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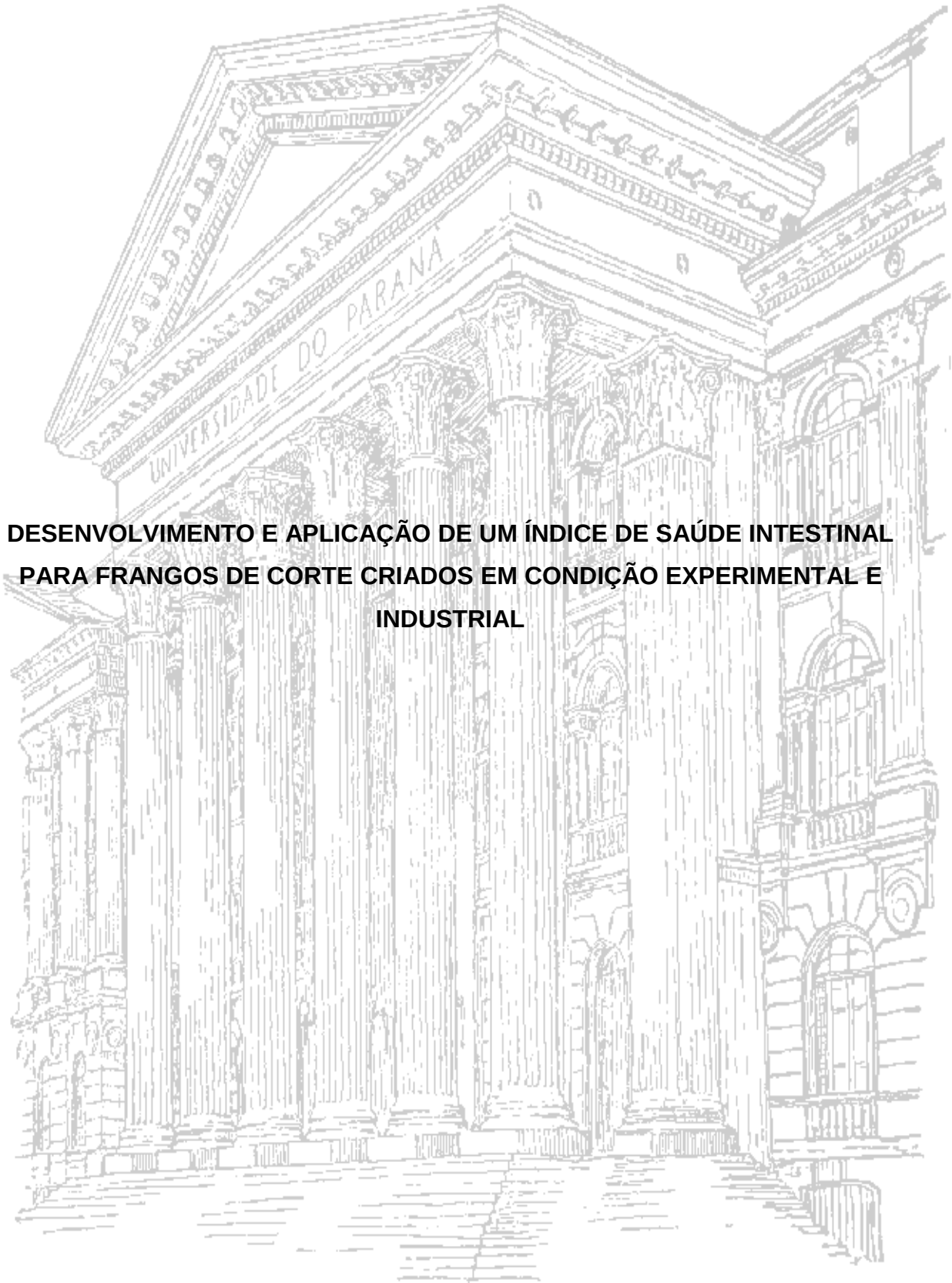

DESENVOLVIMENTO E APLICAÇÃO DE UM ÍNDICE DE SAÚDE INTESTINAL PARA FRANGOS DE CORTE CRIADOS EM CONDIÇÃO EXPERIMENTAL E INDUSTRIAL

CURITIBA

2017

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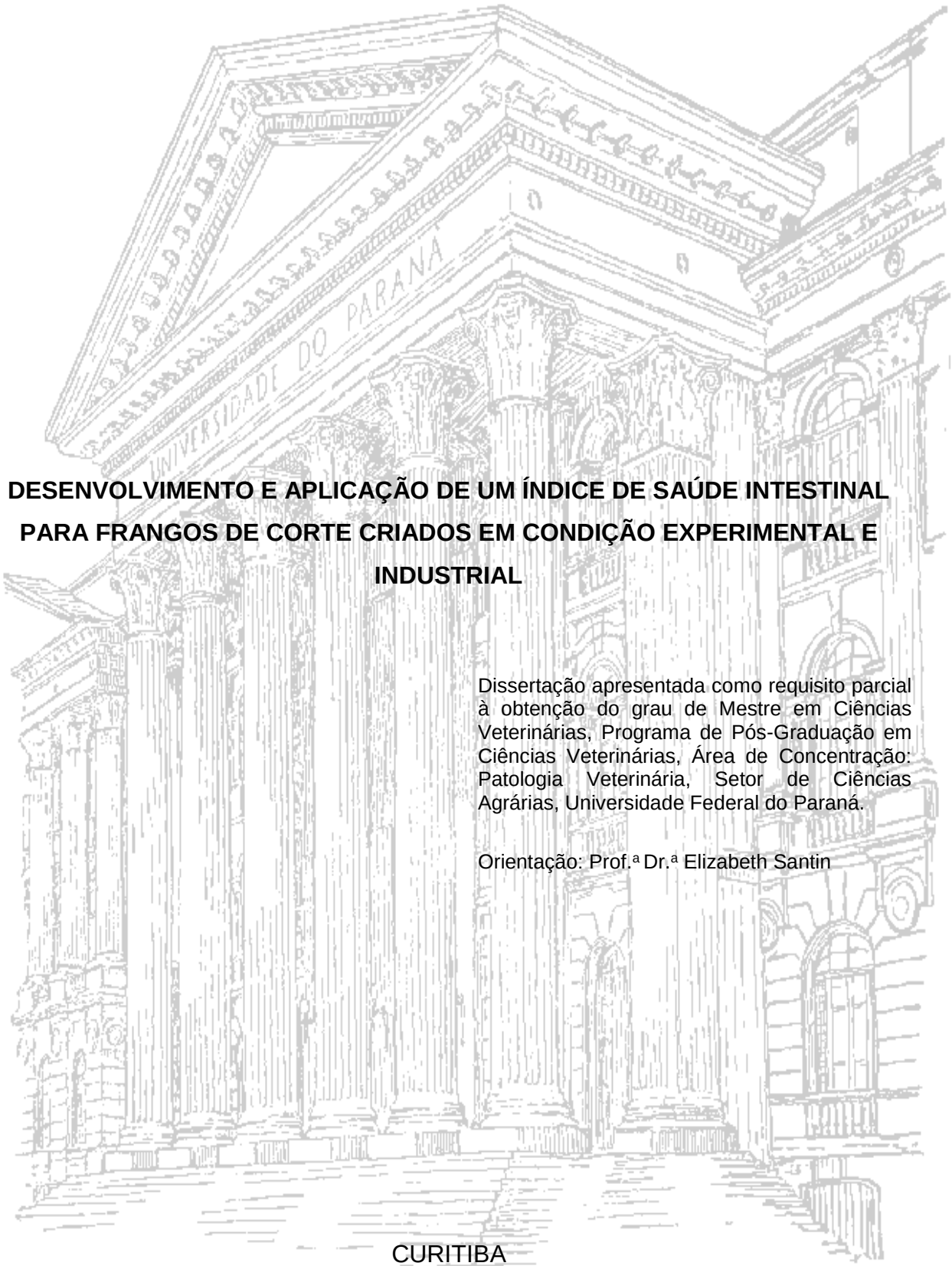

DESENVOLVIMENTO E APLICAÇÃO DE UM ÍNDICE DE SAÚDE INTESTINAL PARA FRANGOS DE CORTE CRIADOS EM CONDIÇÃO EXPERIMENTAL E INDUSTRIAL

Dissertação apresentada como requisito parcial à obtenção do grau de Mestre em Ciências Veterinárias, Programa de Pós-Graduação em Ciências Veterinárias, Área de Concentração: Patologia Veterinária, Setor de Ciências Agrárias, Universidade Federal do Paraná.

Orientação: Prof.ª Dr.ª Elizabeth Santin

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PARECER

A Comissão Examinadora da Defesa da Dissertação intitulada **"DESENVOLVIMENTO E APLICAÇÃO DE UM ÍNDICE DE SAÚDE INTESTINAL PARA FRANGOS DE CORTE CRIADOS EM CONDIÇÃO EXPERIMENTAL E INDUSTRIAL"** apresentada pelo Mestrando **ANTONIO LEONARDO KRAIESKI** declara ante os méritos demonstrados pelo Candidato, e de acordo com o Art. 79 da Resolução nº 65/09–CEPE/UFPR, que considerou o candidato Apto para receber o Título de Mestre em Ciências Veterinárias, na Área de Concentração em Ciências Veterinárias.

Curitiba, 18 de janeiro de 2017

Professora Dra. Elizabeth Santin
Presidente/Orientadora

Dr. Mário Sérgio Assayag Junior
Membro

Professor Dr. Fabiano Montiani Ferreira
Membro

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RESUMO

A saúde intestinal de aves de produção está diretamente relacionada a bons resultados de desempenho zootécnico. Avaliar a saúde intestinal é o primeiro passo para direcionar a tomada de decisão em um sistema de produção. No entanto, não existe um método científico padrão bem estabelecido, que converta alterações patológicas em dados quantitativos e permita fazer comparações entre diferentes fatores. A presente dissertação tenta trazer uma abordagem baseada em um índice matemático, para avaliar a saúde intestinal de frangos de corte, com objetivo de facilitar a comunicação entre veterinários patologistas e outros profissionais que atuam na área avícola, e permitir comparações entre fatores de interesse. Para validar esse índice foram feitos dois experimentos, os quais constituem capítulos da dissertação. O primeiro experimento foi conduzido em ambiente controlado, onde foi avaliado o efeito de inclusão de aflatoxina B1 e desafio vacinal com *Eimeria* sp., em conjunto ou separado, sobre desempenho, imunidade e alterações histológicas em frangos de corte. O segundo experimento foi conduzido em uma empresa de frangos de corte, onde foram comparados frangos criados em aviário convencional e em aviário climatizado (tipo túnel), com relação as alterações histológicas, expressão de mRNA de citocinas IL-10 e IL-12, desempenho e condenação de carcaças no abatedouro. Nos dois experimentos o índice aplicado na histologia apresentou correlação negativa forte com parâmetros de desempenho, o que indica que pode ser aplicado na produção industrial de aves.

Palavras-chave: Índice de saúde intestinal. Histopatologia. Imunidade. Frango de corte. Sistema de escores.

