei + Cp/HS35 groups. Thus, it seems reasonable to suggest a relationship between the variations in free and total corticosterone levels in the infected and/or stressed birds and changes in their intestinal health and immunity.

The data presented indicate that heat stress increased the cfu counts of *C. perfringens* in the cecum of stressed animals (Cp/HS35 group) when compared to the unstressed control animals (Cp group). This result is in agreement with previous studies that reported an increase in the number of *C. perfringens* cfu in the cecum of poultry stressed by cold (Tsiouris et al., 2015a,b) or overcrowding (Gomes et al., 2014). According to the literature, heat stress increases the elimination of *C. perfringens* into the feces of infected birds thereby predisposing the animals to NE reinfection as a consequence of increased bacterial load. The gross and histopathological intestinal analysis revealed that the number of cfu found in the cecum was positively correlated with the injury scores observed in the small intestines of animals in the Cp and Cp/HS35 groups. The increased macroscopic lesion scores of animals in the Cp/HS35 group relative to the Cp group may be a consequence of heat stress.

The present data contrast with those previously reported (Calefi et al., 2014). We reported decreased scores of intestinal injuries in heat stressed animals infected with *C. perfringens*. Differences between the current and previous studies seem related, at least in terms of two main factors: the presence of thioglycolate broth medium as a predisposing factor for *C. perfringens* development in our previous study and the current application of intermittent heat stress. We reported that the application of long-term and continuous heat stress in an NE experimental model using thioglycolate broth medium as a predisposing factor for infection development reduced the intestinal inflammation triggered by the irritant, thereby decreasing *C. perfringens* pathogenesis. Furthermore, the intermittent heat stress applied to chickens in the current model may have allowed heterophil migration into the site of infection during the periods of thermal comfort, which may not have happened after continuous and long-term heat stress used in our previous study.

Changes in heterophil migration are known to enhance tissue injuries (Calefi et al., 2014; Harmon, 1998). Heat stress increased the lesion scores induced in chickens by *C. perfringens*. Interestingly, it produced the opposite effects during *Eimeria* spp. induced infection. Indeed, the lesion scores in the duodenum, jejunum, and ileum of Ei/HS35 animals were decreased when compared to those of the Ei group. Banfield et al. (1998) reported that heat stress decreases *Eimeria acervulina* oocyst load and elimination in broiler chickens. A decrease in the pathogenesis of *Eimeria* after the application of heat stress may explain the reduction in macroscopic and microscopic lesion scores observed in the Ei + Cp/HS35 group relative to the Ei + Cp group. Thus, it is reasonable to hypothesize that the decrease in lesions observed in the intestinal epithelium of birds in the Ei + Cp/HS35 group changed *C. perfringens* development in the infected birds and as a consequence, the course of NE.

The primary determinant of the development and progression of infections is the immunity of the host. After initial *Eimeria* spp. infection B cells present in GALT were shown to initiate the production of specific antibodies against the parasite that induced lymphocyte activation to ensure mucosal immunity (Lillehoj et al., 2004; Yun et al., 2000). In turn, C. *perfringens* infection was reported to produce tissue injury by releasing toxins that produce tissue necrosis in a continuous process that ends in the extensive mobilization of heterophils and lymphocytes into the infection site (Cooper and Songer, 2009; Parish, 1961; Thompson et al., 2006).

The use of histopathological examination in this study leads us to valuable insights that are lost when tissues are macerated for analysis of cytokine expression. The description of morphological changes related to inflammatory processes and tissue damage are crucial for the understanding of cytokine production (Hong et al., 2006). During an infectious process, in addition to the direct reaction of the immune system against the pathogen, cellular damage, and type of inflammatory infiltrate helps us to understand the expression of cytokines and to understand the pathogenesis of the disease in multifactorial processes such as the one presented in this study. Besides, due to the small

amount of information on the cytokine balance in broilers under different field conditions, it is essential to verify whether the mammalian cytokine pattern interpretation is effectively applied in poultry studies.

*Eimeria* spp. and *C. perfringens* were reported to activate cytokine release and the migration of inflammatory cells that modulate the host immune system in different ways. Stressors were also reported to modulate the production and release of cytokines and other relevant host inflammatory mediators via HPA-axis activation and corticosterone release (Shini et al., 2010). Thus, it is reasonable to maintain that the immunomodulatory phenomena of the different experimental groups in the current study are distinct. It should not be forgotten, however, that this different profile of cytokine and inflammatory activation might also rely on *Eimeria* spp. strains and/or on the genotypic characteristics of *C. perfringens*.

