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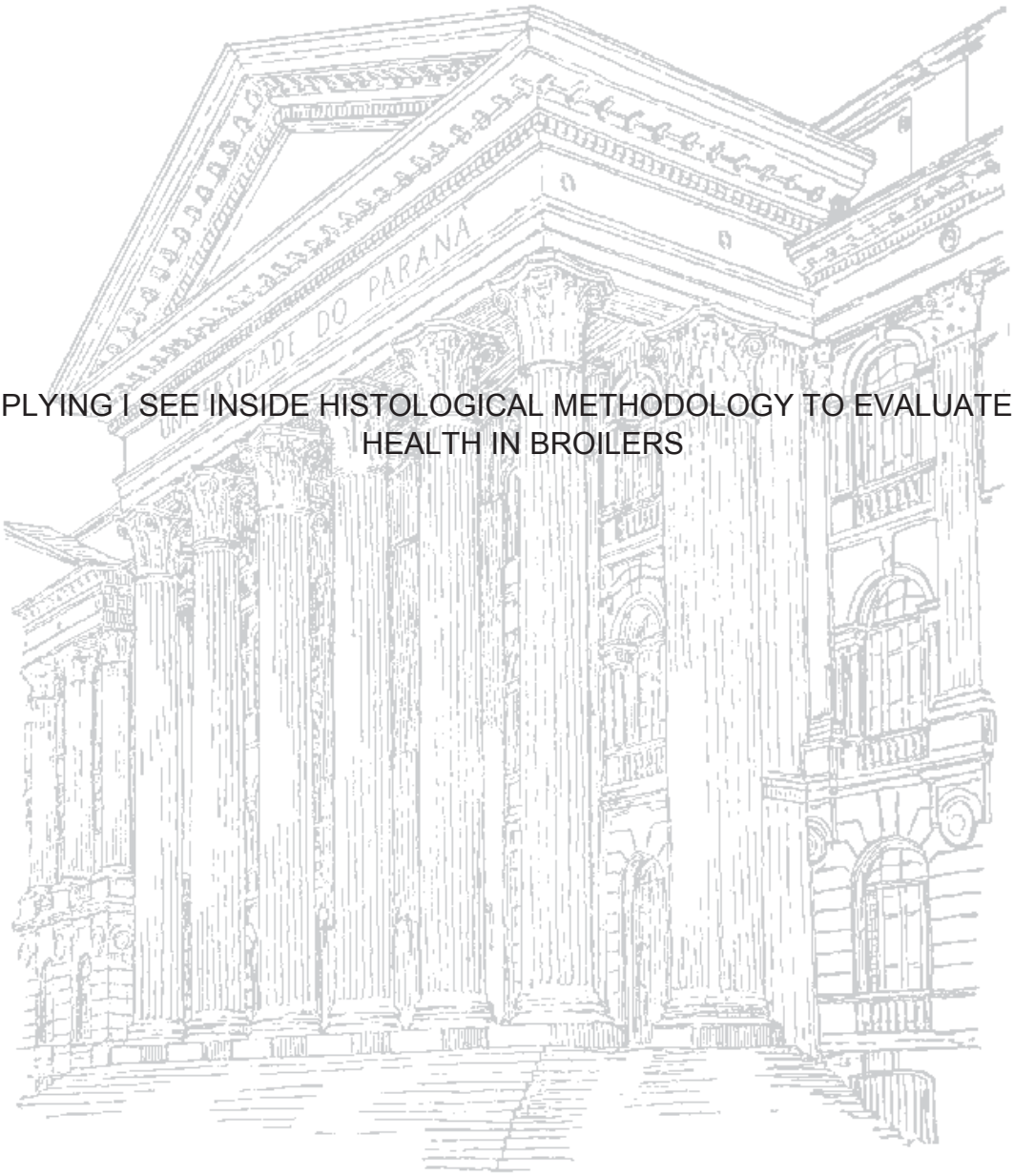

APPLYING I SEE INSIDE HISTOLOGICAL METHODOLOGY TO EVALUATE GUT HEALTH IN BROILERS

CURITIBA
2018

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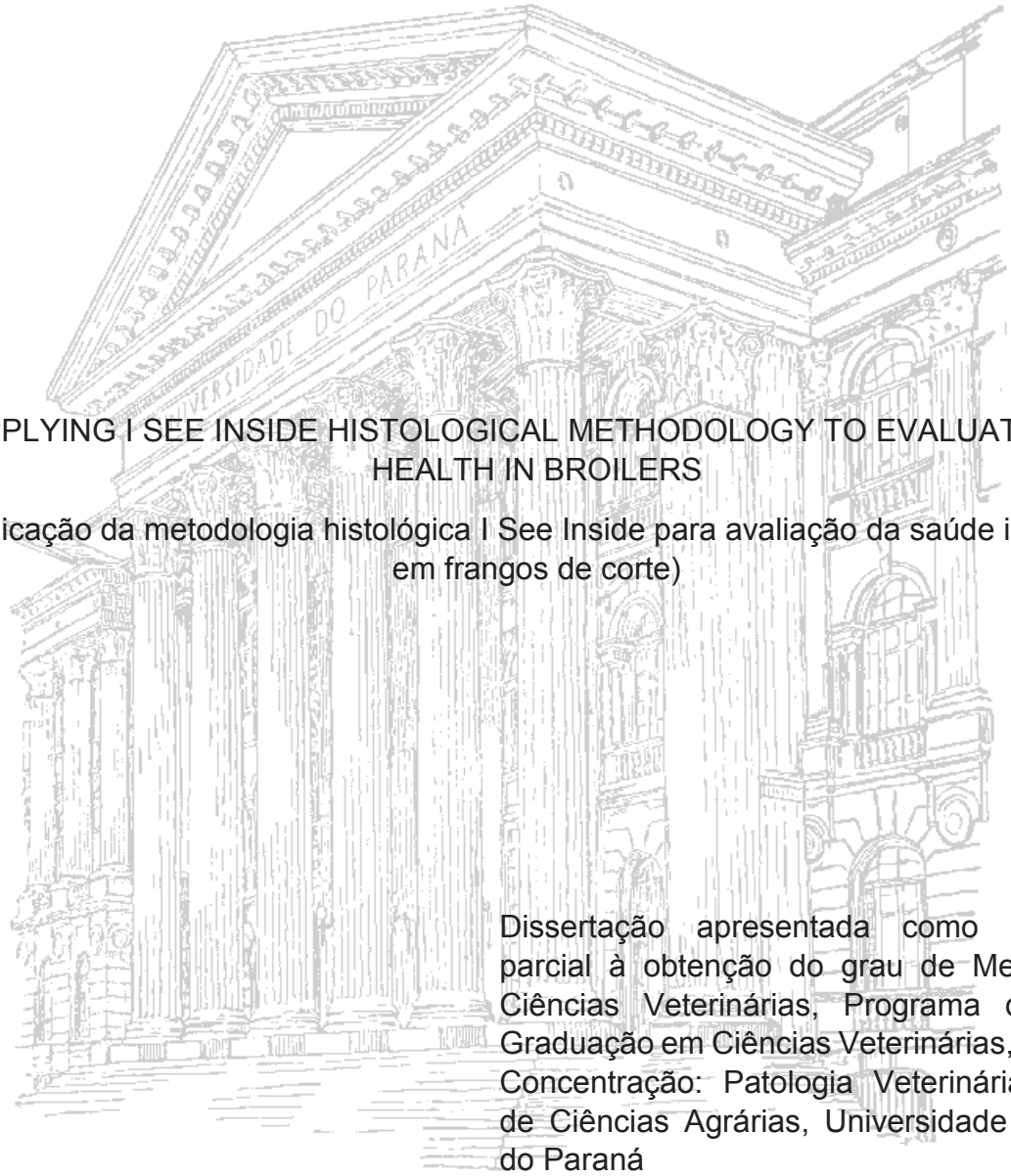

APPLYING I SEE INSIDE HISTOLOGICAL METHODOLOGY TO EVALUATE GUT HEALTH IN BROILERS

(Aplicação da metodologia histológica I See Inside para avaliação da saúde intestinal em frangos de corte)

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: Profa. Dra. Elizabeth Santin

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS VETERINÁRIAS DA Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **BRUNA LUIZA BELOTE** intitulada: **APPLYING I SEE INSIDE HISTOLOGICAL METHODOLOGY TO EVALUATE GUT HEALTH IN BROILERS**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua Aprovação no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 02 de Março de 2018.

ELIZABETH SANTIN
Presidente da Banca Examinadora (UFPR)

MARIANA LEMOS DE MORAES
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IVOMAR OLDONI
Avaliador Externo (BRF S.A)

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RESUMO

Na produção de frangos de corte, o desenvolvimento do trato gastrointestinal está altamente correlacionado com o desempenho animal. No entanto, a manutenção da saúde intestinal é mais complexa do que apenas a modulação do microbiota intestinal através de aditivos. A saúde intestinal é uma combinação de microbiologia, imunologia e nutrição. Se o trato gastrointestinal está danificado, a digestão e a absorção de nutrientes serão afetadas e o desempenho e o bem-estar da ave serão comprometidos, levando a perda econômica e aumentando a susceptibilidade de doenças. Nesta dissertação, foi apresentado dois capítulos, apresentando dois experimentos que se utilizou a metodologia I See Inside (ISI) para avaliar os parâmetros da saúde intestinal e do fígado. Aplicando a metodologia ISI no experimento apresentado no primeiro capítulo, foi possível avaliar a eficiência da enramicina como promotor de crescimento em frangos de corte desafiados com *Eimeria* sp. e *Clostridium perfringens* e seu efeito na mucosa intestinal. Foram avaliados o desempenho zootécnico e a histologia de fígado e íleo de acordo com a metodologia ISI e foi observado que o uso de enramicina minimizou significativamente o escore total do ISI no íleo em 21 e 28 dias quando comparado ao grupo de desafiado não suplementado. Estes resultados sugerem que as alterações de espessura da lâmina própria e a infiltração de células inflamatórias podem ser um padrão comparativo de saúde intestinal. No segundo capítulo foi aplicada a metodologia do ISI fígado e no intestino e foi usada a correlação de Pearson para demonstrar que os danos intestinais podem afetar negativamente o desempenho dos frangos de corte. A presença de oocisto observado na análise histológica de jejuno e íleo aos 7d indica um efeito negativo no desempenho nas próximas duas semanas (14d e 21d). Quando o score do ISI total é mensurado no íleo aos 14d, nós observamos um forte efeito negativo no desempenho zootécnico a partir desse período aos 14d e nas semanas seguintes de 21d e 28d. Os resultados demonstram que a metodologia ISI é uma ferramenta interessante para melhor avaliação da saúde intestinal.

Paravras-chave: Coccidiose, *Clostridium perfringens*, Desafio, Enramicina, Índice histológico.