ABSTRACT

The intestinal health of broilers is directly related to good performance results. The evaluation of intestinal health is the first step to take decision in a production system. However, there is not a well established scientific standard method, that turn pathological alterations into quantitative data and allow to make comparisons between different flocks. This dissertation tried to bring an approach based in a mathematical index to evaluate intestinal health of broilers, with the objective of facilitate the communication between veterinarian pathologists and other professionals that work with poultry and make comparisons between factors of interest. In order to validate this index two experiments were carried out, which constitute the chapters of this dissertation. The first experiment was conducted in controlled environment, where it was evaluated the effect of inclusion of aflatoxin B1 and *Eimeria* vaccine challenge, combined or separated, over performance, immunity and histological modifications of broilers. The second experiment was carried out in a poultry company, where broilers reared in conventional and tunnel houses were compared, in relation to histological alterations, mRNA of IL-10 and IL-12 expression, performance and carcass condemnation at slaughterhouse. In both experiments, the index applied in histology was strong negatively correlated with performance parameters, which indicates that it can be applied in the poultry industry production.

Key-words: Intestinal health index. Histopathology. Immunity. Broilers. Scoring system.

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SUMMARY

GENERAL INTRODUCTION

Veterinarians that work with poultry are supposed to deal with population of birds, and it requires an adequate approach that differs from those who deal with individuals. It is important to understand the main factors that affect performance, welfare and health of broilers, to take better decisions. However, due the great number of poultry farms and broilers involved, it is difficult to isolate the factors affecting animal health and well-being.

Once the majority of the diseases present very similar clinical signals in live animals, the laboratory support is essential. The use of tools like histopathology, hematological and biochemical analysis, serology, and search for infectious agents, may lead to a specific diagnostic. When sampling birds for analysis, it is important to select adequately, choosing from different parts of the rearing environment, evaluating the mean animal size and health appearance. In order to isolate factors, the stratification of sample collection is a useful way to reach the main cause of losses by analyzing the data statistically.

In broiler production, the gastrointestinal tract development is highly correlated with animal performance (MAIORKA et al., 2000; SANTIN et al., 2001). There are many factors that impair intestinal health, such as nutritional (composition of diet, quality of ingredients, toxins, water quality), environmental (management, house type, litter type) and infectious agents. Evaluating these factors in a numerical way could be useful for monitoring and taking decisions when appropriated. Scoring methods have been object of various studies, as applied to evaluate coccidiosis (Johnson and Reid, 1970), macroscopic (KEIRS et al., 1991; TEIRLYNCK et al., 2011) and microscopic changes (GHOLAMIANDEHKORDI et al., 2007), tibial dyscondroplasia (PELICIA et al., 2012), and welfare (BLATCHFORD; FULTON; MENCH, 2015). In our Lab, we

developed an index, “I See Inside” (ISI), to evaluate macro and microscopic alterations in the same approach, which is the object of this study.

The protozoa of the generous *Eimeria* and the mycotoxin Aflatoxin B1 produce severe impact over the gut health and performance of broilers. Many studies (ELLAKANY et al., 2011; GIRGIS et al., 2010; HUFF; RUFF, 1982; STOEV; KOYNARSKY; MANTLE, 2002) showed that the combination of mycotoxicosis and coccidiosis generates greater decrease in body weight, increase in feed conversion and decrease in plasma carotenoid levels, than the coccidiosis by itself. Due this impact, it was used as a model to validate the index.

The environment where broilers are reared is important to ensure a good performance, once heat stress may impair performance, intestinal integrity, and immunological balance (QUINTEIRO-FILHO et al., 2010). Environmental conditions are directly influenced by the type of the structure house. Tunnel ventilated house (TVH) may reduce more efficiently the temperature in heat environment regions (LACY; CZARICK, 1992), and consequently, the performance may be better than conventional house (CH) (LOTT; SIMMONS; MAY, 1998). In addition, the TVH allow to increase the stocking density with no effect on mortality and carcass quality when ventilation rate and air circulation are adequate (DAWKINS; DONNELLY; JONES, 2004; FEDDES; EMMANUEL; ZUIDHOFT, 2002). Nevertheless, in terms of intestinal health, there is no study comparing the structure housing conditions.

Therefore, in an attempt to contribute to a better communication between veterinarians and managers, and to facilitate to make comparisons between different factors, we developed and applied an index to evaluate macro and microscopic alterations in broiler. The objective of this dissertation was to validate the index applying it in two experiments. The first was in controlled environment, in which we

evaluated the effect of aflatoxin B1 and *Eimeria* vaccine challenge and their interaction on intestinal immune response. The second experiment was conduct in a poultry company, where we evaluated broilers reared in conventional and tunnel ventilated houses. In both experiments, we tried to correlate the index with performance parameters.

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CHAPTER 1:

EFFECT OF AFLATOXIN EXPERIMENTAL INGESTION AND *EIMEIRA* VACCINE CHALLENGES ON INTESTINAL HISTOPATHOLOGY AND IMMUNE CELLULAR DYNAMIC OF BROILERS: APPLYING AN INTESTINAL HEALTH INDEX

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EFFECT OF AFLATOXIN EXPERIMENTAL INGESTION AND *EIMEIRA* VACCINE CHALLENGES ON INTESTINAL HISTOPATHOLOGY AND IMMUNE CELLULAR DYNAMIC OF BROILERS: APPLYING AN INTESTINAL HEALTH INDEX

ABSTRACT

The present study evaluated the effect of aflatoxin B1 and *Eimeria* vaccine challenges and their interaction on intestinal morphology, applying the morphometric index ¨I See Inside¨ (ISI). Immune cellular response and broiler chickens performance were also studied. A total of 240 broiler chickens were divided in 2x2 factorial arrangement with 04 treatments, T1: Control diet and no challenge (CON), T2: Aflatoxin B1 (AFLA), T3: Control diet and *Eimeria* challenge (COC), and T4: Aflatoxin B1 and *Eimeria* challenge (AFLA+COC). The mathematical morphometric index ISI was applied to evaluate macro and microscopic alterations. Samples of liver and jejunum were analyzed for macrophages, CD4+ and CD8+ cells counting by immunohistochemistry at 7, 14 and 21 days of age. Chickens challenged with *Eimeria* presented higher ISI of macroscopic alterations associated to *Eimeria* lesion at medium small intestine, lower body weight gain (BWG) and feed intake (FI) and worse feed conversion ratio compared to non-challenged birds. Both *Eimeria* and aflatoxin challenges modulated the immune cells in jejunum and liver, generally increasing the number of macrophages, CD4+ and CD8+ cells in relation to control group. Birds from COC and COC+AFLA groups presented higher ISI histological score in jejunum at 7 and 14 days of age compared to CON and AFLA groups. The reduction of FI and BWG were correlated to high histological ISI and resulted of high presence of immune cells in tissues, suggesting immune response demand. The histological ISI had statistical correlation to broilers performance.

Key-words: Immune cost. Pro-inflammatory response. Intestinal health. Gut immunity. Health index

INTRODUCTION

Regarding broilers production, gastrointestinal tract development is highly correlated with animal performance (MAIORKA et al., 2000; SANTIN et al., 2001). Epithelium of intestinal mucosa has the function of digestion and absorption, also represents an important barrier between the intestine lumen and the animal against a wide spectrum of harmful and immunogenic substances (GOTTARDO et al., 2016). Damages and losses of the epithelium barrier result in inflammation, uncontrolled immune responses and unbalanced organic homeostasis (KITESSA et al., 2014).

Gut health could be affected by nutritional and infectious agents such as mycotoxins (SANTIN et al., 2002, 2003) and *Eimeria* (JANG et al., 2011; LILLEHOJ; TROUT, 1996), respectively, increasing the intestinal mucosa inflammatory process. The acute phase of immune response is the first mechanism of defense and it is related to systemic and metabolic changes (KLASING, 2004; KOGUT; KLASING, 2009). Animal performance could be affected by the association between inflammatory process and intestinal damage (TEIRLYNCK et al., 2011).

In order to evaluate the alteration promoted by nutritional, infectious and environmental agents on animal health scoring methods have been object of various studies. They were applied to evaluate coccidiosis (Johnson and Reid, 1970), macro (KEIRS et al., 1991; TEIRLYNCK et al., 2011) and microscopic alterations (GHOLAMIANDEHKORDI et al., 2007), tibial dyscondroplasia (PELICIA et al., 2012), and welfare (BLATCHFORD; FULTON; MENCH, 2015). There are many researchers and producers looking for some gut health index to be applied in the poultry industry and correlated to animal performance.

Many studies (ELLAKANY et al., 2011; GIRGIS et al., 2010; HUFF; RUFF, 1982; STOEV; KOYNARSKY; MANTLE, 2002) showed that the combination of

mycotoxins and *Eimeria* generates greater decrease in body weight, increase in feed conversion and decrease in plasma carotenoid levels, than the coccidiosis by itself. The objective of this study was to evaluate the effect of aflatoxin B1 and *Eimeria* vaccine challenges and their interactions on intestinal immune response in addition to apply and evaluate a mathematical morphometric index – ISI (I See Inside) in broilers using these nutritional and infectious models.

MATERIALS AND METHODS

This experiment was approved by the Institutional Animal Use Ethics Committee of Agricultural Sciences of the Federal University of Parana (Protocol 028/2015).

ANIMALS, EXPERIMENTAL DESIGN, DIET AND HOUSING

A number of 240 male broiler chickens, Cobb 500, were used from 1 to 21 days of age. The experiment was completely randomized using a 2x2 factorial arrangement with four treatments and four replicates each, with 15 birds in each repetition. T1: Control diet (no detectable concentration of Aflatoxin) and no challenge (CON), T2: Aflatoxin B1 400 ppb (AFLA), T3: Control diet and *Eimeria* challenge (COC), and T4: Aflatoxin B1 400 ppb and *Eimeria* challenge (AFLA+COC). Purified aflatoxin B1 was mixed in the feed and given to the T2 and T4 chickens during all experimental period. Diet samples were analyzed for aflatoxin by LC-MS/MS (Liquid Chromatography coupled to Mass Spectrometry/Mass Spectrometry) methodology and the result were 468 and 393 ppb of aflatoxin for treatments T2 and T4, respectively. *Eimeria* vaccine challenge was performed orally on the third day of age using a live commercial vaccine, 10 times higher than the manufacturer recommended dose (± 3.3

x 10^5 oocysts per bird). The vaccine included seven species of *Eimeria* (*E. acervulina, E.brunetti, E. maxima, E. necatrix, E. praecox, E. tenella* and *E. mitis*).

The experiment was conducted in previously disinfected isolated rooms, with negative pressure, containing vertically stacked cages (replications) with sterilized wood shaving litter (to avoid external contamination), nipple drinkers and temperature and lighting automatic control. Animals were maintained in comfortable temperature according to their age, with feed and water *ad libitum*. The diet was based on corn and soymeal, following Brazilian nutritional recommendations for poultry (ROSTAGNO, 2011).

SCORING SYSTEM METHODOLOGY – ISI

The ¨I See Inside¨ (ISI) methodology in process of patent (INPI BR 1020150036019) was developed based on a numeric score of alteration. In this methodology, an impact factor (IF) is defined for each alteration in macro and microscopic analysis according to the reduction of organ functional capacity, based on previous knowledge of literature and background research. The IF range from 1 to 3, where 3 is the most impactful for the organ function; e.g. necrosis has the highest IF because the functional capacity of affected cells is totally lost. In addition, the extent of each lesion (intensity) or observed frequency compared to non-affected organ is evaluated in each organ/tissue per animal and the score ranges from 0 to 3: score 0 (absence of lesion or frequency), score 1 (alteration up to 25% of the area or observed frequency), score 2 (alteration ranges from 25 to 50% of the area or observed frequency), and score 3 (alteration extent more than 50% of the area or observed frequency). To reach the final value of the ISI index the IF of each alteration is

multiplied by the respective score number, and the results of all alterations are summed.