The current study demonstrated that cytokine expression in the different groups varied depending on the intestinal segment. Thus, increased expression of cytokines, such as IFN-γ, was observed in the duodenum whereas the expression of the same cytokines was noticed in the jejunum and ileum. Furthermore, the cytokine expression measured in the current study exhibited distinct correlations between them in the different segments of the intestine. Therefore, it is difficult to propose a single and direct role for cytokines in the different groups in the current study.

An important finding in this study was the lack of well-defined correlations between the expressions of the different cytokines in the control group. However, heat stress (C/HS35 group) leads to high indexes of positive correlations among the cytokines IFN-α, IL-2 and IL-10 suggesting that heat stress increased the Th2 response in birds. Thus, it is possible that IL-10 expression decreased the Th1 response while simultaneously increasing the B cell response as observed in the jejunum of chickens in the C/H35 group. It has been demonstrated previously that the Th2 cytokines are related to changes in immunoglobulin secretion and mucus production by Globet cells (Honda et al., 2015).

Heat stress modulates the Th1/Th2 cytokine profile in the intestines of chickens, as has already been shown in mammals (Min et al., 2001). This may be related to the increased number of Globet cells (Kondo et al., 2002) and mucus production (Calefi et al., 2014) already demonstrated after stress (Calefi et al., 2014). These changes may have changed the pathogenesis of *C. perfringens*. Data from the cecal tonsils of birds in the C/HS35 group support the reported increase in the Th2 response after heat stress. Furthermore, IL-12 was found to be correlated with IL-13, IL-16 and TNFSF15 in the cecal tonsils of heat-stressed animals. These cytokines can stimulate Th2 responses and macrophage activation.

Decreased Th2 responses were observed in the Ei group. However, each segment of the *Eimeria* spp. infected bowel presented a specific pattern of cytokine correlation. Such variations may reflect responses to the diversity of parasite strains employed in this study. Indeed, it is known that different strains of coccidia colonize different segments of the intestine and in a particular way. However, the application of heat stress in birds (Ei/HS35 group) increased the participation of cytokines in the Th2 responses of animals as evidenced by the increased indexes of positive correlations found among IL-4, IL-10, IL-12, and IL-13 expression. In particular, the stress produced high indexes of correlation between TGF-β and other cytokines analyzed in the Ei/HS35 group, a finding not observed in the non-stressed animals (Ei group). TGF-β is known to be involved in the repair of the intestinal mucosa and has also been reported to inhibit *Toxoplasma gondii* infection (Hunter et al., 1995; Robinson et al., 2000). Thus, the expression of cytokines in the Ei/HS35 group indicates an increase in the immune response against *Eimeria* spp. infection. The cytokine data reported in the current study are consistent with reports on the reduction of the number of *Eimeria* spp. oocysts induced by heat stress and with the effects of corticosterone on immunomodulation (Banfield et al., 1998).

Data from chickens in the Cp group demonstrated that *C. perfringens* infection *per se* stimulated the Th2 response in the duodenum and jejunum. Indeed, a high rate of positive correlation was found between the expression of IL-2 and IL-10 in these tissues. Nevertheless, other cytokines seem to be involved with the inflammatory intestinal processes, such as IL-2/IL-10, IFN-α/ IFN-γ/IL-12 in the duodenum and IL-16/ LITAF and TGF-β in the jejunum of the Cp group. However, there were no clear definitions in the patterns of cytokines expressed in the jejunum of stressed birds in the Cp/HS35 group. This response imbalance induced by heat stress may be related to a decreased immune response to *C. perfringens*. If so, then it would be possible to explain the currently observed tendency to increase intestinal lesion scores in the Cp/HS35 group relative to those of the Cp group. The significant reduction in T-bet expression in the Cp/HS35 group reinforces the findings discussed above. T-bet is known to coordinate the relationships between innate and adaptive immunity (Lazarevic et al., 2013). Together, these data suggest that animals in the Cp/HS35 group might have experienced a reduction in their adaptive immune response and, as a consequence in their ability to fight *C. perfringens*-induced infection.