ABSTRACT

In broiler production, the GIT development is highly correlated with animal performance. However, the maintenance of gut health is more complex than just modulation of the gut microbiome through additives. The gut health is a combination of microbiology, immunology and nutrition. If the GIT is unhealthy, digestion and nutrient absorption will be affected, then bird´s performance and welfare will be compromised, leading to economic loss and increase the susceptibility to disease. In this dissertation, was presented two studies applying the methodology I See Inside (ISI) to evaluate parameters of the gut health. In the first chapter, the ISI was applied to evaluate the efficiency of enramycin as a growth promoter in broilers challenged with *Eimeria* sp. and *Clostridium perfringens* and its effect in intestinal mucosa. The zootechnical performance and the histology by ISI were evaluated on liver and ileum samples. We observed that enramycin significantly minimized the ISI score in the ileum at 21 and 28 days when compared to challenge group. With this study we could suggest that alteration of lamina propria thickness and inflammatory cell infiltration could be standards parameters to compare gut health. In the second chapter was applied the ISI methodology in the liver and intestine, and the Pearson's correlation was used to demonstrates how the damages to the gut, could affect negatively the performance of broilers. The presence of oocysts in histological analysis of the jejunum and ileum at 7d indicate a negative effect on performance over the next 2 weeks (14d and 21d). When the ISI total score was measured in the ileum at 14d, we observed a strong negative effect on zootechnical performance from this period on 14d, and the over next weeks 21d and 28d. The results demonstrate that ISI methodology is an interesting tool to evaluate intestinal health.

Key words: Coccidiosis, *Clostridium perfringens*, Challenge, Enramicyn, Histology index.

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GENERAL INTRODUCTION

Gut health is a major topic for research both in humans and animals today. In humans, it is related with functions of the gut microbiome including metabolic activities that result in salvage of energy and absorbable nutrients, important trophic effects on intestinal epithelium, immune structure and function and protection of the colonized host against invasion by agents (GUARNER; MALAGELADA, 2003). It is also an essential factor in certain pathological disorders including multisystem organ failure (KLINGENSMITH N.J; COOPERSMITH, 2016), colon cancer (ARUMUGAM, 2017) and inflammatory bowel diseases such as Crohn´s Disease (GEVERS, et al., 2017).

In broiler production, the gastrointestinal tract (GIT) development is highly correlated with animal performance (MAIORKA et al., 2000; SANTIN et al., 2001, KRAIESKI et al.,2017). In fact, formulating diet for effects on intestinal health is turning into a reality in the monogastric animal industries (CHOCT, 2009). If the GIT is unhealthy, digestion and nutrient absorption will be affected, then bird´s performance and welfare will be compromised, leading to economic loss and increase the susceptibility to disease (EDENS, 2003).

It is now recognized that maintenance of gut health is more complex than just modulation of the gut microbiome through additives (DAMASKOS; KOLIOS, 2008). The gut health is a combination of microbiology, immunology and nutrition, in other words, it depends on the maintenance of the balance between the host, the intestinal microbiota, the intestinal environment and diet.

In addition, the gastrointestinal tract has the most extensive body surface constantly exposed to a wide variety of potentially harmful substances (YEGANI; KORVER, 2008).

The evaluation of intestinal health is also constantly object of research. There are many research groups worldwide looking for analytical patterns that can characterize gut health but, until now, none of these patterns can be used for evaluation in a myriad of situations.

The traditional intestinal morphometry, using the length of the villi and width of crypts might not be enough to evaluate the real status of gut health, once do not consider histological parameters linked to digestive physiology or inflammation process.

In this study, to evaluate parameters of the gut and liver health at both chapters, it was used the methodology I See Inside (ISI) adapted from Kraieski et al (2017). It is based on a numeric score of changes such as lamina propria and epithelial thickness, enterocyte proliferation, lamina propria and epithelial inflammatory cells infiltration, goblet cells proliferation, congestion and presence of oocysts in GIT. In liver, the evaluated histological alterations are congestion, cell vacuolization, bile-duct proliferation, immune cells infiltration, necrosis, pericholangitis and lymphocytic aggregate. Therefore, it is possible to observe the structure of these organs. If these parameters are changed, the structure and functionality of the evaluated organ is modified too. In this methodology, an impact factor (IF) is defined for each parameter in the microscopic analysis, according to the impairment in the tissue functional capacity, based on previous knowledge from literature and background research (e.g. necrosis has the highest IF because the functional capacity of affected cells is completely lost).

In both chapters, it was possible to observe and numerically evaluate the lesions caused by *Eimeria* on intestinal mucosa and histological alterations in liver due to challenge, using the ISI methodology.

The protozoa of the generous *Eimeria*, in single infections or coinfections with *Clostridium perfringens* when the host is submitted an immunosuppression conditions, produce several impacts over the gut health and performance of broilers (LEE et al., 2013; STANLEY et al., 2014; KALDHUSDAL; BENESTAD; LØVLAND, 2016).

Applying this methodology in the study presented in the first chapter, it was possible to evaluate the efficiency of enramycin as a growth promoter in broilers challenged with *Eimeria* sp. and *Clostridium perfringens* and its effect in intestinal mucosa. When applied the ISI methodology in the study presented in the second chapter, it was possible to measure the efficiency of coccidiosis vaccine.

The objective of this both studies was to demonstrate the validity of the ISI methodology and its elements, opening new possibilities for better evaluation of the intestinal health.

CHAPTER 1:

HISTOLOGICAL PARAMETERS TO EVALUATE INTESTINAL HEALTH ON BROILERS CHALLENGED WITH *EIMERIA* AND C*LOSTRIDIUM PERFRINGENS* WITH OR WITHOUT ENRAMYCIN AS GROWTH PROMOTER

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HISTOLOGICAL PARAMETERS TO EVALUATE INTESTINAL HEALTH ON BROILERS CHALLENGED WITH *EIMERIA* AND C*LOSTRIDIUM PERFRINGENS* WITH OR WITHOUT ENRAMYCIN AS GROWTH PROMOTER

ABSTRACT

The maintenance of integrity of the gastrointestinal tract is an important aspect for animal productivity, since it is able to absorb nutrients more efficiently and serves as a barrier against microorganisms. To control agents detrimental to intestinal integrity, growth-promoting antibiotics (AGP) are used, which reduce the number of toxinproducing microorganisms in the intestinal lumen, acting as anti-inflammatory agents. There is a demand for restriction of use of AGP in animal feed, but there are few studies showing what parameters we should observe to search for alternative additives. The aim of this study was to establish histological parameters that explain the effect of enramycin as growth promoter on intestinal health in broilers challenged with *Eimeria* and *Clostridium perfringens*. The zootechnical performance and the histology by I See Inside (ISI) methodology were evaluated on liver and ileum samples. Chickens challenged without AGP have the worst BWG, FCR, and histological ISI score (ISI score 9) in the ileum compared to non-challenged (ISI score 5). The use of enramycin on challenged group significantly minimized the ISI score in the ileum at 21 and 28 d (ISI score 7.4 and 8.0, respectively) compared with the challenged group not fed with enramycin (ISI score 9.2 and 9.9, respectively), associated with reduced lamina propria thickness and inflammatory cell infiltration. We suggest these 2 histological parameters as a standard to compare products for gut health.

Key words: intestinal health, ISI, histology gut, performance.

1 INTRODUCTION

An important aspect regarding animal productivity is the maintenance of integrity of the gastrointestinal tract (GIT) since it is responsible for absorbing nutrients efficiently as well as it serves as barrier against microorganisms (NEISH, 2002). Thus, maintaining the integrity of the GIT prevents the establishment of enteric diseases, improves performance and reduces mortality (DROLESKEY et al., 1994). Gut microbiota have a significant effect on host nutrition, health, and growth performance (MOUNTZOURIS et al., 2010). This host-microbiome interaction is influenced by the composition and function of the gut microbiota, affecting positively or negatively the health and growth of birds. To control agents detrimental to intestinal integrity, growth promoting antibiotics (AGP) which promote significant benefits to animal performance are therefore used (ESCELI; DEMIR.,2010).

The performance improvement caused by AGP is associated with a modification of the gut microbiota that promotes a greater balance of the microbial

population, since it reduces the number of microorganisms that produce toxins in the intestinal lumen (DIBNER; RICHARDS, 2005), but it was interpreted by Niewold (2007) as a direct anti-inflammatory agent.

Despite these benefits to animal production, some countries have banned the use of these AGP due to risks to human health caused by the presence of residues in products with animal origin and due to the possibility of inducting bacterial resistance (TOROK et al., 2011). Although the idea that withdrawing AGP could reduce the risk of creating antibiotic resistant bacteria is quite attractive, Jensen and Hayes (2014) Jensen and Hayes (2014) demonstrated that although the withdrawal of AGP improved health and welfare problems in swine in Denmark, it also resulted in a 10% increase in the use of therapeutic antibiotic on the first year after AGP banned.

On the other hand, a meta-analysis study from Laxminarayan et al (2015) has shown that efficiency of antibiotics as growth promoters is not as evident as thought in studies from the years 2000's compared to literature from 1980's for poultry production. According to the authors, the production response to use of AGPs are reduced when production conditions are optimized (good housing, hygiene and optimal nutrition).

The AGP ban in Europe has highlighted the growing need for alternatives to antibiotic supplementation as a way of controlling necrotic enteritis (NE) in poultry. Despite decades of research, NE remains one of the major challenges in the poultry industry and it has been associated with extensive production losses worldwide. Necrotic enteritis it is a type of enterotoxaemia caused by an anaerobic, Gram-positive and spore-forming bacterium from the specie *Clostridium perfringens*, a common but health threatening commensal in poultry (Van Immerseel et al., 2004). An outbreak of NE in broilers often results in high mortality rates and reduces growth performance (MCDEVITT et al., 2006). This disease causes a loss in world poultry industry of US $ 2 billion per year in performance and in medicine cost (LEE et al., 2011). The acute form leads to sudden increases in mortality rates, while the subclinical form causes loss of performance due to intestinal damage such as focal necrosis of the mucosa (VAN IMMERSEEL et al., 2004; WILLIAMS, 2005).

Although *Clostridium perfringens* is clearly the pathogen involved in NE, both field experience and experimental efforts to reproduce the disease have shown that the onset of NE is a complex process requiring one or a number of predisposing factors rather than the solo presence of the pathogenic *C. perfringens* strains (NEISH, 2002). In experimental disease challenges, it is necessary to introduce predisposing factors,

such as *Eimeria* co-infection, control immunosuppression or deliberately stress the birds in order to produce clinical symptoms in a substantial number of challenged birds (COLLIER et al., 2008; SHOJADOOST et al., 2012, MESA et al., 2014; PRESCOTT et al., 2016).

The importance of coccidiosis triggering NE is due to the lesions in the intestinal mucosa caused by the parasite that facilitates the adhesion and replication of *C. perfringens* and the production of toxins (VAN IMMERSEEL et al., 2004). In addition, infection with *Eimeria* sp. strains causes an elevation of mucogenesis. Since *C. perfringens* can use mucus as substrate, growth is then enhanced (VAN IMMERSEEL et al., 2004; COLLIER et al., 2008). After the AGP ban in European countries, the incidence of NE has increased on broiler farms (CASEWELL et al., 2003)

The aim of this study was to establish histological parameters that explain the effect of enramycin as growth promoter on intestinal health in broilers challenged with *Eimeria* and *Clostridium perfringens*.