For macroscopic analysis, broilers were euthanized by cervical dislocation and the carcasses were systematically evaluated following a division into five system groups: locomotor, gastrointestinal associated organs (GIT), intestine, coccidiosis typical lesion and respiratory system, according to Table 1. The sum of all alterations could reach 174, representing the worst health status. The methodology described above is based in the different relations among other organs and the intestinal health. For instance, the poor intestinal health affects the quality of litter which will affect the locomotor system and the tegument (MONTAGNE; PLUSKE; HAMPSON, 2003; WIDEMAN; PRISBY, 2013), indirectly affecting the feeding and drinking accesses worsening the gut status. The presence of yolk sac in birds older than 7 d of age could be associated with a delayed access to feed during first days of life. Respiratory tract is evaluated since it can be used as a “sentinel” tissue for the environmental quality, such as ammonia levels which also will be affected by intestinal health (FENG-XIAN et al., 2012) and it is important to bear in mind that the airs sacs are anatomically related to gut.

For histological alterations, the same calculation was applied. Table 2 describes the histological alterations evaluated in intestine and liver. The scales range from 0 to 54 for intestine, and from 0 to 39 for liver.

Table 1 – ISI macroscopic alterations of different systems and organs evaluated at necropsy

System	Organ	Alteration observed	Impact Factor	Maximum score for system[1]
Locomotor system (Locomotor)	Skin and adnexas	Foot pad lesion/ pododermatitis	1	21
		Pigmentation	1	
		Oral mucosa lesion	1	
	Locomotor system	Hemorrhage presence	1	
		Femoral head necrosis	1	
		Tibial dyscondroplasia	1	
		Bone resistance	1	
Other Gastrintestinal organs (GIT)	Pancreas	Hypertrophic	2	33
		Hypotrophic	2	
	Kidney	Hypertrophic	1	
	Proventriculus	Inflammation	1	
	Gizzard	Erosion	1	
	Yolk	Persistence	1	
	Liver	Red color (congestion)	2	
		Yellow and hypertrophic	2	
		Yellow and hypotrophic	3	
Intestine (Intestine)	Duodenum	Necrosis	3	72
		Inflammatory process on serosa or mucosa layer	1	
		Cell debris and thick mucus on mucosa	2	
	Jejunum	Necrosis	3	
		Inflammatory process on serosa or mucosa layer. Peyer patches pattern	1	
		Cell debris and red thick mucus	2	
		Thickness of intestine wall	2	
	Ileum	Necrosis	3	
		Inflammatory process on serosa or mucosa layer. Peyer patches visible	1	
		Cell debris and red thick mucus	2	
	Caeca	Inflammatory process on mucosa	2	
		Presence of gas	1	
Coccidiosis Lesions (Coccidiosis)	Duodenum	*Eimeria* Lesion	2	30
	Jejunum	*Eimeria* Lesion	3	
	Ileum	*Eimeria* Lesion	3	
	Caeca	*Eimeria* Lesion	2	
Respiratory System (Respiratory)	Trachea	Trachea infection	1	18
	Heart	Hydropericardium	2	
	Airsacs	Airsacculitis	3	

[1]Maximum score for system represents the results considering an observation of score 3 for each alteration, multiplied by the impact factor (fixed value for each alteration) and summed at the final.

Table 2 – ISI histological alterations evaluated in intestine and liver

Organ	Alterations	Impact factor (IF)	Maximum score[1]
Intestine	Lamina propria thickness	2	54
	Epithelial thickness	1	
	Enterocytes proliferation	1	
	Epithelial plasma cell infiltration	1	
	Mixed inflammatory infiltration in the lamina propria	3	
	Goblet cells proliferation	2	
	Congestion	2	
	Necrosis (apical karyolysis)	3	
	Presence of oocysts	3	
Liver	Congestion	1	39
	Hydropic degeneration	1	
	Cell vacuolation	2	
	Bile-duct proliferation	3	
	Immune cells infiltration	1	
	Pericholangitis	3	
	Lymphocytic aggregate	2	

[1]Maximum score represents the results considering an observation of score 3 for each alteration, multiplied by the impact factor (fixed value for each alteration) and summed at the final.

NECROPSY, HISTOLOGY AND IMMUNOHISTOCHEMISTRY

At 7, 14 and 21 days of age, 5 birds per treatment were euthanized by cervical dislocation. These chickens were necropsied for ISI macroscopic analysis, and samples of jejunum (2 cm proximal to Meckel's diverticulum) and liver (central lobe) were fixed in 10% buffered formalin solution for ISI histological analysis. The same samples were collected and conserved in Tissue-Tek O.C.T. gel, immediately frozen in dry ice and then stocked in a -20°C freezer for immunohistochemistry analysis.

For ISI histological analysis, samples were embedded in paraffin and 5 µm sections were cut and stained with hematoxylin and eosin plus Alcian Blue. For intestinal morphology, a slide per bird was evaluated and villi were divided into merged and normal. Ten intestinal villi per bird were evaluated proportionally to the morphological distribution (merged and normal), in 10X objective (also used 40X

objective to confirm alterations) of an optical microscope (Nikon Eclipse E200). Liver samples were evaluated in 5 fields per bird in 10X objective.

For the immunohistochemical analysis, samples were processed for CD4+, CD8+ T-lymphocyte and macrophage counting according to Lourenço et al. (2015). Immunohistochemistry slides were horizontally placed in a humid incubation chamber and incubated with 100-500 µL of primary specific antibody for macrophages, CD4+ or CD8+ T-lymphocyte. Each antibody was placed in a different slide, washed three times with PBS. The slides were incubated for 30-60 min with HRP-conjugated antibody specific for the primary antibody , then peroxidase activity was blocked using DAB kit for immunocytochemistry (HRP-conjugated rabbit anti-mouse Ig, Dako North America). The slides were counterstained with hematoxylin solution. The labeled cells were counted in an optical microscope (100X magnification objective). Five fields per bird totalizing 25 fields per treatment of jejunum and liver were measured.

PERFORMANCE

Birds were randomly allocated at 1 day of age in order to obtain equal average initial body weight per cage. Birds and feed were weighed weekly (0, 7, 14 and 21 days) to evaluate feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR). All euthanized birds for tissue sampling, as well as by other mortalities causes, were individually weighed for estimating corrected feed conversion for mortality and withdrawn chickens.

STATISTICAL ANALYSIS

The experimental unit for performance was the cage, and for other analysis, the bird. Data were submitted to ANOVA, and significantly different averages ($P < 0.05$)

were compared using the Tukey test at the level of 5% probability using the statistical software Statistix 9 for Windows. Data were presented as means with their standard errors. For correlation analysis, it was used Pearson's correlation coefficient (r) and the software provided the P-values.

RESULTS AND DISCUSSION

Birds challenged with *Eimeria* had high scores of coccidiosis lesions, mainly observed at medium small intestine, and non-challenged birds did not present any lesions (Table 3). ISI score of GIT was high in birds challenged with *Eimeria*. Aflatoxin did not statistically affect any evaluated macroscopic parameter and there was no interaction between the factors in macroscopic analysis. Although aflatoxin could cause macroscopic alterations in various organs and specially in the liver (GIAMBRONE et al., 1985; HUFF et al., 1986; MONSON; COULOMBE; REED, 2015; PANDEY; CHAUHAN, 2007), no difference was observed between the groups, which could be probably related to the level of aflatoxin used in the present study. According to Dalvi and McGowan (1984) in experimental conditions, only levels of 10 ppm of aflatoxin were able to cause significant difference in broilers performance. Broilers fed with low level of aflatoxin (50 to 250 ppb) in experimental conditions did not present any macroscopic lesions in target organs, but had histopathological changes (ORTATATLI et al., 2005; SANTIN et al., 2003). In Figure 1, it is possible to observe the interaction between COC and AFLA in jejunum and liver histology.

Table 3 – ISI macroscopic scores mean and standard error in systems of different treatments at 21 days of age.

Treatment		Locomotor	Respiratory	Other GIT	Intestine	Coccidiosis	Total score
Aflatoxin	No	1.7	0.0	4.7	6.6	0.4	13.5
	Yes	2.2	0.2	3.6	6.4	1.5	14.0
Eimeria	No	1.7	0.2	2.5[b]	7.6	0.0[b]	12.1
	Yes	2.2	0.0	5.9[a]	5.4	1.9[a]	15.4
SEM		0.27	0.12	0.75	0.79	0.45	1.13
				Probabilities			
Aflatoxin (P_1)		0.403	0.337	0.369	0.874	0.143	0.835
Eimeria (P_2)		0.403	0.337	0.016	0.172	0.022	0.192
Interaction (P_1*P_2)		0.999	0.337	0.103	0.221	0.143	0.755

[a,b] Different letters in the same column indicate a significant difference (P < 0.05).

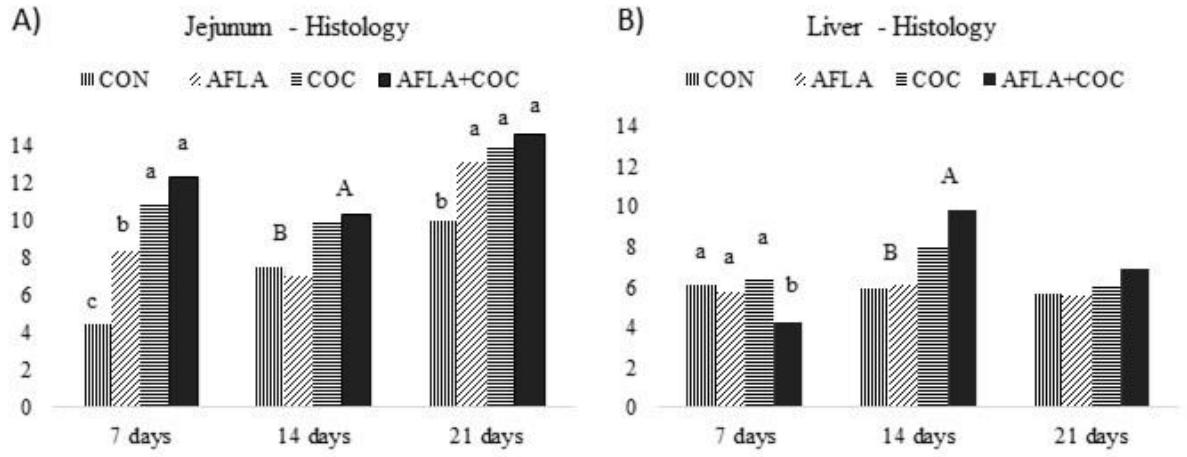

Figure 1 – ISI histological alteration scores in jejunum (A) and liver (B) in different groups of 7, 14 and 21 days of age. Error bars represent standard errors of the mean.
CON: control, AFLA: aflatoxin B1, COC: *Eimeria* challenge, AFLA+COC: aflatoxin B1 and *Eimeria* challenge.
[a,b,c] Different letters indicate a significant difference (P < 0.05).
[A,B] Different letters indicate a significant difference (P < 0.05), in coccidiosis challenge factor.

Eimeria challenge reduced FI and BWG and negatively affected FCR. FI was 11% and 12% lower in *Eimeria* challenged birds from day 1 to 21 and also from day 7 to 21, respectively, compared to non-challenged group (Table 4). There was also an interaction between *Eimeria* and aflatoxin challenge on FI from 14 to 21 days. It was observed higher FI in the group fed with aflatoxin and without *Eimeria*, compared to the group fed with aflatoxin and *Eimeria.* No difference was observed in groups without aflatoxin.