Chickens in the co-infected groups (Ei + Cp and Ei + Cp/HS35) presented a more pronounced and aggressive process of infection as inferred by the gross and histopathological evaluation of their small intestine. The Ei + Cp/HS35 group exhibited high rates of correlation between LITAF and Th2 cytokines. It was reported that *Eimeria* spp.-induced infection increases the expression of LITAF in interepithelial lymphocytes (IELs) (Hong et al., 2006). Thus, it is possible that heat stress application induced an IEL response and as a consequence, a subsequent increase in the expression of LITAF and TNFSF15 macrophages. In addition, the current data showed that the Ei + Cp/HS35 group exhibited decreased intestinal lesion scores as a consequence of the reported reduction in *Eimeria* spp. infection. Together, these findings might have contributed to the observed decrease in *C. perfringens* infection. Indeed, *Eimeria* spp.-induced infection is a predisposing factor of *C. perfringens* development in chickens.

In short, it is reasonable to suggest that in chickens, heat stress, via activation of the HPA axis and corticosterone release, directs Th2 cytokine responses toward a reduction of *Eimeria* spp. pathogenicity, thereby allowing *C. perfringens* infection in chickens. The complexity of the interactions between heat stress and the induced responses of infected and non-infected chickens may explain the diversity of effects on cytokine expression, intracellular signaling pathways, and membrane receptor activation. Furthermore, although the experimental model used in the current study was effective in NE settlement, the necessity of proper controls and histopathology analysis emerged as undisputable to analyze and understand the data. Finally, it should not be forgotten that possible gut-brain axis activation during infection and heat stress may have also contributed to the neuroimmunomodulatory effects induced by chronic stress. Together, these data indicate the relevance of heat stress control in the host response of birds not only for animal welfare and health but also for their response to pathogens and vaccines and, therefore, successful poultry production.

## Acknowledgments

The authors wish to express their sincere thanks to Costola, C. C., Namazu, L. B., Margatho, R. and Fonseca, J. G. S. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (Processes: 2009/51886-3, 2012/03103-2, 2013/17408-2) and by Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (Processes: 474690/2013-0, 152725/2016-4, 434848/2016-6). These foundations had no role in our study design, data collection and analysis and preparation of the manuscript for publication.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetimm.2019.03.001.

## References


Abdul-Careem, M.F., Hunter, B.D., Sarson, A.J., Mayameei, A., Zhou, H., Sharif, S., 2006. Marek's disease virus-induced transient paralysis is associated with cytokine gene expression in the nervous system. Viral Immunol. 19, 167–176. https://doi.org/10.1089/vim.2006.19.167.

Banfield, M.J., Ten Doeschate, R.A.H.M., Forbes, J.M., 1998. Effect of whole wheat and heat stress on a coccidial infection in broiler chickens. Br. Poult. Sci. 39, 25–26. https://doi.org/10.1080/00071669888179.

Burkholder, K.M., Thompson, K.L., Einstein, M.E., Applegate, T.J., Patterson, J.A., 2008. Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to Salmonella enteritidis colonization in broilers. Poult. Sci. 87, 1734–1741. https://doi.org/10.3382/ps.2008-00107.

Calefi, A.S., Honda, B.T., Costola-de-Souza, C., de Siqueira, A., Namazu, L.B., Quinteiro-Filho, W.M., Fonseca, J.G., Aloia, T.P., Piantino-Ferreira, A.J., Palermo-Neto, J., 2014. Effects of long-term heat stress in an experimental model of avian necrotic enteritis. Poult. Sci. 93, 1344–1353. https://doi.org/10.3382/ps.2013-03829.

Calefi, A.S., de Siqueira, A., Namazu, L.B., Costola-de-Souza, C., Honda, B.B.T., Ferreira, A.J.P., Quinteiro-Filho, W.M., da Silva Fonseca, J.G., Palermo-Neto, J., 2016a. Effects of heat stress on the formation of splenic germinal centres and immunoglobulins in broilers infected by *Clostridium* perfringens type A. Vet. Immunol. Immunopathol. 171, 38–46. https://doi.org/10.1016/j.vetimm.2016.02.004.

Calefi, A.S., Fonseca, J.G., da, S., Cohn, D.W.H., Honda, B.T.B., Costola-de-Souza, C., Tsugiyama, L.E., Quinteiro-Filho, W.M., Ferreira, A.J.P., Palermo-Neto, J., 2016b. Gut-brain axis interactions during heat stress and avian necrotic enteritis. Poult. Sci. 95, 1005–1014.