2 MATERIALS AND METHODS

This trial was approved by the Institutional Animal Use Ethics Committee of Agricultural Sciences of the Federal University of Parana (Protocol 041/2016).

2.1 ANIMALS, EXPERIMENTAL DESIGN, DIET, AND HOUSING

A total of 240 male broiler Cobb 500 with age from one to 28 days were used. The experiment followed a randomized design, with 10 replicates of 8 birds for each of the three treatments: T1) negative control non–challenge group (NC), T2) positive control group challenged with *Eimeria* sp. and *Clostridium perfringens* without antibiotic (PC) and T3) *Eimeria* sp. and *Clostridium perfringens* challenged with antibiotic growth promoter (Enramicyn 10ppm) (PC+AGP).

The trial was conducted in disinfected isolated rooms, under negative pressure, containing vertically stacked cages (replications) with sterilized wood shaving as litter (to avoid external contamination), nipple drinkers, and controlled temperature and photoperiod. Animals were maintained in comfortable temperature according to their age, with feed and water *ad libitum*. The diet was a corn and soybean

based mash that followed Brazilian nutritional recommendations for poultry (ROSTAGNO et al., 2011).

2.2 CHALLENGE

On the first d of age, all birds of the challenge groups (PC and PC+AGP) received anti-coccidial vaccine. The doses were administrated by gavage and were 15 times higher than the manufactured recommendation [7.1 ± × 10^4 oocysts per bird Bio-Coccivet R® - Biovet Brazilian Laboratory, *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. praecox, E. tenella* e *E. mitis*, isolated in Brazilian field and grown in specific pathogen-free (SPF) birds]. At 10, 11, and 12 d of age, an inoculum of 10^8 cfu/mL/bird of *Clostridium perfringens* was also administered by gavage.

2.3 PERFORMANCE

At one d of age, birds were separated into treatments in a way that initial body weight average was similar in all cages selected for each treatment, in order to obtain equal initial body weight average per cage. Birds and feed were weighed weekly (zero, 7, 14, 21, and 28 d) to evaluate feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR).

2.4 HISTOLOGICAL ANALYSIS

On d 14, 21, and 28, 10 birds per treatment were euthanized by cervical dislocation. Samples of liver and ileum were collected and fixed in Davidson's solution (100 mL glacial acetic acid, 300 mL 95% ethyl alcohol, 200 mL 10% neutral buffered formalin, and 300 mL distilled water) for at least 24 hours. All samples were dehydrated, infiltrated, and embedded in paraffin following common histological routine. Blocks were cut in 5 µm sections and stained with hematoxylin and eosin associated with Alcian Blue for goblet cells staining (RAPP; WURSTER, 1978). For intestinal morphology, one slide and 20 intestinal villi per bird were observed in 10X magnification (using 20X and 40X magnification to confirm alterations) under optical microscope (Nikon Eclipse E200, Sao Paulo, Brazil). For liver samples, 10 fields in 10X objective per bird were evaluated. The I See Inside (ISI) methodology was adapted

from Kraieski et al. (2017), and evaluation parameters are presented in Table 1. The ISI methodology in process of patent (INPI BR 1020150036019) is based on a numeric score of alteration. In this methodology, an impact factor (IF) is defined for each alteration in macroscopic and microscopic analysis, according to the reduction of organ functional capacity, based on previous knowledge from the literature and background research (i.e., necrosis has the highest IF because the functional capacity of affected cells is completely lost). The IF ranges from 1 to 3, with 3 being the most impacting to organ function. In addition, the extent of each lesion (intensity) or the observed frequency compared to non-affected organ is evaluated in each organ/tissue with score (S) ranging 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 extends to more than 50% of the area or observed frequency). To obtain 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 according to the formula $ISI = \Sigma(IF * S)$, where IF = impact factor and S = Score. For example, the lamina propria thickness has IF = 2, and this number will be multiplied by the observed score (ranging from 1 to 3); if a score S = 3 (maximum score) was observed for lamina propria thickness in the villi, so the ISI for this parameter in the villi will be $ISI = (2 * 3) = 6$. The average of 20 villi observed in each bird will reach the final value for this parameter, and the sum of the average of all parameters presented in Table 1 will give the total ISI value for this specific bird (each bird is a replicate for statistical analysis).

Table 1. ISI histological alterations evaluated in intestine and liver.

Organ	Alteration	Impact Factor (IF)		Score	Final score	Maximum Score[1]
Ileum	Lamina propria thickness	2	X	3	6	45
	Epithelial thickness	1	X	3	3	
	Enterocytes proliferation	1	X	3	3	
	Epithelial plasma cell infiltration	1	X	3	3	
	Lamina propria Inflammatory infiltration	3	X	3	9	
	Goblet cells proliferation	2	X	3	6	
	Congestion	2	X	3	6	
	Presence of oocysts	3	X	3	9	
Liver	Congestion	1	X	3	3	42
	Cell vacuolation	2	X	3	6	
	Bile-duct proliferation	2	X	3	6	
	Immune cells infiltration	1	X	3	3	
	Necrosis	3	X	3	9	
	Pericholangitis	3	X	3	9	
	Lymphocytic aggregate	2	X	3	6	

[1]Maximum score represents the sum of all alteration according to with the formula ISI =Σ(IF*S) where IF = impact factor (previous fixed) and S=Score (observed) considering the maximum observed S. For example, the lamina propria thickness has IF = 2, this number will be multiplied by observed score (range from 1 to 3), if in a villus it was observed a score S=3 (maximus score) to lamina propria thickness, so the ISI for this parameter in this villi will be ISI = (2*3) = 6. The average of 20 villi in ileum or 10 fields in the liver for each bird will be the final ISI value for each bird.

2.5 STATISTICAL ANALYSIS

Data were presented as mean ± standard error. At first, data normality was verified using Shapiro-Wilk normality test. Rates were compared using one-way analysis of variance (ANOVA) followed by Tukey test ($P<0.05$) for parametric data. For performance, each cage was used as a sample while for the remaining analysis, each bird was used as a sample. All analysis were performed at Statistix 9 software for Windows.

3 RESULTS

The feed intake was not significantly different among treatments (data not shown). The NC group presented significantly higher BWG in comparison with PC group at periods 1 to 14 d, 1 to 21 d and 1 to 28 d of age ($P < 0.05$), and there was no significant difference between PC+AGP and the other groups (Figure 1). The NC group showed significantly better results at FCR compared with the PC group at periods 1 to 7 d and 1 to 21 d of age ($P < 0.05$), and there was no significant difference between

PC+AGP and the other groups (Figure 2). There was no difference among the treatments during the other periods.

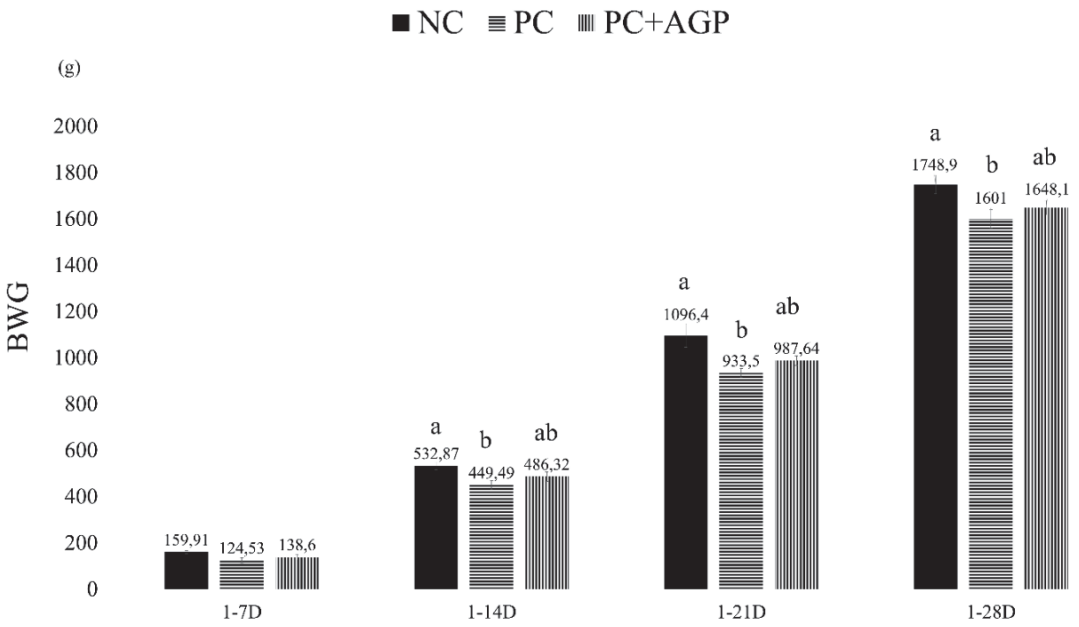

Figure 1. Mean and standard error body weight gain (BWG) in grams for different treatments at each period. NC: non-challenged group, PC: *Eimeria sp*. and *Clostridium perfringens* challenge, PC+AGP: *Eimeria* sp. and *Clostridium perfringens* challenge + antibiotic growth promoter. Different superscript letters indicate significant difference (P < 0.05).