Table 4 – Feed intake (FI, grams) mean and standard error in different periods and treatments

Treatment		FI 1-14 d	FI 7-14 d	FI 1-21 d	FI 7-21 d	FI 14-21 d
Aflatoxin	No	606	462	1120	1014	562
	Yes	642	503	1178	1078	583
Eimeria	No	608	461	1214[a]	1114[a]	601
	Yes	640	504	1083[b]	978[b]	544
Aflatoxin	*Eimeria*					
No	No	597	453	1143	1029	553[ab]
Yes	No	620	469	1285	1199	648[a]
No	Yes	615	472	1096	998	570[ab]
Yes	Yes	665	536	1071	957	519[b]
SEM		15.3	16.4	30.1	33.0	18.4
				Probabilities		
Aflatoxin (P_1)		0.260	0.227	0.243	0.233	0.492
Eimeria (P_2)		0.333	0.198	0.017	0.020	0.090
Interaction (P_1*P_2)		0.673	0.457	0.103	0.062	0.034

[a,b] Different letters in the same column indicate a significant difference ($P < 0.05$).

BWG was affected only by *Eimeria* challenge. The challenged group had 20%, 28%, 17%, 19% and 13% lower BWG in the periods of 1 to 14, 7 to 14, 1 to 21, 7 to 21 and 14 to 21 days, respectively, compared to non-challenged group (Table 5).

Table 5 – Body weight gain (BWG, grams) mean and standard error in different periods and treatments

Treatment		BWG 1-14 d	BWG 7-14 d	BWG 1-21 d	BWG 7-21 d	BWG 14-21 d
Aflatoxin	No	332	224	658	588	370
	Yes	338	223	663	582	356
Eimeria	No	371[a]	260[a]	722[a]	647[a]	388[a]
	Yes	298[b]	187[b]	599[b]	523[b]	338[b]
SEM		10.7	10.4	18.1	17.8	9.0
				Probabilities		
Aflatoxin (P_1)		0.520	0.939	0.774	0.721	0.320
Eimeria (P_2)		0.001	0.001	0.001	0.001	0.003
Interaction (P_1*P_2)		0.052	0.074	0.106	0.191	0.881

[a,b] Different letters in the same column indicate a significant difference ($P < 0.05$).

Feed conversion ratio was worse in *Eimeria* challenged birds on periods from 1 to 14 and 1 to 21 days compared to non-challenged group (Table 6). There was a significant aflatoxin and *Eimeria* challenge interaction in FCR from 7 to 14 days, where AFLA+COC had the worst FCR compared to other treatments. This effect of coccidiosis on performance could be associated to inflammation, which reduce feed

intake and increase requirements demand (JANG et al., 2010; KLASING, 2007). The highest impact on FI was from 7 to 21 days and BWG was from 7 to 14 days, which was associated to the acute phase of immune response linked to the *Eimeria* cycle (ALLEN; FETTERER, 2002). As described in others studies (ELLAKANY et al., 2011; GIRGIS et al., 2010; HUFF; RUFF, 1982; STOEV; KOYNARSKY; MANTLE, 2002), aflatoxin had a boosting effect on coccidiosis in some periods.

Table 6 – Feed conversion ratio (FCR) mean and standard error in different periods and treatments

Treatment		FCR 1-14 d	FCR 7-14 d	FCR 1-21 d	FCR 7-21 d	FCR 14-21 d
Aflatoxin	No	1.84	2.10	1.70	1.73	1.53
	Yes	1.95	2.39	1.78	1.85	1.64
Eimeria	No	1.64[b]	1.77	1.68[b]	1.72	1.56
	Yes	2.15[a]	2.72	1.81[a]	1.87	1.61
Aflatoxin	*Eimeria*					
No	No	1.66	1.79[c]	1.62	1.61	1.42
Sim	No	1.61	1.75[c]	1.74	1.83	1.70
No	Yes	2.01	2.42[b]	1.79	1.85	1.65
Yes	Yes	2.29	3.03[a]	1.82	1.87	1.57
SEM		0.07	0.14	0.02	0.04	0.05
				Probabilities		
Aflatoxin (P_1)		0.185	0.050	0.140	0.104	0.292
Eimeria (P_2)		0.001	0.001	0.018	0.053	0.624
Interaction (P_1*P_2)		0.064	0.027	0.383	0.163	0.077

[a,b] Different letters in the same column indicate a significant difference ($P < 0.05$).

According to the results, *Eimeria* challenge efficacy was verified not only by performance but also by macroscopic (table 3), immunohistochemistry (Figure 2) and histological (figure 1) analysis. In the naive host, coccidia activates local dendritic cells and macrophages allowing various pro-inflammatory chemokines (DALLOUL et al., 2003). Following primary and secondary infections with *Eimeria* an increased percentage of CD8+ T-lymphocytes (CHOI; LILLEHOJ, 2000) and CD4+ T-lymphocytes (BESSAY et al., 1996) is observed 8 days post infection (CHOI; LILLEHOJ, 2000), but the proportion of CD8+ cells is high (ROTHWELL et al., 1995).

In the present study, it was observed a higher number of macrophages in jejunum and liver 4 days after challenge in AFLA+COC compared to other groups, and higher CD8+ cells 11 days after challenge in COC and AFLA+COC compared to other groups. For CD4+ cells at 7 days, it was observed a lower number of this lymphocyte in jejunum and liver when aflatoxin was present (AFLA and AFLA+COC), compared to groups not supplemented with aflatoxin (CON and COC). The role of CD4+ cell in coccidiosis could involve the production of soluble cytokines (YUN; LILLEHOJ; LILLEHOJ, 2000) to increase or reduce the immune response according to the period of infection. The intake of aflatoxin contaminated diet can induce broilers lymphoid organs impairment, characterized by histological lesions (PENG et al., 2015). The present study is in accordance to Peng et al. (2014), who also observed high number of CD8+ cells in broilers fed with diets containing aflatoxin, and low CD4+:CD8+ ratio. CD8+ T cells carry out their protecting role against specific host target cells which are compromised with infection or oncogenic transformation (PRADO-GARCIA et al., 2012; WILLIAMS; BEVAN, 2007). Aflatoxin that has carcinogenic effects (WILD et al., 2000; WOGAN, 1992) and *Eimeria* as intracellular infectious agent, could be related to the increased amount of CD8+ cells in liver and jejunum found in the present study.

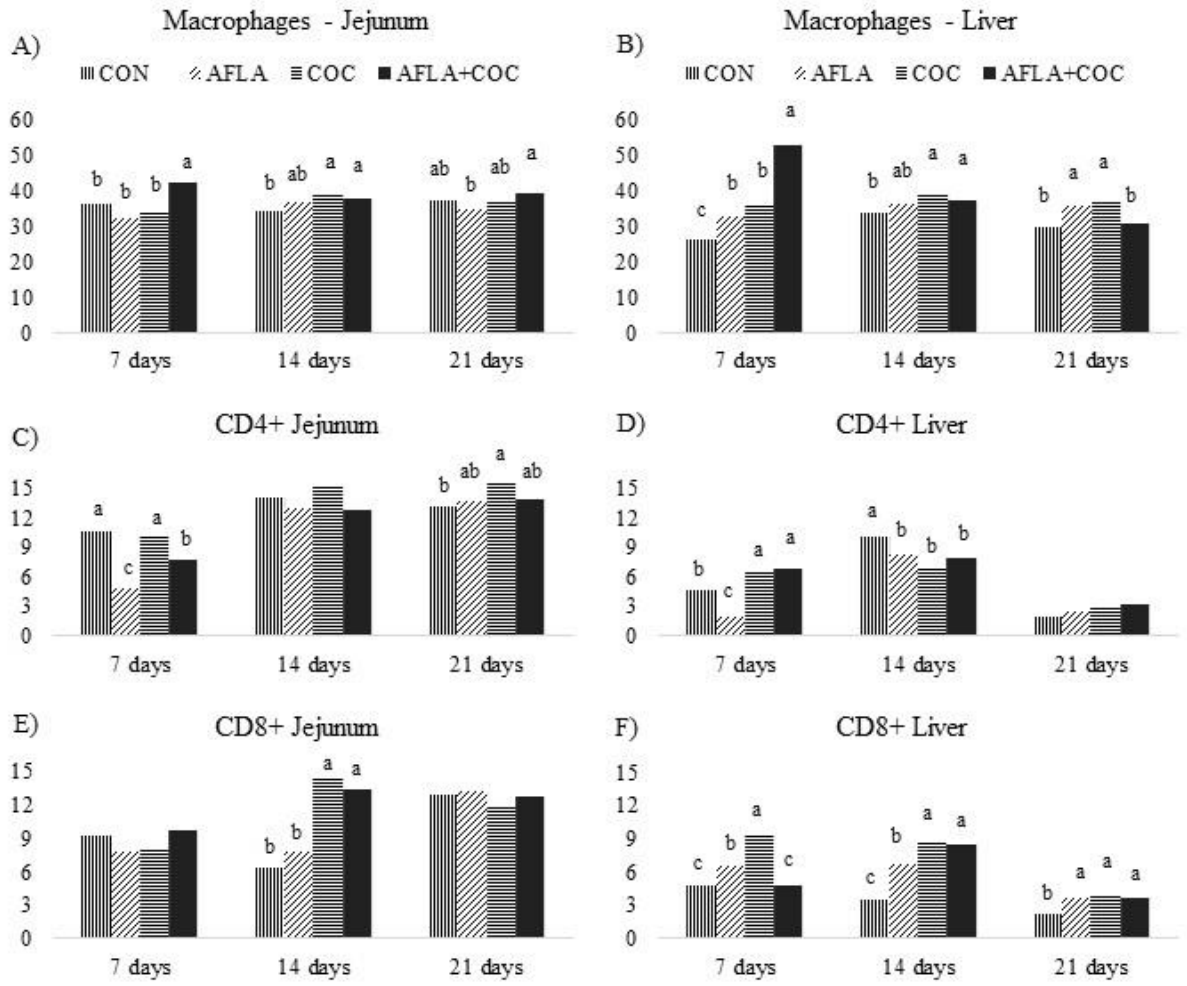

Figure 2 – Changes in immune cell subpopulations (macrophages, CD4+ and CD8+ T-lymphocytes stained by immunohistochemistry) in jejunum and liver in different groups of 7, 14 and 21 days of age. Error bars represent standard errors of the mean.

CON: control, AFLA: aflatoxin B1, COC: *Eimeria* challenge, AFLA+COC: aflatoxin B1 and *Eimeria* challenge.

[a,b,c] Different letters indicate a significant difference ($P < 0.05$).

It was possible to observe that in histological evaluation, at 7 days of age, the groups challenged with *Eimeria* (COC and AFLA+COC) had higher ISI histological alterations in jejunum compared to non-challenged groups (CON and AFLA), mainly because of the presence of mixed inflammatory infiltration in the lamina propria (MILP), goblet cells proliferation, and lamina propria thickness. These scores are correlated to high number of immune cells in the COC groups on immunohistochemistry. It could be associated to the inflammatory response against *Eimeria*, but only the lamina propria thickness had negative statistical correlation with FCR ($r = -0.59$) and no correlation

with IF and BWG was found (Table 7). The acute inflammatory response induces anorexia in birds, characterized by less feeding and small body size, specially observed on the first days post-challenge (JIANG et al., 2010).