Cooper, K.K., Songer, J.G., 2009. Necrotic enteritis in chickens: a paradigm of enteric infection by *Clostridium* perfringens type A. Anaerobe 15, 55–60. https://doi.org/10.1016/j.anaerobe.2009.01.006.

Cooper, K.K., Songer, J.G., 2010. Virulence of *Clostridium* perfringens in an experimental model of poultry necrotic enteritis. Vet. Microbiol. 142, 323–328. https://doi.org/10.1016/j.vetmic.2009.09.065.

Gomes, A.V.S., Quinteiro-Filho, W.M., Ribeiro, A., Ferraz-de-Paula, V., Pinheiro, M.L., Baskeville, E., Akamine, A.T., Astolfi-Ferreira, C.S., Ferreira, A.J.P., Palermo-Neto, J., 2014. Overcrowding stress decreases macrophage activity and increases *Salmonella enteritidis* invasion in broiler chickens. Avian Pathol. 43, 82–90. https://doi.org/10.1080/03079457.2013.874006.

Harmon, B., 1998. Avian heterophils in inflammation and disease resistance. Poult. Sci. 77, 972–977. https://doi.org/10.1093/ps/77.7.972.

Honda, B.T.B., Calefi, A.S., Costola-De-Souza, C., Quinteiro-Filho, W.M., Da Silva Fonseca, J.G., De Paula, V.F., Palermo-Neto, J., 2015. Effects of heat stress on peripheral T and B lymphocyte profiles and IgG and IgM serum levels in broiler chickens vaccinated for Newcastle disease virus. Poult. Sci. 94, 2375–2381. https://doi.org/10.3382/ps/pev192.

Hong, Y.H., Lillehoj, H.S., Lee, S.H., Dalloul, R.A., Lillehoj, E.P., 2006. Analysis of chicken cytokine and chemokine gene expression following *Eimeria* acervulina and *Eimeria* tenella infections. Vet. Immunol. Immunopathol. 114, 209–223. https://doi.org/10.1016/j.vetimm.2006.07.007.

Hunter, C.A., Bermudez, L., Beernink, H., Waegell, W., Remington, J.S., 1995. Transforming growth factor-beta inhibits interleukin-12-induced production of interferon-gamma by natural killer cells: a role for transforming growth factor-beta in the regulation of T cell-independent resistance to *Toxoplasma gondii*. Eur. J. Immunol. 25, 994–1000. https://doi.org/10.1002/eji.1830250420.

Hurley, C.B., 2004. Clustering visualizations of multidimensional data. J. Comput. Graph. Stat. 13, 788–806. https://doi.org/10.1198/106186004X12425.

Johnson, J., Reid, W.M., 1970. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. Exp. Parasitol. 28, 30–36. https://doi.org/10.1016/0014-4894(70)90063-9.

Kondo, M., Tamaoki, J., Takeyama, K., Nakata, J., Nagai, A., 2002. Interleukin-13 induces goblet cell differentiation in primary cell culture from Guinea pig tracheal epithelium. Am. J. Respir. Cell Mol. Biol. 27, 536–541. https://doi.org/10.1165/rcmb.4682.

Laboratory Animal Research (US), I., (US), N.A.P, 2010. Guide for the Care and Use of Laboratory Animals. National Academies Press.

Lambert, G.P., 2009. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. J. Anim. Sci. 87, E101–8. https://doi.org/10.2527/jas.2008-1339.

Lazarevic, V., Glimcher, L.H., Lord, G.M., 2013. T-bet: a bridge between innate and adaptive immunity. Nat. Rev. Immunol. 13, 777–789. https://doi.org/10.1038/nri3536.

Lee, K.W., Lillehoj, H.S., Jeong, W., Jeoung, H.Y., An, D.J., 2011. Avian necrotic enteritis: experimental models, host immunity, pathogenesis, risk factors, and vaccine development. Poult. Sci. 90, 1381–1390. https://doi.org/10.3382/ps.2010-01319.

Lillehoj, H.S., Min, W., Dalloul, R.A., 2004. Recent progress on the cytokine regulation of intestinal immune responses to Eimeria. Poult. Sci. 83, 611–623. https://doi.org/10.1093/ps/83.4.611.

McFarlane, J.M., Curtis, S.E., 1989. Multiple concurrent stressors in chicks.: 3. effects on plasma corticosterone and the Heterophil: Lymphocyte ratio. Poult. Sci. 68, 522–527. https://doi.org/10.3382/ps.0680522.