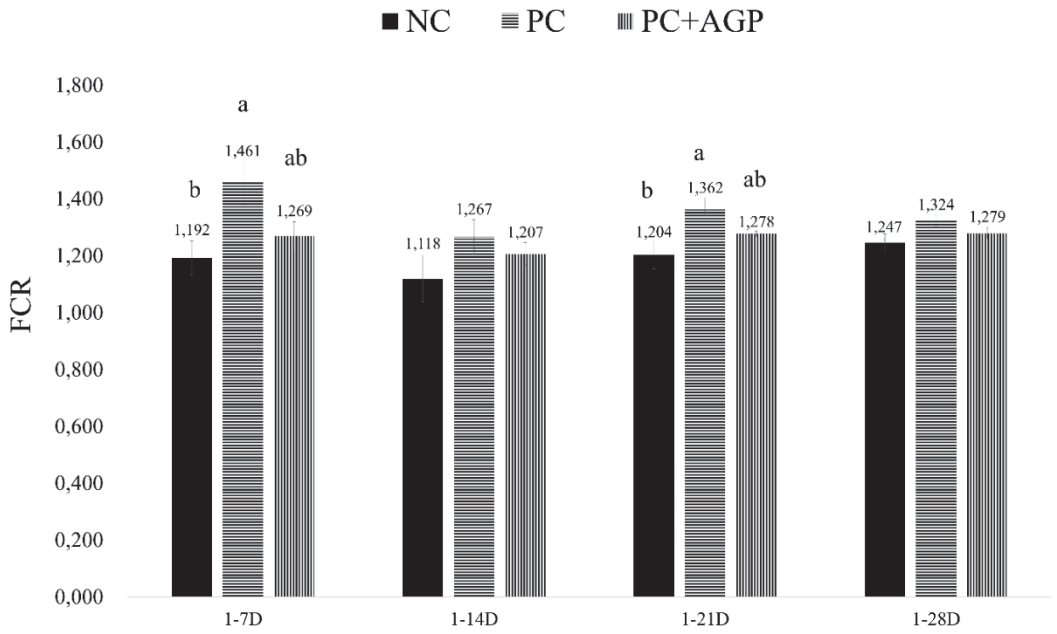

Figure 2. Mean and standard error feed conversion ratio (FCR) in grams for different treatments at each period. NC: non-challenged group, PC: *Eimeria* sp. and *Clostridium perfringens* challenge, PC+AGP: *Eimeria* sp. and *Clostridium perfringens* challenge + antibiotic growth promoter. Different superscript letters indicate significant difference (P < 0.05)

According to the results, the efficacy of the *Eimeria* sp. and *C. perfringens* challenge was verified not only by performance but also by histological analysis (Figure 3). Using ISI histological evaluation in the liver, the NC group presented lower total ISI score for all periods when compared with the challenged groups (P <0.05) (Figure 3 A). The PC and PC+AGP groups presented higher ISI scores (P<0.05) as a consequence of immune cell infiltration and congestion (Figure 4). In the ileum at the age of 14 d, the NC group presented a lower ISI score when compared with PC and PC+AGP groups (P<0.05). However, at d 21, NC and PC+AGP groups had no significant difference between them, and both presented significantly lower ISI total scores when compared to the PC group (P<0.05). At 28 d, the PC+AGP group presented a higher ISI total score compared with the NC group (P<0.05), but lower ISI total score compared with the PC group (P<0.05) (Figure 3 B). The PC group presented the highest ISI total scores (i.e., more tissue lesion) due to cell plasma infiltration on the epithelium and due to in ileum at the age of 14 days, the NC group presented lower ISI score when compared with PC and PC+AGP groups (P<0.05). However, at day 21, NC and PC+AGP groups had no significant difference between them, and both presented significantly lower ISI total score when compared to PC group (P<0.05). At 28 days the PC+AGP group presented higher ISI total score compared with NC group (P<0.05), but lower ISI total score compared with PC group (P<0.05) (Figure 3 B). The PC group presented the highest ISI total scores (i.e. more tissue lesion) due to cell plasma infiltration on epithelium and due to lamina propria inflammatory cell infiltration, causing an increase of goblet cells and presence of oocysts, at all periods (P<0.05) (Figure 5).

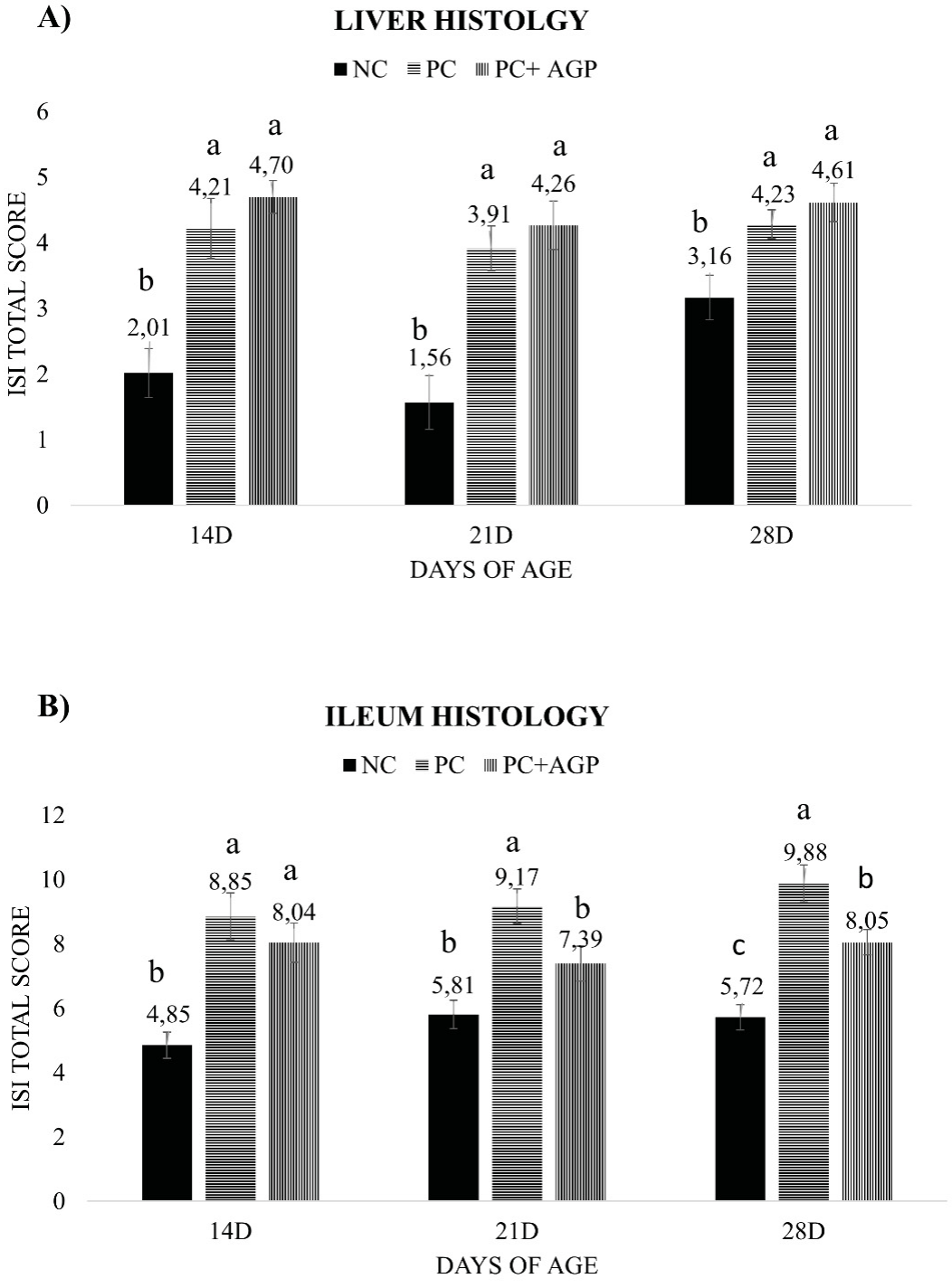

Figure 3. ISI total histological alteration scores in liver (A) and ileum (B) in different groups at 14, 21, and 28 d of age. Error bars represent standard error of the mean. NC: non-challenged group, PC: *Eimeria* sp. and *Clostridium perfringens* challenge, PC+AGP: *Eimeria* sp. And *Clostridium perfringens* challenge + antibiotic growth promoter. Different superscript letters indicate significant difference ($P < 0.05$).

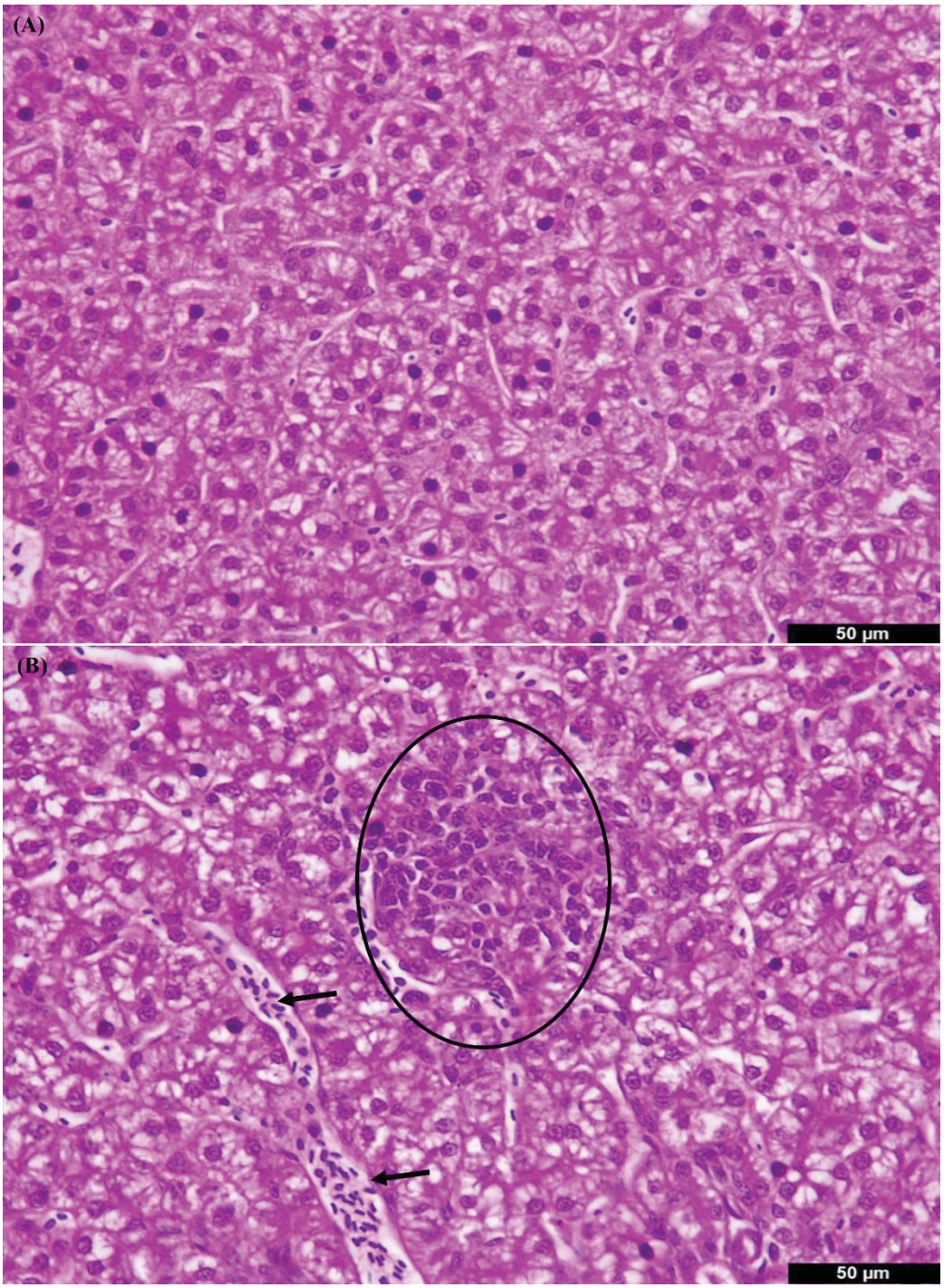

Figure 4. Photomicrographs of hematoxylin and eosin-stained chicken liver sections. A) Normal liver histological structure of nonchallenged (NC) group (400X); B) inflammatory cell infiltration (circle) and congestion (arrows) of the liver in the challenged group (PC) (400X). These changes contributed to the highest ($P < 0.05$) ISI index in *Eimeria* sp. and *C.perfringens* challenged group at 21 d of age.