In liver histology at 7 days, AFLA+COC group showed the lowest ISI compared to other groups. At this age, birds showed a high physiologic steatosis due to yolk absorption. It was observed that at this age it could be difficult to apply the ISI histology on the liver, once that vacuolation IF is usually high at this period (Figure 3, F). There was also no statistical correlation between the evaluated parameters in liver histology with the performance data (Table 7).

Table 7 – Pearson´s correlation coefficient (r) between evaluated parameters in ISI histological analysis and performance (FI, BWG, and FCR[1]) in the different

ISI histological analysis	r for FI[2]			r for BWG[2]			r for FCR[2]		
	7 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d
Evaluated parameters in jejunum:									
Lamina propria thickness	-0.44	0.29	0.05	0.41	-0.55*	-0.29	-0.59*	0.59*	0.55*
Epithelial thickness	0.02	0.04	0.56*	0.22	-0.44	0.73*	-0.11	0.42	-0.34
Enterocytes proliferation	0.00	0.00	0.27	0.25	-0.62*	0.51*	-0.14	0.52*	-0.41
Epithelial plasma infiltration	-0.19	-0.13	-0.54*	-0.11	0.11	-0.65*	-0.08	-0.10	0.23
Mixed inflammatory infiltration in the lamina propria	-0.24	-0.08	-0.07	-0.05	-0.59*	-0.45	-0.16	0.39	0.62*
Goblet cells proliferation	-0.36	0.21	0.55*	-0.32	0.04	0.68*	-0.13	0.09	-0.27
Congestion	-0.12	-0.12	0.46	0.41	-0.20	0.45	-0.32	0.08	-0.04
Necrosis / apical karyolysis	-	0.01	0.34	-	0.20	0.35	-	-0.19	-0.04
Presence of oocysts	-0.10	0.00	-0.37	-0.17	-0.76*	-0.74*	0.00	0.61*	0.67*
Total score	-0.39	0.08	-0.01	0.13	-0.72*	-0.48*	-0.39	0.62*	0.78*
Evaluated parameters in liver:									
Congestion	0.09	-0.03	-0.14	-0.21	-0.75*	-0.31	0.19	0.53*	0.29
Hydropic degeneration	0.19	0.02	0.29	-0.37	0.07	0.38	0.37	-0.05	-0.16
Cell vacuolation	0.27	0.07	-0.03	-0.04	-0.06	-0.42	0.25	0.09	0.66*
Bile-duct proliferation	-0.11	0.31	0.02	0.15	0.00	-0.01	-0.19	0.15	0.10
Immune cells infiltration	-0.48*	0.03	0.30	0.06	-0.72*	0.33	-0.42	0.58*	-0.10
Pericholangitis	-	0.11	0.09	-	-0.29	-0.01	-	0.28	0.14
Lymphocytic aggregate	-0.25	0.44	0.35	-0.32	-0.34	0.26	-0.02	0.55*	0.10
Total score	0.27	0.27	0.13	-0.26	-0.43	-0.19	0.37	0.48	0.57*

*Indicate significant Pearson's coefficient correlation ($P < 0.05$).
[1]FI: feed intake; BWG: body weight gain; FCR: feed conversion ratio.
[2]For the correlation analysis, performance parameters were obtained in the periods of 1 to 7, 1 to 14 and 1 to 21 days and histological results were evaluated at 7, 14 and 21 days, respectively.

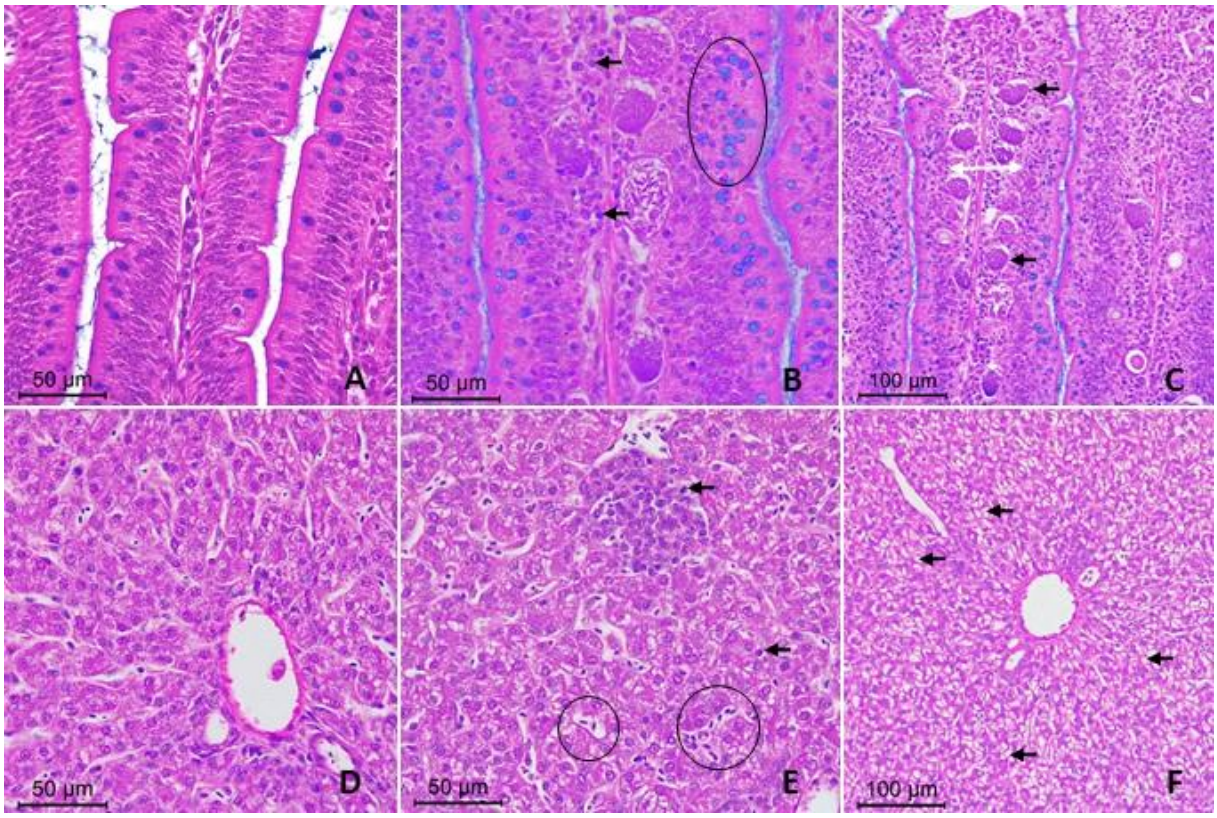

Figure 3 – Photomicrographs of hematoxylin and eosin stained chicken jejunum and liver sections. Alcian Blue was used to stain the goblet cells in jejunum. A) and D) show normal histological structure of control group jejunum and liver, respectively (400X); B) shows inflammatory infiltration cells in the lamina propria (arrows) and increased amount of goblet cells (Circle) (400X); C) shows *Eimeria* oocysts (black arrows – also observed in B) and lamina propria thickness (white arrow) (200X). These alterations were the most statistically correlated to performance data and contributed to the highest ISI index in coccidiosis challenged groups at 21 days of age. E) shows presence of inflammatory (arrows) and red blood (circles) cells in the interstitial liver space of birds challenged with *Eimeria* at 14 days of age (400X); F) shows cell vacuolation (arrows) in liver of 7 days old chickens (200X).

At 14 days, the histology of jejunum showed that challenged birds had higher histological ISI compared to non-challenged group, due to oocyst presence and MILP. When the correlation of histology ISI with performance was evaluated, it was observed negative statistical correlation of lamina propria thickness, proliferation of enterocytes, MILP and presence of oocyst with BWG ($r = -0.55$, $r = -0.62$, $r = -0.59$, and $r = -0.76$, respectively) and FCR ($r = 0.59$, $r = 0.52$, $r = 0.39$ and $r = 0.61$, respectively). No correlation with FI was observed at 14 days (Table 7). These results were expected because the histological parameters are associated to inflammation (LILLEHOJ; TROUT, 1996) and the presence of parasites. Enterocytes cells are responsible for absorption (UNI; SMIRNOV; SKLAN, 2003), but perhaps not totally mature to have a

positive relation with performance. However, more studies should be done to evaluate the interaction between enterocytes proliferation and animal performance.

In liver histology, *Eimeria* challenged groups had high scores as consequence to immune cell infiltration and congestion (Figure 3, D) which is related to the immunohistochemistry data. In fact, liver in birds has a role as ectopic lymphoid tissue, mainly in the beginning of life (OLÁH; VALVERDE, 2008). There is a negative statistical correlation between immune cell infiltration in liver and BWG (r = -0.72) and FCR (r = 0.58) at 14 days, showing once again the inflammation effect on performance.

At 21 days, in jejunum histology, challenged birds had high scores due to oocysts (Figure 3), which is correlated to the macroscopic observations of coccidiosis lesions. At this age, epithelial plasma cell infiltration was negatively correlated to FI (r = -0.54) and BWG (r = -0.65), MILP was correlated to FCR (r = 0.62), and the presence of oocysts with BWG (r = -0.74) and FCR (r = 0.67) (table 7). These results suggested that the mobilization of immune cells could affect the performance parameters. Other histological parameters, as epithelial thickness, enterocytes and goblet cells proliferation were positively correlated with FI (r = 0.56, r = 0.27, and r = 0.55, respectively) and BWG (r = 0.73, r = 0.51, and r = 0.68, respectively). In fact, epithelial thickness could be associated to goblet cells and enterocytes proliferation or inflammatory cells infiltration. *Enterocytes* are absorption cells, and as observed at 14 days of life, their proliferation have different correlation with performance which could be related to the different stage of cells maturation.

Goblet cells synthesize and secret mucin glycoproteins that form a mucus layer in the small intestine and it is important to protect and transport nutrients between lumen and enterocyte brush border membrane (UNI; SMIRNOV; SKLAN, 2003). There are differences in the types of mucin glycoproteins synthesized along the growth period

(SHUB et al., 1983). More studies about the mucins types should be done in order to better understand the correlation between goblet cells proliferation and animal performance.

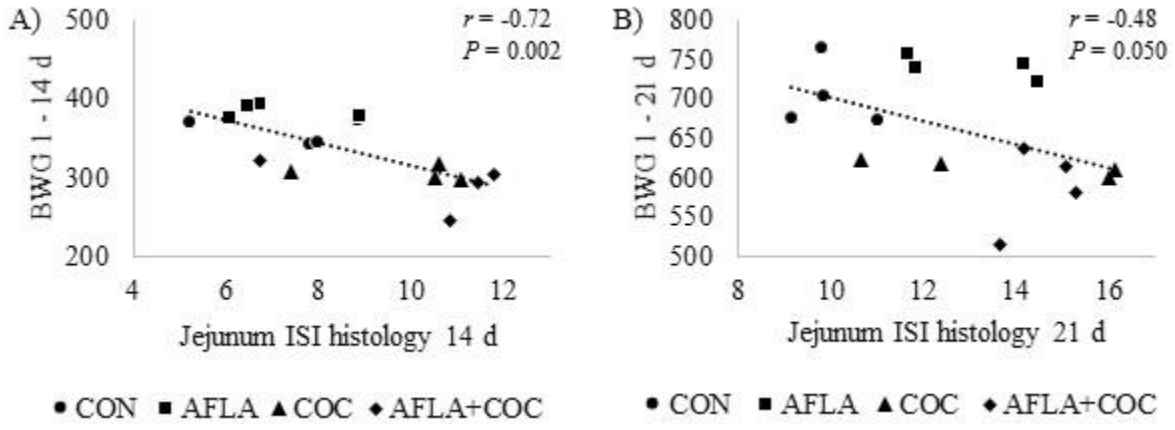

Figure 4 – Correlation of jejunum ISI histologic alterations and body weight gain (BWG) at 14 d (A) and 21 d (B).