McReynolds, J.L., Byrd, J.A., Anderson, R.C., Moore, R.W., Edrington, T.S., Genovese, K.J., Poole, T.L., Kubena, L.F., Nisbet, D.J., 2004. Evaluation of immunosuppressants and dietary mechanisms in an experimental disease model for necrotic enteritis. Poult. Sci. 83, 1948–1952.

Min, W., Lillehoj, H.S., Burnside, J., Weining, K.C., Staeheli, P., Zhu, J.J., 2001. Adjuvant effects of IL-1β, IL-2, IL-8, IL-15, IFN-α, IFN-γ TGF-β4 and lymphotactin on DNA vaccination against Eimeria acervulina. Vaccine 20, 267–274. https://doi.org/10.1016/S0264-410X(01)00270-5.

Parish, W.E., 1961. Necrotic enteritis in the fowl (Gallus gallus domesticus). I. Histopathology of the disease ans isolation of a strain of Clostridium welchii. J. Comp. Pathol. 71, 377–393.

Quinteiro-Filho, W.M., Gomes, A.V.S., Pinheiro, M.L., Ribeiro, A., Ferraz-de-Paula, V., Astolfi-Ferreira, C.S., Ferreira, A.J.P., Palermo-Neto, J., 2012. Heat stress impairs performance and induces intestinal inflammation in broiler chickens infected with *Salmonella* Enteritidis. Avian Pathol. 41, 421–427. https://doi.org/10.1080/03079457.2012.709315.

Robinson, P., Okhuysen, P.C., Chappell, C.L., Lewis, D.E., Shahab, I., Lahoti, S., White, A.C., 2000. Transforming growth factor beta1 is expressed in the jejunum after experimental Cryptosporidium parvum infection in humans. Infect. Immun. 68, 5405–5407.

Shane, S., 2005. Re-emergence of necrotic enteritis in the broiler industry. Int. J. Poult. Sci. 4, 604–611.

Shini, S., Huff, G.R., Shini, A., Kaiser, P., 2010. Understanding stress-induced immunosuppression: exploration of cytokine and chemokine gene profiles in chicken peripheral leukocytes. Poult. Sci. 89, 841–851. https://doi.org/10.3382/ps.2009-00483.

Star, L., Nieuwland, M.G., Kemp, B., Parmentier, H.K., 2007. Effect of single or combined climatic and hygienic stress on natural and specific humoral immune competence in four layer lines. Poult. Sci. 86, 1894–1903. https://doi.org/86/9/1894.

Thompson, D.R., Parreira, V.R., Kulkarni, R.R., Prescott, J.F., 2006. Live attenuated vaccine-based control of necrotic enteritis of broiler chickens. Vet. Microbiol. 113, 25–34. https://doi.org/10.1016/j.vetmic.2005.10.015.

Truscott, R.B., Al-Sheikhly, F., 1977. Reproduction and treatment of necrotic enteritis in broilers. Am. J. Vet. Res. 38, 857–861.

Tsiouris, V., Georgopoulou, I., Batzios, C., Pappaioannou, N., Ducatelle, R., Fortomaris, P., 2015a. High stocking density as a predisposing factor for necrotic enteritis in broiler chicks. Avian Pathol. 44, 59–66. https://doi.org/10.1080/03079457.2014.1000820.

Tsiouris, V., Georgopoulou, I., Batzios, C., Pappaioannou, N., Ducatelle, R., Fortomaris, P., 2015b. The effect of cold stress on the pathogenesis of necrotic enteritis in broiler chicks. Avian Pathol. 44, 430–435. https://doi.org/10.1080/03079457.2015.1083094.

Vandenborne, K., De Groef, B., Geelissen, S.M.E., Kühn, E.R., Darras, V.M., Van der Geyten, S., 2005. Corticosterone-induced negative feedback mechanisms within the hypothalamo-pituitary-adrenal axis of the chicken. J. Endocrinol. 185, 383–391. https://doi.org/10.1677/joe.1.05969.

Yun, C.H., Lillehoj, H.S., Lillehoj, E.P., 2000. Intestinal immune responses to coccidiosis. Dev. Comp. Immunol. 24, 303–324.

Zulkifli, I., Abdulllah, N., Azrin, N.M., Ho, Y.W., 2000. Growth performance and immune response of two commercial broiler strains fed diets containing Lactobacillus cultures and oxytetracycline under heat stress conditions. Br. Poult. Sci. 41, 593–597.