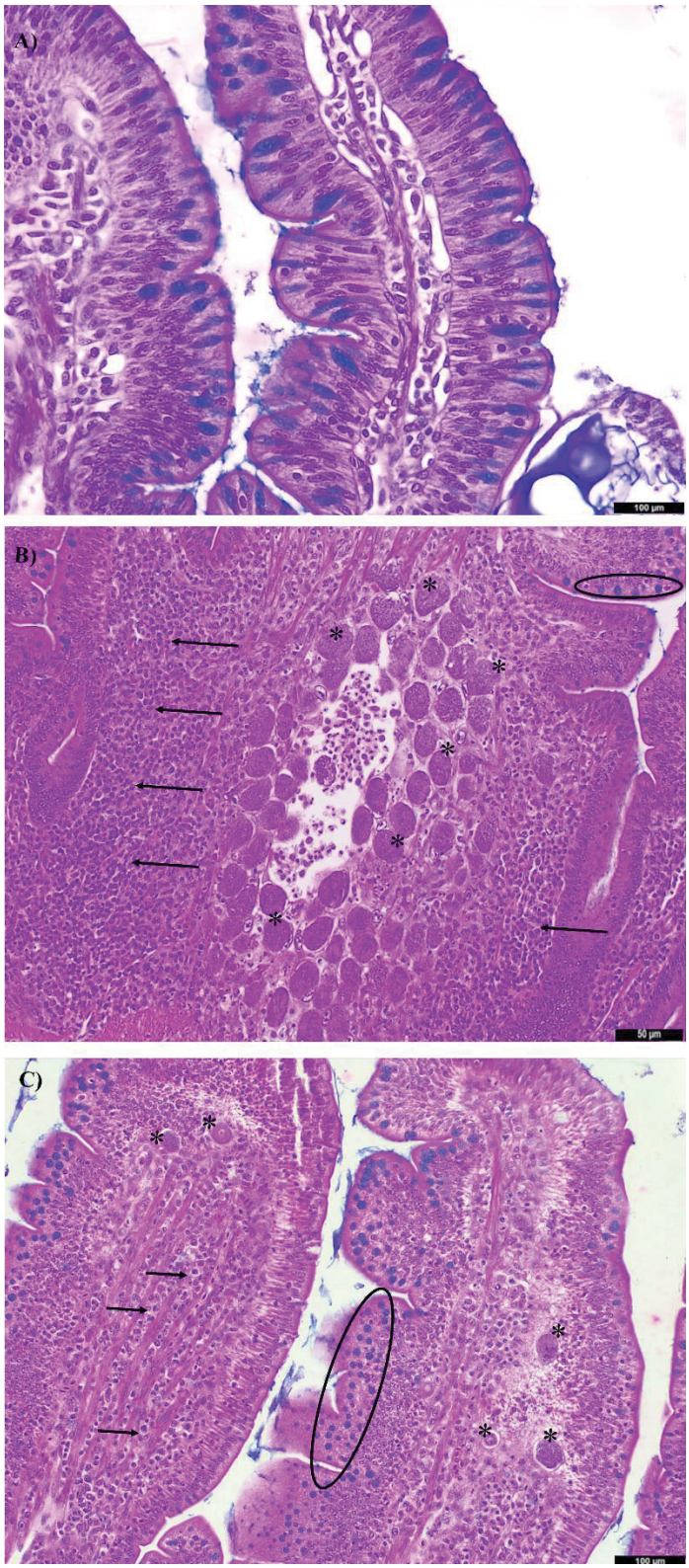

Figure 5. Photomicrographs of hematoxylin and eosin-stained chicken ileum sections. Alcian Blue was used to stain the goblet cells. A) Normal histological structure of non-challenged (NC) group in ileum (200X); B) increase of goblet cells on epithelium (circle), lamina propria inflammatory infiltration cells (arrows), and presence of oocysts (*) in the challenged group (PC) (400X; C) increase of goblet cells on epithelium (circle), lamina propria inflammatory infiltration cells (arrows), and presence of oocysts (*) in the challenged group with AGP (PC+AGP) (200X). These alterations contributed to the highest ISI ($P < 0.05$) index in *Eimeria* sp. and *C. perfringens* challenged groups at 14 d in comparison to the non-challenged group.

4 DISCUSSION

The acute phase of the immune response is related to systemic and metabolic changes and acts as the first defense mechanism (KLASING, 2004; KOGUT; KLASING, 2009). Self-maintenance entails defending molecules from damage, removing deteriorated or misplaced molecules and cells, preventing excessive proliferation of cells and defending tissues from pathogens. The consequences of self-maintenance include decrease in animal productivity (KLASING, 2009). Immunological stress caused by a disease or infection challenge has a rather profound and significant effect on feed intake, consequently affecting body weight gain and feed conversion ratio (JIANG et al., 2010; FERKET; GERNAT, 2006). In the present study, although we did not observed difference on feed intake, we did observe that the challenged group presented lower BWG from 1-14d, 1-21 and 1-28d as well as worst FCR at 1 to 7 and 1 to 21 day's periods when compared to the non-challenged group; 10 ppm of enramycin in diet did not show difference between NC and PC groups. These results could be linked to the *Eimeria* sp. cycle (ALLEN; FETTERER, 2002; SHIRLEY; LILLEHOJ, 2012) used as a challenge. The damage in the intestinal mucosa caused by *Eimeria* sp. and *C. perfringens* challenge leads to a decrease in digestion and absorption (HOFACRE et al, 2003), but is also associated with inflammation that reduces feed intake and increases energy demands (KOGUT; KLASING, 2009). Kraieski et al (2017) found a significant ($P<0.05$) negative correlation ($r=-0.72$) between immune cell infiltration on liver and BWG on *Eimeria* sp. challenge broilers. This may explain the inflammatory process on liver and ileum and the lower productivity performance of broilers observed at the present study.

The enramycin is a polypeptide antibiotic that acts in the Gram-positive bacteria cell wall biosynthesis and it is not expected to have an effect on *Eimeria* sp. control. This could explain the mild effect of this AGP on animal productivity in *Eimeria* sp. challenged birds. Both Kamran et al. (2013) and El-Husseiny et al. (2008) have shown that broilers fed with enramycin as AGP in their diet, significantly increased weight gain, feed intake and improve feed conversion of broilers in birds not challenged with *Eimeria* sp. or *C. perfringens*.

Niewold (2007) suggested that the antibiotic growth promoters actually reduce inflammatory response by sparing the immune system and, in our study, we intended to stablish this effect by evaluating histological sections. Microscopic examination of

intestine of broilers at early stages of necrotic enteritis showed strong inflammatory reactions to *C. perfringens*. The lamina propria is hyperaemic and infiltrated with numerous inflammatory cells, mainly heterophilic granulocytes. Most of the significant early changes are seen at the interface of the basal domain of enterocytes and lamina propria. These areas are extensively edematous, allowing for substantial disturbance of the structural integrity between the lamina propria and the enterocytes (OLKOWSKI et al., 2006). In our study, we evaluated and observed similar histopathological alterations (increase of lamina propria thickness, lamina propria inflammatory infiltration and congestion) but we translated these histologic observations into numbers. In challenged groups, we observed higher ISI histological scores in the liver and in ileum associated to inflammatory process as an increase of immune cells infiltration and edema.

In previous study in our laboratory applying ISI methodology, where *Eimeria sp.* vaccine and aflatoxin challenge was used, the highest ISI associated to inflammatory reaction was observed at day 14 (KRAIESKI et al., 2017). In the present study, the Figure 5 showed ileum mucosa of birds at day 14. It was possible to observe the presence of coccidian parasites in different developmental stages in the challenges groups (Figure 5 B and C), inflammatory cell infiltration on mucosa and epithelium and increase in goblet cell, which indicate that ISI methodology would also be a good tool to evaluate *Eimeria* sp. immune reaction. There is no effect of enramycin for the ISI histological parameters at this period of evaluation.

In fact, enramycin was not expected to have a direct effect on *Eimeria* sp., but the 10 ppm of enramycin in the diet reduced significantly ($P<0.05$) the inflammatory cell infiltration on the lamina propria and the lamina propria thickness (Figure 6) on ileum at 21 and 28 days, compared with challenged group without AGP (PC group). In the PC group, we did observe (Figure 6) an increase in lamina propria and epithelial thickness, inflammatory cell infiltration on epithelium and lamina propria at 21 days, and all these alterations plus an increase of goblet cell and the presence of oocysts at 28 days, in comparison to non-challenge group. The use of AGP is consistent with reduced inflammation because of reduced influx and accumulation of inflammatory cells (LARSSON et al., 2006).