It was observed at liver histological analysis that aflatoxin and *Eimeria* challenge caused more lesions (e.g. cell vacuolation), but there were no interactions between these factors. The vacuolation was the only parameter that showed negative correlation to FCR (r = 0.66) (Table 7). Santin et al. (2002) showed that mycotoxins can increase this histological changes in birds and it is associated to hepatocyte damage.

When ISI histological result was correlated to BWG (Figure 4) and *Eimeria* challenge, it was observed that at 14 and 21 days the highest ISI value was from the group with the lowest BWG (r = -0.72 and r = -0.48, respectively). These results suggest the possibility to apply ISI methodology in poultry industry to evaluate and correlate gut health with animal performance. However, more studies are necessary to adjust ISI index according to the correlation data for performance parameters from this

study. In conclusion, ISI histological index applied to jejunum could be correlated to BWG and FCR using the *Eimeria* and aflatoxin models.

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CHAPTER 2

FIELD APPLICATION OF A SYSTEM TO EVALUATE INTESTINAL HEALTH THROUGH NECROPSY AND HISTOPATHOLOGY AND ITS CORRELATION WITH PERFORMANCE IN BROILERS REARED IN CONVENTIONAL OR TUNNEL HOUSE

FIELD APPLICATION OF A SYSTEM TO EVALUATE INTESTINAL HEALTH THROUGH NECROPSY AND HISTOPATHOLOGY AND ITS CORRELATION WITH PERFORMANCE IN BROILERS REARED IN CONVENTIONAL OR TUNNEL HOUSE

ABSTRACT

Broilers spend almost all the lifetime inside the poultry house and have to deal with infectious, nutritional, immunological and environmental challenges. Environmental challenges, mainly related to thermic control and airborne contaminants, affect performance, welfare, and health status of broilers. The objective of this study was to apply a system to evaluate intestinal health of broilers reared in conventional house (CH) and tunnel ventilated house (TVH), and its correlation with performance. Were selected 40 poultry farms, in a poultry company in mid-west Brazil, from which were gathered six healthy broilers of each one. At necropsy, broilers were evaluated systematically by the “I See Inside” (ISI) methodology, and samples of liver, duodenum, jejunum and ileum were gathered for ISI histologic analysis and samples of liver for IL-10 and IL-12 mRNA expression. At the final of rearing period, the company provided the flock data of performance and carcass condemnation. Broilers from TVH presented 5.75% lower feed conversion ratio (FCR), 4.10% higher daily body weight gain (BWG), and higher percentage of partial carcass condemnation due to abscess compared to those from CH. At macroscopic analysis, broilers from TVH present higher ISI index in respiratory system alterations compared to CH. At histology, broilers from CH had higher ISI index in ileum, which was correlated with BWG (r = -0.56) and FCR (r = 0.53). Our results showed that it is possible to apply a mathematical index for macroscopic and histological alterations to compare flock in the poultry industry.

Key-words: Gut health index. Ambience. Broiler. Histopathology.

INTRODUCTION

During the production cycle, broilers have to deal with challenges that impair immunity, health, performance and welfare (YEGANI; KORVER, 2008). Environmental conditions are important in all this aspects, as the ambient temperature and moisture, stocking density, airborne contaminants, litter materials, and the type and age of the building (CAMPE et al., 2013). Measuring the effect of these factors on the health and its correlation with performance could be useful for decision taking.

The environmental temperature, air velocity (LOTT; SIMMONS; MAY, 1998; OLIVEIRA et al., 2006) and moisture (YAHAV et al., 1995) are important for a good performance. Heat stress may impair performance, intestinal integrity, and immunological balance (QUINTEIRO-FILHO et al., 2010), and it is directly related to structure and management of the broiler house.

Environmental conditions are influenced by the type of the structure house. Tunnel ventilated house (TVH) may reduce more efficiently the temperature in heat environment regions (LACY; CZARICK, 1992), and consequently, the performance may be better than conventional house (CH) (LOTT; SIMMONS; MAY, 1998). In addition, the TVH allow to increase the stocking density with no effect on mortality and carcass quality when ventilation rate and air circulation are adequate (FEDDES; EMMANUEL; ZUIDHOFT, 2002). Nevertheless, in terms of intestinal health there is no study comparing the structure housing conditions.

This study aimed to apply a mathematical index to evaluate intestinal health of broilers through necropsy and histopathology, in broilers reared in conventional and tunnel ventilated houses, and also its correlation with performance data.

MATERIAL AND METHODS

This experiment was approved by the Institutional Animal Use Ethics Committee of Agricultural Sciences of the Federal University of Parana (Protocol 015/2016).

SCORING SYSTEM METHODOLOGY – ISI

The ¨I See Inside¨ (ISI) methodology in process of patent (INPI BR 1020150036019) is based on a numeric score of alteration according with our previous experiment (KRAIESKI et al., 2016). Briefly, in this methodology, an impact factor (IF) is defined for each alteration in macro and microscopic analysis according to the reduction of organ functional capacity, based on previous knowledge of literature and background research. The IF range from 1 to 3, where 3 is the most impactful for the organ function; e.g. necrosis has the highest IF because the functional capacity of affect cells is totally missing. Furthermore, the extent of each lesion (or intensity) or observed frequency compared to non-affected organ is evaluated in each organ/tissue per animal and the score ranges from 0 to 3: score 0 (absence of lesion or frequency), score 1 (alteration up to 25% of the area or observed frequency), score 2 (alteration range from 25 to 50% of the area or observed frequency), and score 3 (alteration extent more than 50% of the area or observed frequency). To reach the final value of the ISI index the IF of each alteration is multiplied by the respective score value and the results of all alterations are summed.

For macroscopic analysis, broiler carcasses were systematically evaluated following a division into five system groups: locomotor, gastrointestinal (GIT)

associated organs, intestine, coccidiosis typical lesion and respiratory system. The sum of all alterations could reach 174, representing the worst health status.

For histological changes, the same calculation was applied and the scales range from 0 to 54 for intestine, and from 0 to 39 for liver. Figures 1 and 2 present examples of the changes evaluated in liver and intestine, respectively. To calibrate the observer effect, 50 squares were evaluated by 3 veterinarian pathologists.

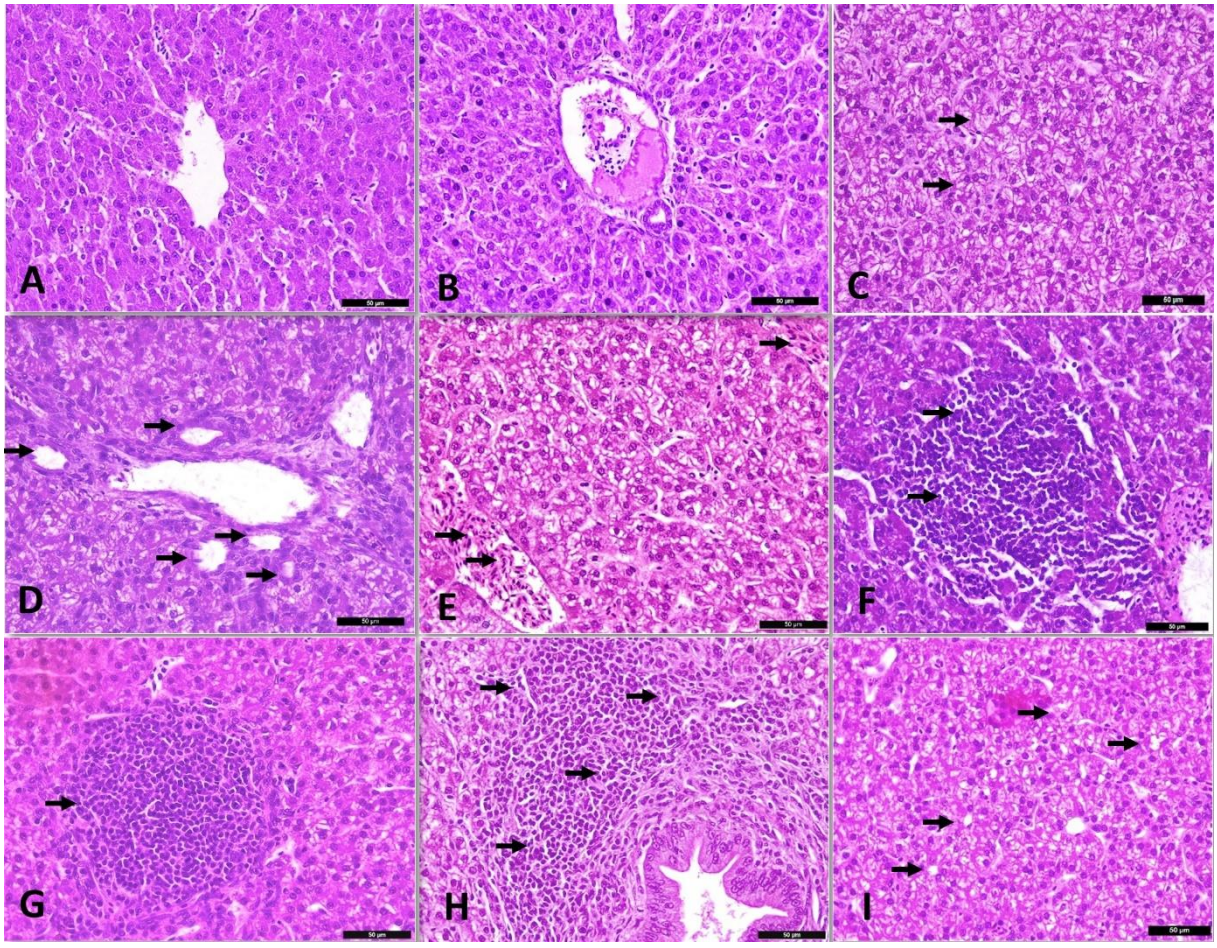

Figure 1 – Photomicrographs of changes evaluated in liver of broilers (hematoxylin and eosin stained). A) normal tissue (lobular central vein); B) normal tissue (portal tract); C)Hydropic degeneration, score 3; D) Bile-duct proliferation, score 3; E) Congestion, score 3; F) Immune cells infiltration, score 3; G) Lymphocytic aggregate, score 3; H) Pericholangitis, score 3 I) Cell vacuolation, score 3. 400X, bar = 50 µm.