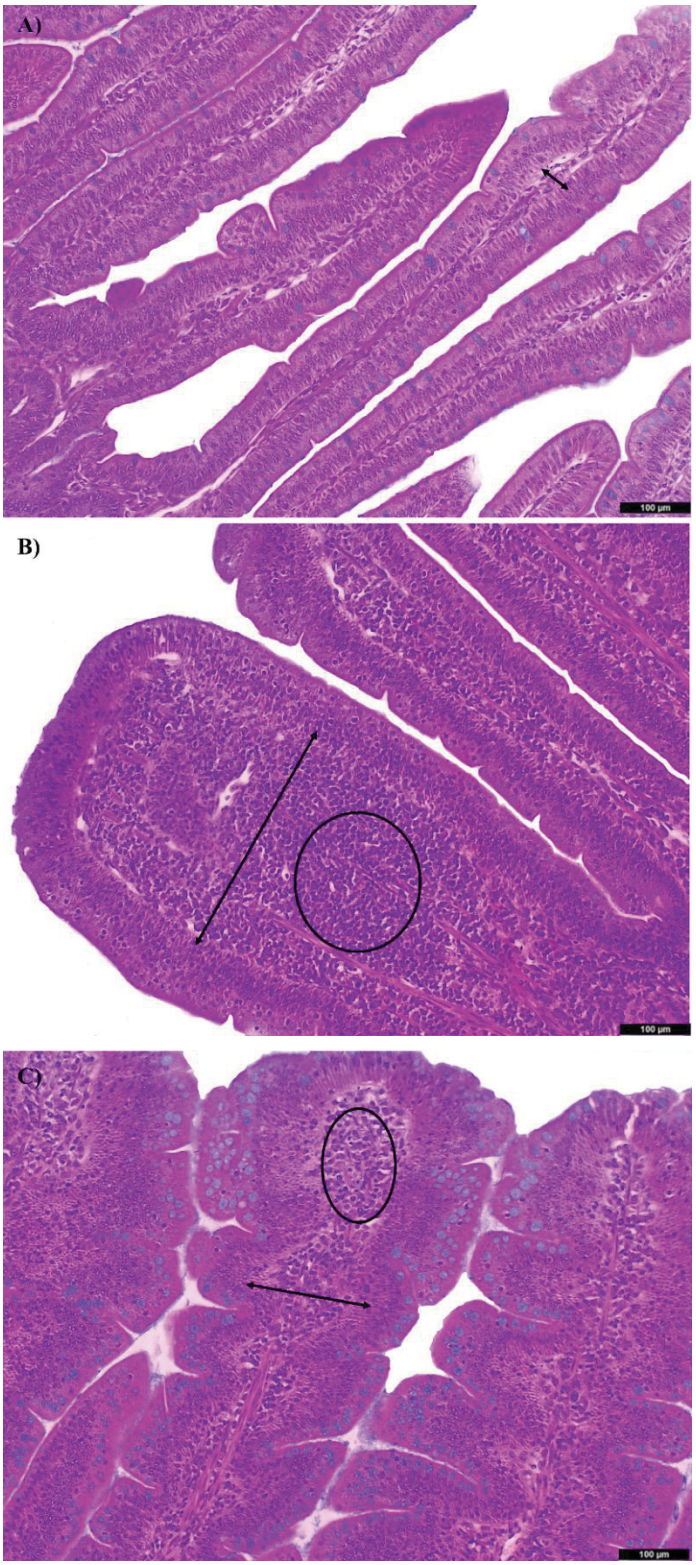

Figure 6. Photomicrographs of hematoxylin and eosin-stained chicken ileum sections. Alcian Blue was used to stain the goblet cells. A) Normal histological structure of non-challenged (NC) group in ileum; arrows indicate increase of lamina propria thickness, and no inflammatory infiltration cells in the lamina propria (200X); B) increase of lamina propria thickness (arrows) and lamina propria inflammatory infiltration cells (circle) in challenged group (PC) with high ISI score (200x); C) increase of lamina propria thickness (arrows) and lamina propria inflammatory infiltration cells (circle) in challenged group with AGP (PC+AGP) with low ISI score (200x). These alterations contributed to the highest ISI ($P < 0.05$) index in *Eimeria* sp. and *C. perfringens* challenged (PC) group at 21 days.

Despite other segments of small intestine possibly being affected by *Eimeria* sp., the ileum was chosen to this evaluation because it was supposed to be the segment most affected by the association of *Eimeria* sp. + *C. perfringens* challenge.

In the liver, we did not observe any effect of the enram-*ycin from the diet on the inflammation process as we observed in the ileum, which suggested a local effect. *Clostridium. perfringens* induces hepatitis (LOVLAND; KALDHUSDAL, 2001) and we did not observe reduction on hepatitis on challenge group fed with AGP. Although we did not evaluate the Minimum Inhibitory Concentration (MIC) of enramycin for *C. perfringens* strain used, Redondo et al (2015) described a MIC of 2-8 ppm of enramycin for poultry isolated *C. perfringens* strain from Argentina.

The challenge used in this study was an association of *Eimeria* sp. and *C. perfringens,* and many alterations on the microbiome and its relationship with host immune response are expected. It is important to have more studies on this area to establish if the effect of enramycin is due to a single antimicrobial, anti-inflammatory effect or an association of both mechanisms.

We could observe that our challenge and ISI methodology allowed us to numerically compare the effect of different treatments on gut health. The use of enramycin as AGP reduced the lamina propria thickness and inflammatory cell infiltration at the lamina propria compared to challenged birds without AGP. We can infer that the lamina propria thickness and inflammatory cell infiltration at the lamina propria are good parameters to be evaluated when we intend to compare the effect of AGP and other AGP alternative products on intestinal mucosa.

CHAPTER 2:

APPLYING I SEE INSIDE HISTOLOGICAL METHODOLOGY TO EVALUATE GUT HEALTH IN BROILERS CHALLENGED WITH *EIMERIA*

APPLYING I SEE INSIDE HISTOLOGICAL METHODOLOGY TO EVALUATE GUT HEALTH IN BROILERS CHALLENGED WITH *EIMERIA*

ABSTRACT

The present study evaluated the effects of coccidiosis on histological parameters and performance of broilers submitted to a mild challenge with *Eimeria* sp. A total of 132 broilers were randomly divided into two groups with 6 replicates of 11 birds each: negative control (NC) – birds uninfected and challenge (CH) - birds infected by gavage at day-one with 10x the manufacturer recommended dose of an *Eimeria* sp. Vaccine. From 1 to 28 d of age, weekly the zootechnical performance was evaluated and samples of the liver, duodenum, jejunum and ileum were collected and submitted to histological analysis by the I See Inside methodology (ISI). The ISI methodology is a metric evaluation of histological alterations in the intestine and liver. Which translates macroscopic and microscopic alterations into numbers and allows their correlation with the animal zootechnical performance. Pearson's correlation was used to demonstrates how the damages to the gut, evaluated by ISI method, could affect negatively the performance of broilers. The presence of oocysts in histological analysis of the jejunum and ileum at 7d indicate a negative effect on performance over the next 2 weeks (14d and 21d). When the ISI total score was measured in the ileum at 14d, we observed a strong negative effect on zootechnical performance from that period on 14d, 21d and 28d. The obtained data demonstrates that the higher the ISI scores, the worse are the zootechnical performance results of broilers challenged with *Eimeria* sp and the I See Inside scores could be applied to evaluate coccidiosis effect on performance in future.

1 INTRODUCTION

Chicken coccidiosis is a disease caused by several protozoan parasites of the genus *Eimeria*; its economic impacts could reach up to 3 billion dollars per year worldwide (DALLOUL; LILLEHOJ, 2006). Seven *Eimeria* species have been recognized to infect chickens: *Eimeria acervulina*, *E. maxima*, *E. tenella*, *E. necatrix*, *E. mitis* and *E. praecox*. Each species has its own characteristics regarding lesion, site of infection in the intestine and immunogenicity (VERMEULEN et al., 2001). The infectious parasite invades intestinal epithelial cells, eliciting a variety of clinical manifestations, including necrotic gut lesion, inefficient feed utilization, impaired growth rate and, in several cases, mortality (MIN et al., 2013).

The most common zootechnical aspects observed during infection are poor growth rate, reduction in weight gain and worse feed conversion without mortality (LEE et al., 2013) even in the absence of clinical symptoms (sub-clinical coccidiosis). The pathological changes vary from local disintegration of the mucosa barrier (associated

with inflammation on the underlying tissue) to systemic effects such as blood loss, shock syndrome and even death (VERMEULEN et al., 2001).

The diagnosis of sub-clinical coccidiosis is very difficult, provided that there are only mild, non-specific macroscopic histological alterations. These are sometimes labeled as “non important lesions” during pathological analysis and therefore neglected. The I See Inside methodology (ISI), described by Kraieski et al (2017) and adapted by Belote et al (2018), is a metric evaluation of histological alterations in the intestine and liver. ISI translates macroscopic and microscopic alterations into numbers and allows their correlation with the animal zootechnical performance. For this reason, we believe that the ISI methodology could be a good tool to determine, in raw numbers, mild tissue alterations caused by this disease and their correlation with performance results.

The objective of the present study was to determine the macroscopic and microscopic alterations in intestine and liver of broilers, both non-challenged and challenged with *Eimeria sp*. and to correlate these results with their performance applying the ISI methodology.

2 MATERIALS AND METHODS

2.1 ETHICS STATEMENT

The study protocol and the use of all animal studies were approved by the Animal Use Ethics Commission (Comissão de Ética no Uso de Animais – CEUA), Agricultural Sciences Sector of the Federal University of Paraná – Brazil (Permission number 032/2015).

2.2 HOUSING CHICKENS

One-day-old male Cobb 500 broilers were housed from 1 to 28 days of age in negative pressure facilities previously cleaned and disinfected. Sterile litter was used, as well as nipple drinkers. Automatic temperature control was set according to comfort conditions for each life stage. The temperature was gradually decreased from 34°C on day 1 to 24°C on day 28 and then kept constant. During the whole experiment, birds received water and feed *ad libitum* and the diet followed the Brazilian nutritional recommendations for poultry (Rostagno et al., 2011), without any anticoccidials drug or antibiotic growth promoter.

2.3 EXPERIMENTAL DESIGN

A total of 132 one-day-old broilers were randomly divided in two treatments with 6 replicates of 11 birds each. The treatments were negative control (NC) – uninfected, untreated and challenge birds (CH) – birds infected with 10x the manufactured recommended dose of commercial *Eimeria* vaccine (in order to induce experimental sub-clinical coccidiosis disease).

The challenge was done at the first day of trial by oral gavage with 10X the manufacturer recommended dose of Bio-Coccivet R® live vaccine by Biovet, with strains of *E. acervulina, E. brunetti, E. maxima, E. necatrix, E. praecox, E. tenella* and *E. mitis*. Each bird from the CH group received 0.5 mL solution with 330.000 oocysts of *Eimeria* spp. Each bird of the NC group received 0.5 mL of physiologic water instead.

2.4 PERFORMANCE PARAMETERS

At one day of age, birds were weighted and distributed in each treatment in order to obtain equal initial body weight average per cage (replicate) in each treatment. Birds and feed were weighed weekly (at zero, 7, 14, 21, and 28 days) to evaluate feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR).

2.5 MACROSCOPIC ANALYSIS AND SAMPLE COLLECTION

At 2, 7, 14, 21 and 28 days of age, 6 birds were euthanized per treatment. Macroscopic alterations were evaluated using ISI methodology following Kraieski et al (2017), by three observers. Then, samples of liver, duodenum, jejunum and ileum were collected and fixed in Davidson's solution (100 mL glacial acetic acid, 300 mL 95% ethyl alcohol, 200 mL 10% neutral buffered formalin and 300 mL distilled water) for at least 24 hours. Then, samples were dehydrated, infiltrated and embedded in paraffin following common histological routine. Blocks were cut in 5 µm sections and stained with hematoxylin and eosin with Alcian Blue for goblet cells staining (Rapp and Wurster, 1978).

2.6 I SEE INSIDE METHODOLOGY (ISI)

The ISI (I See Inside) methodology applied for microscopy was according to Kraieski et al (2017), while the histological routine was according to Belote et al (2018), adapted from Kraieski et al. (2017). The “I See Inside” (ISI) methodology, patent pending (INPI BR 10201500360l9), is based on a numeric score of alteration with the evaluated parameter listed in Table 1. In this methodology, an impact factor (IF) is defined for each alteration in macroscopic and microscopic analysis. The IF is established according to the reduction of an organ’s functional capacity, based on previous knowledge from the literature and background research (e.g. necrosis has the highest IF because the functional capacity of the affected cells is completely lost). It ranges from 1 to 3, with 3 being the highest impact to organ function. In addition, the extent of each lesion (intensity) or the observed frequency of the lesion compared to non-affected organs is evaluated in each organ/tissue with score (S) ranging from 0 to 3, where score 0: absence of lesion or frequency; score 1: alteration in up to 25% of the area or observed frequency; score 2: alteration ranges from 25.1 to 50% of the area or observed frequency and score 3: alteration extends to more than 50% of the area or observed frequency.