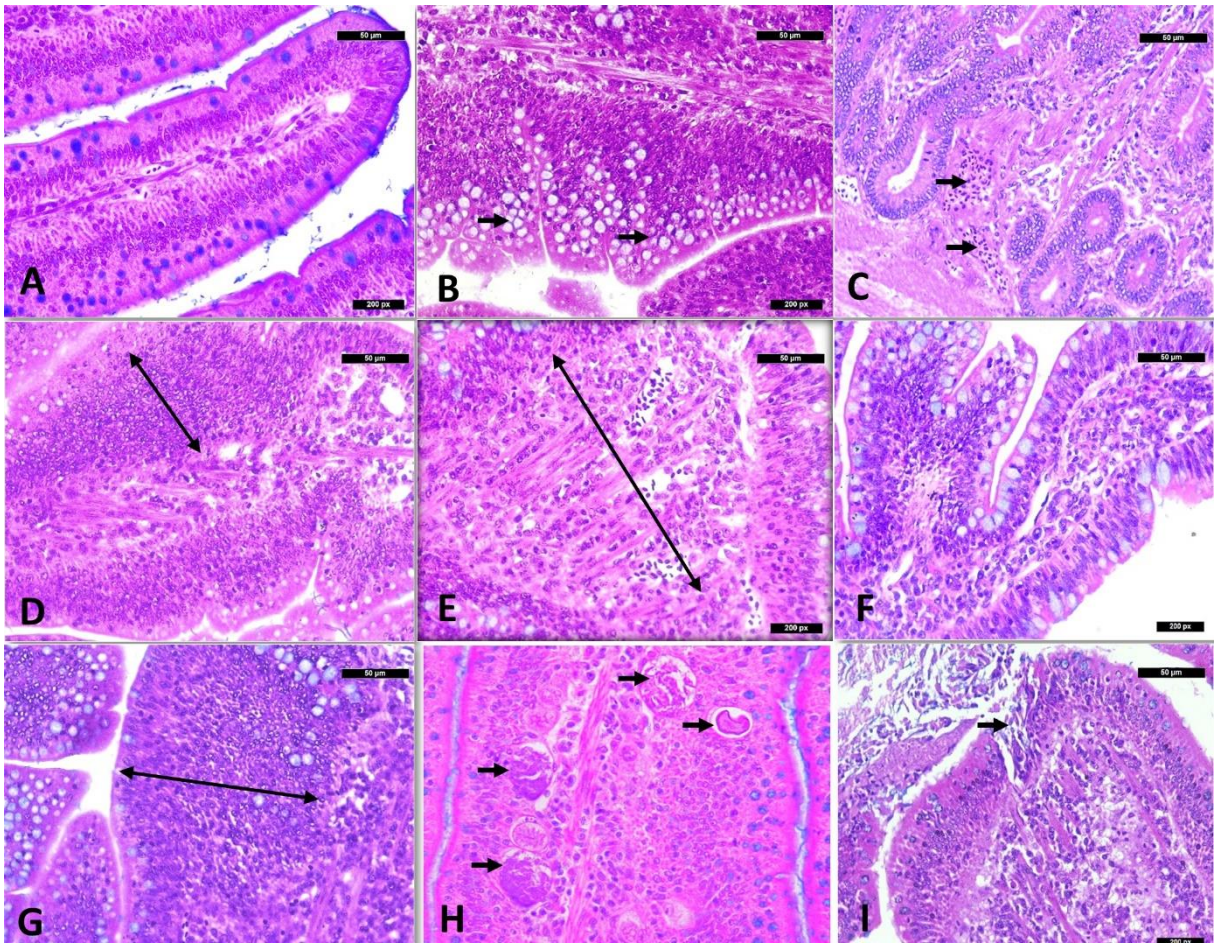

Figure 2 – Photomicrographs of changes evaluated in small intestine of broilers (hematoxylin and eosin, plus Alcian Blue stained), all changes score 3. A) normal tissue (villus); B) Goblet cells proliferation; C) Congestion; D) Epithelial thickness; E) Lamina propria thickness; F) Lamina propria inflammatory cell infiltrate; G) Enterocytes proliferation; H) Presence of oocysts; I) Necrosis / apical karyolysis. 400X, bar = 50 µm.

ANIMALS, FACILITIES AND EXPERIMENTAL DESIGN

The experiment was carried in a poultry company in mid-west Brazil, during two months (September and October) of data collection. Six broilers were took per farm, from 40 poultry farms (1 to 3 stables each one), totalizing 240 sampled broilers. Poultry farms were previously selected according to the type of house, as conventional houses (CH, n = 16 poultry farms) and tunnel ventilated houses (TVH, n = 24 poultry farms). Healthy birds were gathered according to flock average in the house (broiler size and location inside the house), and were not clinically ill. All housed birds were male from ROSS AP91® lineage, hatched in the same hatchery of the poultry company,

and received vaccine against Gumboro and Pox diseases in egg and against infectious bronchitis virus by spray after hatch.

Birds were maintained in comfort temperature, compatible with age according with lineage recommendation (ROSS, 2012), received feed and water *ad libitum* with diet formulated by the poultry company equally during the sampling period. The management activities were performed by the employees of each farm. Average environmental temperature during the experimental period was 24.7 °C (max. 32.5 and min. 17.5 °C) and relative moisture was 56.9 % (max. 82.4 and min. 31.6 %), according to EMBRAPA local measurement service (EMBRAPA, 2016). The average reduction temperature inside the house in relation to external temperature was 1 to 2 °C for conventional house and 3 to 7 °C for tunnel house, according to literature (Lacy and Czanick, 1992).

NECROPSY AND SAMPLING

Birds were selected between days 25 to 30, euthanized by cervical dislocation and necropsied for macroscopic evaluation using ISI methodology. In broilers from 12 poultry farms (5 CH and 7 TVH), additionally to macroscopic evaluation, were collected samples of duodenum (descending handle), jejunum (2 cm proximal to Meckel's diverticulum), ileum (5 cm proximal ileocecal junction) and liver (central lobe) in 10% buffered formalin fixer solution for ISI histology analysis. Samples of liver were gathered in RNA later solution, immediately cooled and stored in -20 °C freezer for further interleukin 10 and 12 (IL-10 and IL-12) mRNA expression quantification.

HISTOLOGY

Samples were embedded in paraffin and 5 µm sections were cut and stained with hematoxylin and eosin plus Alcian Blue. A slide per bird was evaluated and villi

were divided into merged and normal. Ten intestinal villi per bird were evaluated proportionally to the morphological distribution (merged and normal), in 10x objective (also used 40x objective to confirm alterations) of an optical microscope (Nikon Eclipse E200), following the ISI histology alteration. Liver samples were evaluated in 5 fields per bird in 10x objective (KRAIESKI et al., 2016).

RNA ISOLATION AND RT-PCR

The total RNA extraction was performed using Trizol reagent (Invitrogen, Carlsbad, CA) and quantified by spectrophotometry at 260 nm using the NanoDrop 2000 spectrophotometer. After verifying the RNA integrity in agarose gel, this material was submitted to treatment with DNase I (Invitrogen, Carlsbad, CA). The cDNA synthesis was accomplished using a High Capacity cDNA Reverse Transcription kit (Invitrogen, Carlsbad, CA). The quantification of the expression abundance of mRNA for cytokine genes using RT–PCR was performed according to published protocol (Humprey and Klasing, 2005) using the genes described in Table 1. The SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) kit was used, and the gene expression levels were analyzed in a thermocycler StepOnePlus (Applied Biosystems, Foster City, CA) with a protocol of 95 °C for 15 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. The results of real time PCR were analyzed using the comparative 2- ΔΔCT method (Schmittgen and Livak, 2008). This method is based on reducing the values of Ct (threshold cycle or cycle threshold) in the control group compared to the group of interest. Each sample was analyzed in triplicate; the Ct value from each triplicate for the target gene was subtracted from the Ct value average of triplicates for constitutive gene before use in the statistical analysis.

Table 1 – Cytokine primer sequences[a]

Gene	Primer sequence (5'-3')	Gene Bank Accession Nº
ch IL-10	F:*CGGGAGCTGAGGGTGAA* R:*GTGAAGAAGCGGTGACAGC*	AJ621614
ch IL-12p40	F:*AGACTCCAATGGGCAAATGA* R:*CTCTTCGGCAAATGGACAGT*	NM 213571

[a]The listed oligonucleotides were used to analyze liver gene expression via quantitative real-time PCR. F= forward; R= reverse.

PERFORMANCE

In the final of the production cycle, the poultry company provided the flock performance data and percentages of carcass condemnation at the slaughterhouse. Mean daily body weight gain (BWG) was calculated dividing the mean weight at slaughter by the number of rearing days. Livability was calculated by subtracting the number of processed birds from the initial number of housed birds, expressed as percentage. Data of feed conversion ratio (FCR) was corrected to 2.8 standard live weight. Corrected feed conversion ratio (CFCR), was calculated using the following equation, proposed by PATRICIO et al. (2012):

Equation: CFCR SW = (SW-AW) /3.5 + FCR

Where: CFCR SW = feed conversion ratio adjusted for standard weight; SW = standard weight, kg; AW = average flock weight, kg; 3.5 = correction factor (simplification from the 70/20 division); FCR = flock feed conversion ratio.

Carcass condemnation percentages were considered as total and partial condemnation. Partial carcass condemnation was separated by the main causes: abscess, arthritis, cellulitis, contusion and fracture, dermatitis, myopathy, ascites, and others causes, according to federal inspection criteria.

STATISTICAL ANALYSIS

For performance and carcass condemnation, the experimental unit was the poultry farm, and the data were submitted to analysis of variance (ANOVA) and Tukey's test for the means with a significant difference ($P < 0.05$). For necropsy, histology and mRNA expression analysis the experimental unit was the bird and the data, which is based on scores and consequently were not parametric, were compared by Kruskal-Wallis test ($P<0.05$). Histologic analyzed parameters were summarized into one mean for each poultry farm and performed Pearson's correlation analysis with BWG, CFCR and livability. The given scores by each veterinarian pathologist were compared using Spearman's rank correlation test. Data analysis were performed by the statistical software Statistix 9® for Windows.

RESULTS AND DISCUSSION

The housing conditions where birds spent almost the entire life are very important to ensure high performance and welfare (DAWKINS; DONNELLY; JONES, 2004). In our study, we applied a mathematical index (ISI) to evaluate pathologic lesions and its correlation with performance and to compare broilers from different structure house. Our results, from 1,854,521 broilers, showed that broilers housed in tunnel house presented 5.75 % better corrected FCR (1.655 x 1.756, $P = 0.001$) and 4.10% better daily BWG (68.22 x 65.42, $P = 0.001$) compared to conventional house (Table 2). These results are in accordance to literature (LACY; CZARICK, 1992; LOTT; SIMMONS; MAY, 1998), which also presented better BWG in broilers housed in TVH, but did not observe difference in FCR. Numerically, the mean weight at slaughter was higher and the age at time of slaughter was lower in broilers from TVH compared to those from CH.

Table 2 – Effect of structure house on performance and slaughterhouse parameters

Variable	Tunnel ventilated house		Conventional house		P value
	Mean	S.E.M.	Mean	S.E.M.	
Mean daily body weight gain (g/d)	68.22[a]	0.42	65.42[b]	0.60	<0.001
Livability (%)	95.54	0.30	95.42	0.36	0.808
Corrected feed conversion ratio (kg/kg)	1.655[b]	0.01	1.756[a]	0.02	<0.001
Mean live weight at slaughter (kg)	2.82	0.02	2.75	0.03	0.057
Age at time of slaughter (d)	41.47	0.21	42.18	0.32	0.062
Stoking density (kg/m²)	37.33[a]	0.49	34.12[b]	0.88	0.001
Percentage of Total condemnations (%)	0.35	0.04	0.25	0.07	0.214
Percentage of Partial condemnations (%)	3.91[a]	0.16	2.92[b]	0.32	0.005
Condemnation due to abscesses (%)	0.94[a]	0.07	0.54[b]	0.10	0.004
Condemnation due to arthritis (%)	0.40	0.02	0.35	0.03	0.258
Condemnation due to cellulitis (%)	0.57	0.08	0.50	0.12	0.673
Condemnation due to contusion/fracture (%)	0.54	0.05	0.48	0.04	0.436
Condemnation due to dermatitis (%)	0.09	0.03	0.10	0.04	0.930
Condemnation due to myopathy (%)	0.42	0.05	0.37	0.09	0.543
Condemnation due to ascites (%)	0.26	0.02	0.18	0.05	0.159
Other causes of condemnation (%)	0.65	0.11	0.36	0.06	0.060

[a,b] Different letters in the same row are significantly different for P ≤ 0.05 on the Tukey test.

Better performance in broilers from tunnel house may be explained because TVH provide higher air velocities, which contributes to sensible heat loss of the broilers, thus, reducing the expending of energy for panting (Simmons et al., 1997) and the heat stress (TEETER et al., 1985). Livability was not affected by the type of ventilation, and it is in accordance to literature (HEIER; HOGÅSEN; JARP, 2002).

At necropsy evaluation, we observed higher macroscopic alterations in respiratory system in birds from tunnel houses than from conventional houses (2.49 x 1.80, arbitrary units, P = 0.016), being hydropericardium and airsacculitis the most common alterations. However, this alteration did not differ at slaughterhouse observation, which could be due the period of first evaluation and a recovery of the respiratory tract at the end of rearing period. Tunnel house is more efficient in reducing inside temperature compared to conventional houses (LACY; CZARICK, 1992), however the increase in stocking density, as observed in this study, may increase litter moisture and gas emission (KANG et al., 2016), which affect immune response (WEI

et al., 2015), predisposing birds to respiratory infections. The vaccine against infectious bronchitis applied at the hatchery may also influence the respiratory tract in the lifetime of the birds. Although not measured in our study, ammonia exposure may increase the susceptibility to respiratory diseases as a result of biochemical changes in mucus viscosity altering bronchial clearance (KRISTENSEN; WATHES, 2000).

The total average of macroscopic ISI was 15.77 for broilers from TVH and 15.45 for broilers from CH. No difference was observed in the total index and in the other organ systems (Table 3), and neither correlation with performance parameters. However, it was possible to see difference for ISI at respiratory tract between flock. We observed that the mathematical index allowed us to compare the different flock in a scientific manner applying a statistical test. This could be useful for poultry companies once that permit to transform the pathological alteration in a number that could be understood for different professional that work in the field.

When we evaluate the gut health applying the ISI method, only the histological ISI in ileum was higher in broilers from CH in relation to TVH (P = 0.010, table 5), and it was negatively correlated with BWG (r = -0.56) and positively with CFCR (r = 0.53). It means that the higher the histologic index, the lower the BWG and the higher de FCR as observed in other studies in our laboratory (KRAIESKI et al., 2016). The analysis of inter observer correlation showed a moderated (MUKAKA, 2012) agreement (mean r = 0.53) for all the parameters evaluated in histology.

The most impactful lesions were cell plasma infiltration in epithelium and apical necrosis, which contributed to the difference. We also observed higher mRNA expression of IL-10 (P = 0.026) and IL-12 (P = 0.014) in liver of birds reared in CH compared to birds from TVH (table 3). The higher histologic ISI in ileum and cytokines expression in liver suggest that birds reared in CH showed more inflammation process

on GIT, and it could affect animal performance (KLASING, 2004). In liver, duodenum and jejunum was not observed difference in broilers from CH or TVH.

Table 3 – Effect of structure house on macroscopic and microscopic alterations and mRNA cytokine expression of broilers

Variable	Tunnel ventilated house		Conventional house		P value
	Mean	S.E.M.	Mean	S.E.M.	
Macroscopic Evaluation					
Locomotor	1.52	0.10	1.41	0.12	0.494
Respiratory	2.49[a]	0.18	1.80[b]	0.18	0.016
Other GIT organs	5.25	0.24	5.52	0.29	0.488
Intestine	6.06	0.23	6.38	0.33	0.426
Coccidiosis	0.43	0.09	0.33	0.11	0.472
Total score	15.77	0.47	15.45	0.54	0.665
Microscopic Evaluation					
Duodenum	9.93	0.78	10.28	0.96	0.779
Jejunum	16.16	0.46	14.82	0.53	0.062
Ileum	8.36[b]	0.51	10.61[a]	0.96	0.010
Liver	4.00	0.23	4.23	0.28	0.541
Cytokine Analysis					
IL-10	0.20[b]	0.07	0.49[a]	0.10	0.026
IL-12	0.36[b]	0.14	2.21[a]	0.75	0.014

[a,b] Different letters in the same row are significantly different on the Kruskal-Wallis test.

This data indicate that is possible to apply a mathematical index for macroscopic lesions to compare flock in the same form in the poultry industry. The ileum histological ISI could be a mathematical parameter to establish gut health which could be correlated with broilers performance.

At slaughterhouse it was observed that broilers from TVH presented higher percentage of partial condemnation due to abscesses compared to CH, and probably the stocking density (37.33 x 34.12 kg m^{-2}, respectively) could be the cause of it. Higher stocking density (SD) at the final period of rearing may contribute to more scratches on dorsal skin (DOZIER et al., 2005; PROUDFOOT; HULAN, 1985), and then lead to abscesses formation. However, some authors did not observe differences in carcass quality when SD was increased (FEDDES; EMMANUEL; ZUIDHOFT, 2002;

THAXTON et al., 2006; TONG et al., 2012), probably because the better temperature control management practices inside the house (DAWKINS; DONNELLY; JONES, 2004).

CONCLUSION

Broilers housed in TVH showed 5.75 % lower FCR, 4.10 % higher daily BWG and 21,2% lower index ISI for ileum compared to CH, but presented 33,9 % higher partial carcass condemnation due to the occurrence of abscess.

The histological ISI results and cytokines mRNA expression suggested that the difference in performance of broilers could be associated to inflammation in small intestine.

The ileum histological ISI allowed to compare gut health between flock and showed correlation with BWG (r = -0.56) and FCR (r = 0.53).

Our results showed that it is possible to apply a mathematical index for macroscopic and histological alterations to compare flock in the poultry industry.

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FINAL CONSIDERATIONS

Intestinal health of broilers is affected by nutritional, environmental and infectious agents, and to detect and evaluate the impact of these factors is important to take better decision in a production system. The index developed in this study could be a useful tool to evaluate intestinal health, once it converts tissue alterations in numbers that permit to make comparisons and facilitate the communication between professionals involved in poultry production.

For macroscopic changes, the application of the index did not present differences between the groups and neither correlation with performance. It can be due the macroscopic alterations seems very similar in poultry, which also cannot conduct do specific disease diagnostic with only necropsy.

For microscopic alterations, the index presented correlation with performance, showing that the higher the index, the lower was the BWG and higher the FCR. However, for some parameters, the correlation was not significant, suggesting the index could be better considered in order to improve the final correlation coefficient.

The ingestion of aflatoxin and challenge with *Eimeria* vaccine impaired immunity, performance, and histological parameters. These effects contributed to the correlation between the histological index and performance.

Broilers housed in tunnel ventilated house showed 5.75% lower FCR, 4.10% higher daily BWG and 21,2% lower index ISI for ileum histology compared to broilers from conventional house. Tunnel house, when well-managed, provide better environment for broilers, which results in better performance parameters.

In the first trial, a strong challenge was used to induce lesions and affect performance in order to improve the correlation coefficient between the index and performance parameters. The correlation was lower in the second trial, probably because the absence of challenge and the small difference between the groups compared. For a non-specific-challenge situation, the index could be better considered, as the numerical differences are discrete.

Our results showed that it is possible to apply a mathematical index for macroscopic and histological alterations to compare flocks in the poultry industry. In order to improve the index, more comparisons and applications should be done.

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ANNEX

ANNEX 1

Effect of aflatoxin experimental ingestion and *Eimeira* vaccine challenges on intestinal histopathology and immune cellular dynamic of broilers: applying an Intestinal Health Index

A. L. Kraieski, R. M. Hayashi, A. Sanches, G. C. Almeida, and E. Santin[1]

Microbiology and Avian Pathology Laboratory, Department of Veterinary Medicine, Federal University of Parana, Curitiba, Brazil, 80035-050

ABSTRACT The present study evaluated the effect of aflatoxin B1 and *Eimeria* vaccine challenges and their interaction on intestinal morphology, applying the morphometric index "I See Inside" (ISI). Immune cellular response and broiler chicken performance were also studied. A total of 240 broiler chickens were divided in a 2 × 2 factorial arrangement with 4 treatments, T1: Control diet and no challenge (CON), T2: Aflatoxin B1 (AFLA), T3: Control diet and *Eimeria* challenge (COC), and T4: Aflatoxin B1 and *Eimeria* challenge (AFLA+COC). The mathematical morphometric index ISI was applied to evaluate macro and microscopic alterations. Samples of liver and jejunum were analyzed for macrophages, CD4+, and CD8+ cells counting by immunohistochemistry at 7, 14, and 21 d of age. Chickens challenged with *Eimeria* presented higher ISI of macroscopic alterations associated to *Eimeria* lesion at the medium small intestine, lower body weight gain (BWG) and feed intake (FI), and worse feed conversion ratio compared to non-challenged birds. Both *Eimeria* and aflatoxin challenges modulated the immune cells in the jejunum and liver, generally increasing the number of macrophages, CD4+, and CD8+ cells in relation to the control group. Birds from COC and COC+AFLA groups presented higher ISI histological score in the jejunum at 7 and 14 d of age compared to the CON and AFLA groups. The reduction of FI and BWG was correlated to high histological ISI and resulted in a high presence of immune cells in tissues, suggesting immune response demand. The histological ISI had statistical correlation to broiler performance.

Key words: immune cost, pro-inflammatory response, intestinal health, gut immunity, health index

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INTRODUCTION

Regarding broiler production, gastrointestinal tract development is highly correlated with animal performance (Maiorka et al., 2000; Santin et al., 2001). Epithelium of intestinal mucosa has the function of digestion and absorption, and also represents an important barrier between the intestine lumen and the animal against a wide spectrum of harmful and immunogenic substances (Gottardo et al., 2016). Damages and losses of the epithelium barrier result in inflammation, uncontrolled immune responses, and unbalanced organic homeostasis (Kitessa et al., 2014).

Gut health could be affected by nutritional and infectious agents such as mycotoxins (Santin et al., 2002, 2003) and *Eimeria* (Lillehoj and Trout, 1996; Jang et al., 2011), respectively, increasing the intestinal mucosa inflammatory process. The acute phase of immune response is the first mechanism of defense and it is related to systemic and metabolic changes (Klasing, 2004; Kogut and Klasing, 2009). Animal performance could be affected by the association between inflammatory process and intestinal damage.

In order to evaluate the alteration promoted by nutritional, infectious, and environmental agents on animal health, scoring methods have been the object of various studies. They were applied to evaluate coccidiosis (Johnson and Reid, 1970), macro (Keirs et al., 1991; Teirlynck et al., 2011) and microscopic alterations (Gholamiandehkordi et al., 2007), tibial dyscondroplasia (Im et al., 2012), and welfare (Blatchford et al., 2015). There are many researchers and producers looking for some gut health index to be applied in the poultry industry and correlated to animal performance.

Many studies (Huff and Ruff, 1982; Stoev et al., 2002; Girgis et al., 2010; Ellakany et al., 2011) showed that the combination of mycotoxins and *Eimeria* generates a greater decrease in body weight, increase in feed conversion, and decrease in plasma carotenoid levels than the coccidiosis by itself. The objective of this study was to evaluate the effect of aflatoxin B1 and *Eimeria* vaccine challenges and their interactions on intestinal immune response and, in addition, to apply and evaluate

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[1]Corresponding author: besantin@hotmail.com