To obtain the final value of the ISI index referred as ISI total score, the IF of each alteration is multiplied by its respective score number and the results of all alterations are summed according with the formula ISI =Σ(IF*S), where IF = impact factor and S = Score. For example, the increase of lamina propria thickness has an IF = 2. This number is multiplied by the observed score (ranging from 1 to 3). If a S = 3 (maximum score) is given to the increase of lamina propria thickness in a villus, the ISI final value for this parameter in this villus will be ISI = (2 * 3) = 6. An average of 20 intestinal villi per slide was evaluated per bird in 10X objective (using 20X and 40X objective to confirm alterations) of an optical microscope (Nikon Eclipse E200, Sao Paulo-SP- Brazil). Liver samples were evaluated in 10 fields per bird in 10X objective. The ISI scales range from 0 to 45 for the intestine and from 0 to 42 for the liver.

Table 1. ISI histological alterations evaluated in intestine and liver.

Organ	Alteration	Impact Factor (IF)	Maximum[1] Score
Intestine	Lamina propria thickness	2	45
	Epithelial thickness	1	
	Enterocytes proliferation	1	
	Epithelial plasma cell infiltration	1	
	Inflammatory infiltration in the lamina propria	3	
	Goblet cells proliferation	2	
	Congestion	2	
	Presence of oocysts	3	
Liver	Congestion	1	42
	Cell vacuolation	2	
	Bile-duct proliferation	2	
	Immune cells infiltration	1	
	Necrosis	3	
	Pericholangitis	3	
	Lymphocytic aggregate	2	

[1]Maximum score represents the sum of all alterations according to the formula ISI =Σ(IF*S) where IF = impact factor (previously fixed) and S=Score (observed) considering the maximum observed S. For example, the lamina propria thickness has IF = 2, this number is multiplied by the observed score (ranging from 1 to 3). If in a villus, a score S=3 (maximum score) was observed for lamina propria thickness, the ISI for this parameter in this villi will be ISI = (2*3) = 6. The average of 20 villi in ileum or 10 fields in the liver for each bird will indicate the final ISI value for each bird.

2.7 STATISTICAL ANALYSIS

Data were presented as mean plus minus standard error. At first, all data were tested for normality using Shapiro-Wilk normality test. Parametric rates were compared using one-way analysis of variance (ANOVA), followed by Tukey test ($P<0.05$). For performance, each cage was used as a replicate (n = 4) while each bird was used as a sample for the remaining analysis (n = 6). All analysis were performed at Statistix 9 software for Windows. For correlation analysis, Pearson's correlation coefficient (r) was used and the software provided the P values.

3 RESULTS

3.1 PERFORMANCE PARAMETERS

The CH group showed FI reduction ($P<0.05$) at times 1-14d, 1-21d and 1-28d when compared with the NC group (Figure 1), and there was no significant difference among all groups at time 1-7d. The BWG was lower in the CH group for intervals 1-7d, 1-14d, 1-21d and 1-28d when compared to the NC group ($P<0.05$) (Figure 2). The FCR

was worst (P<0.10) in the CH group when compared with the NC group at intervals 1-14d and 1-21d (Figure 3) and there was no significant difference between groups at other periods.

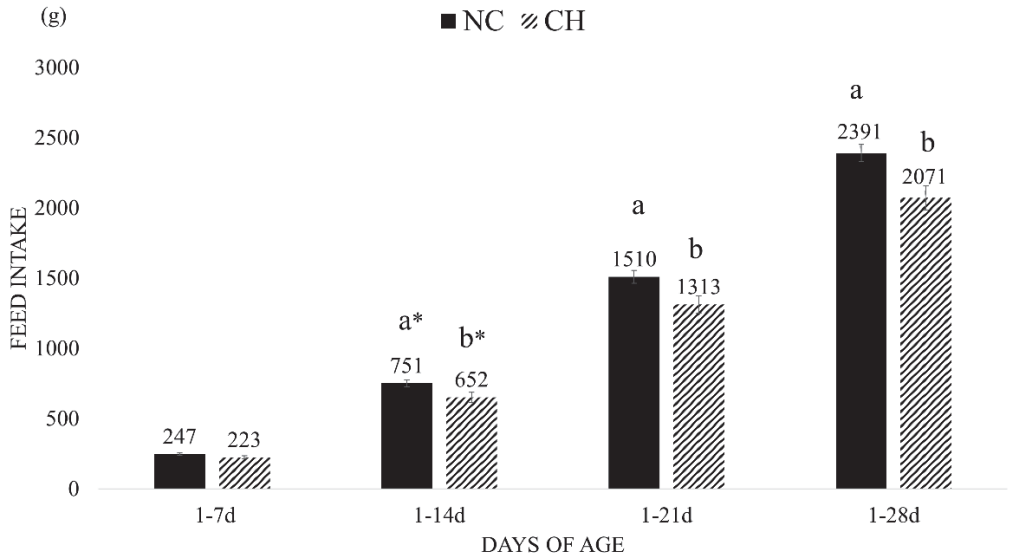

Figure 1. Feed Intake (FI, grams) mean and standard error of treatment at different time periods. Treatments were negative control (NC) –and uninfected, untreated and Challenge (CH) – where animals were infected with 10x the manufactured recommended dose. Different superscript letters indicate significant difference with Tukey test (P < 0.05); * sign on letters indicate a significant level of P<0.07.

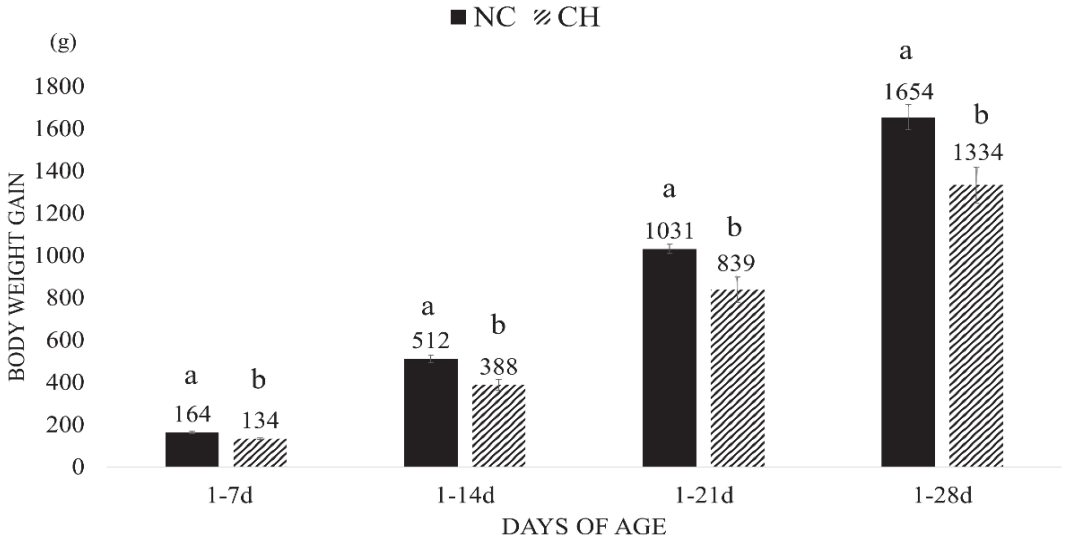

Figure 2. Body weight gain (BWG, grams) mean and standard error of treatment at different time periods. Treatments are negative control (NC) and uninfected, untreated and Challenge (CH), where animals were infected with 10x the manufactured recommended dose. Different supercript letters indicate significant difference with Tukey test (P < 0.05).

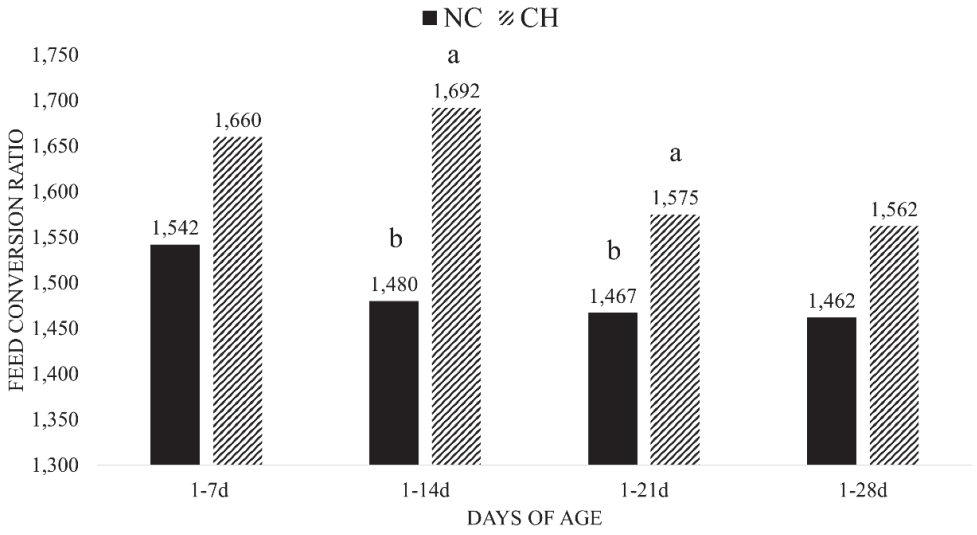

Figure 3. Mean Feed Conversion Ratio (FCR) of treatment at different time periods. Treatments are negative control (NC) and uninfected, untreated and Challenge (CH), where animals were infected with 10x the manufactured recommended dose. Different superscript letters indicate a significant difference with Tukey test (P < 0.10).

3.2 MACROSCOPIC AND HISTOLOGICAL ANALYSES

The CH group presented higher score of coccidiosis macroscopic lesions in comparison to the NC group at time 14d (P<0.05) (CH ISI score1.8±0.7; NC ISI score: 0.0±0.0) and 21 days (NC ISI score 0.0±0.0 and CH ISI score 2.7±0.7). There was no difference for the other macroscopic parameters of ISI between groups at the other periods.

For microscopic results of liver, the CH group showed higher ISI total score in comparison with the NC group at 2d, 21d and 28d of age (P<0.05) (Figure 4) and there was no significant difference at the other periods. The CH group presented higher ISI total score in comparison to other groups due to higher scores at lymphocyte infiltration and cell vacuolization (P<0.05) (Figure 5).

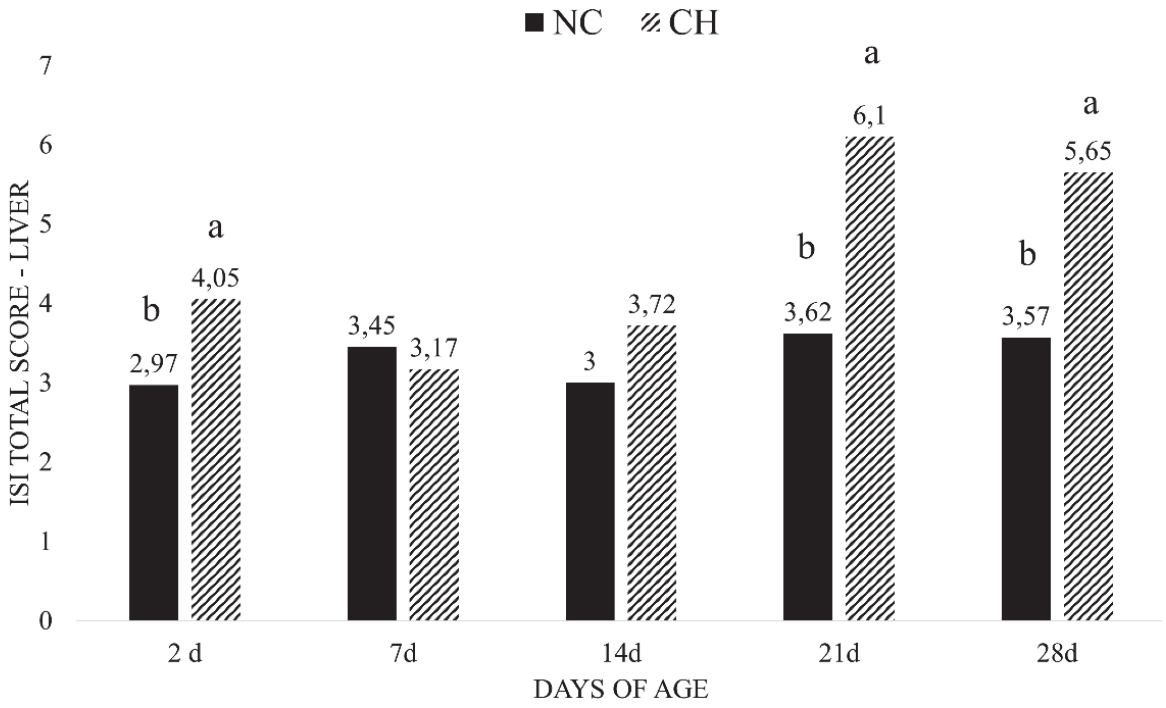

Figure 4. ISI total score of liver lesions at all periods. Data is the mean value of each treatment at different time intervals. Treatments are negative control (NC) and uninfected, untreated and Challenge (CH) – where individuals were infected with 10x the manufactured recommended dose. Different superscript letters indicate significant difference with Tukey test ($P < 0.05$).

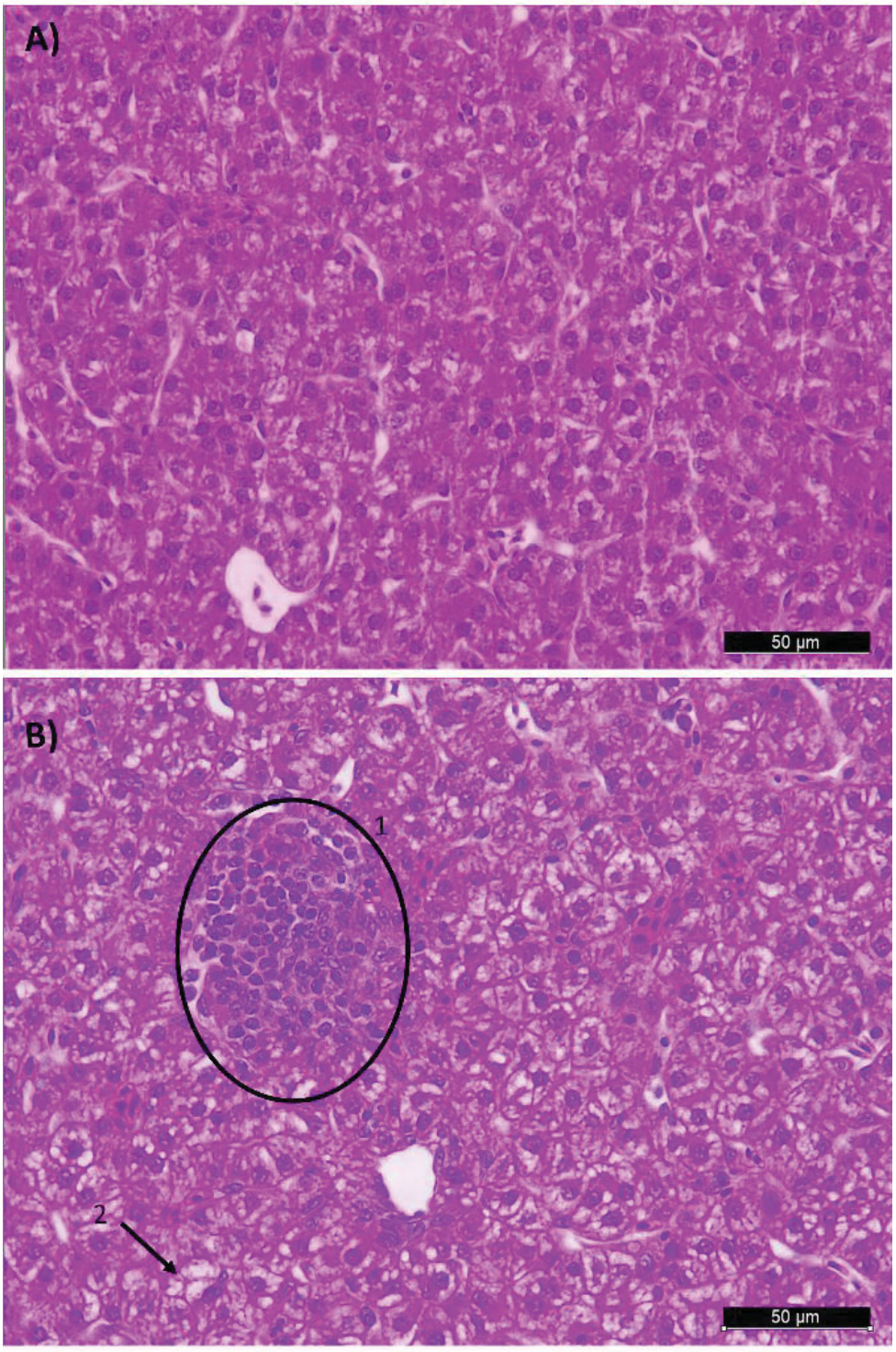

Figure 5. Photomicrographs chicken liver sections stained with hematoxylin and eosin. A) normal liver histological structure of non-challenge (NC) group (400X); B) 1. Inflammatory cell infiltration (circle) and 2. Presence of vacuolization (arrow) in liver of an individual from the Challenged group (CH) (400X). These changes contributed to the highest ($P < 0.05$) ISI index in *Eimeria* sp. challenge group at 21 days of age.

In the duodenum, the CH group showed higher ISI total score compared with NC group (P<0.05) (CH ISI score 16.6±0.9; NC ISI score: 12.6±1.0) at 28d, but no significant statistical difference was observed at the other periods. These results were due to higher scores of inflammatory cell infiltration in the lamina propria and on epithelium and congestion in the CH group when compared to the NC group (Figure 6).

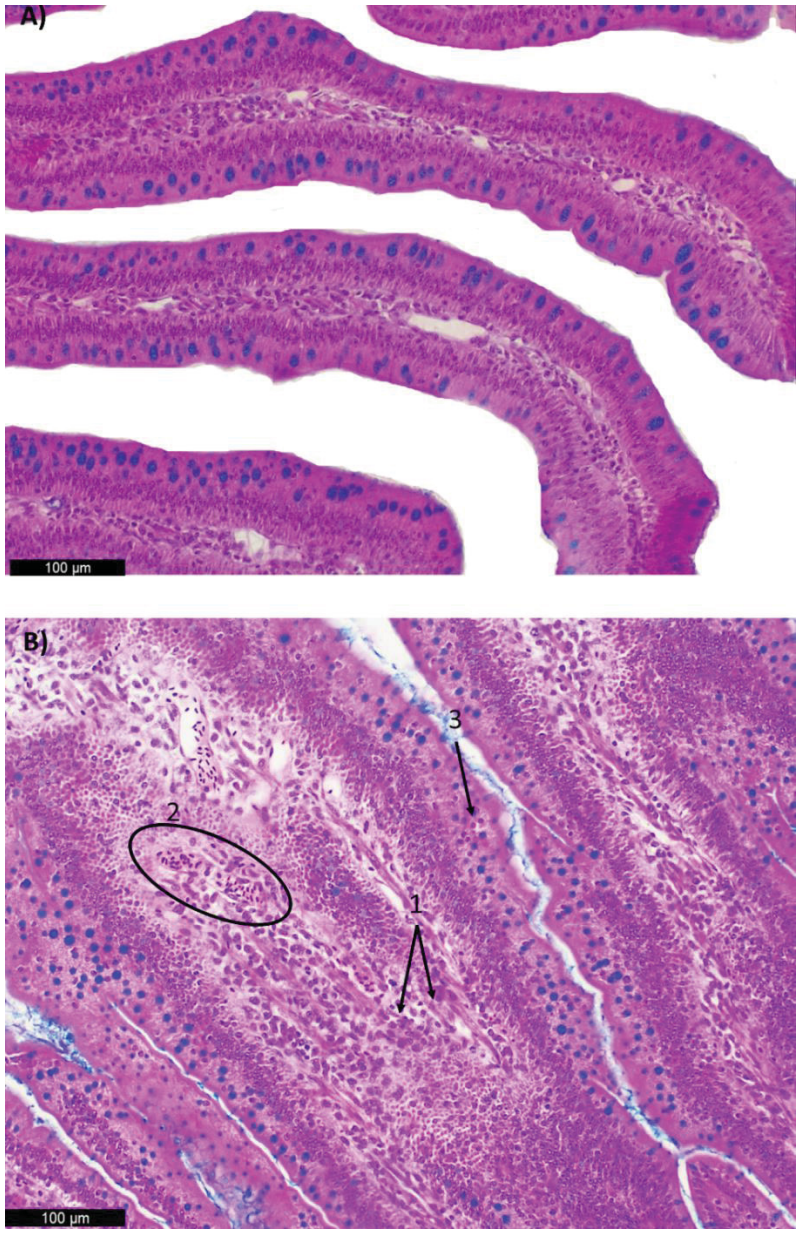

Figure 6. Photomicrographs of chicken duodenum sections stained with hematoxylin and eosin. Alcian Blue was used to stain goblet cells. A) Normal histological duodenum structure of non-challenge treatment (NC) (200X); B) Challenged treatment (CH) (200x) 1. Lamina propria inflammatory infiltration cell; 2. Congestion and 3. Epithelium inflammatory infiltration cell; These alterations contributed to the highest ISI index (P< 0.05) in coccidiosis challenged group at 28 days in comparison to the non-challenged group.

In the jejunum at 21d, the CH group showed higher ISI total score when compared with the NC group (P<0.05) (CH ISI score 16.6±0.6; NC ISI score 10.8±0.6). There was no significant difference between groups at the other periods. The CH group presented higher ISI total score in comparison to other groups due to higher scores (P<0.05) caused by inflammatory cell infiltration in the lamina propria and epithelium, increase of lamina propria thickness and presence of oocysts (Figure 7).

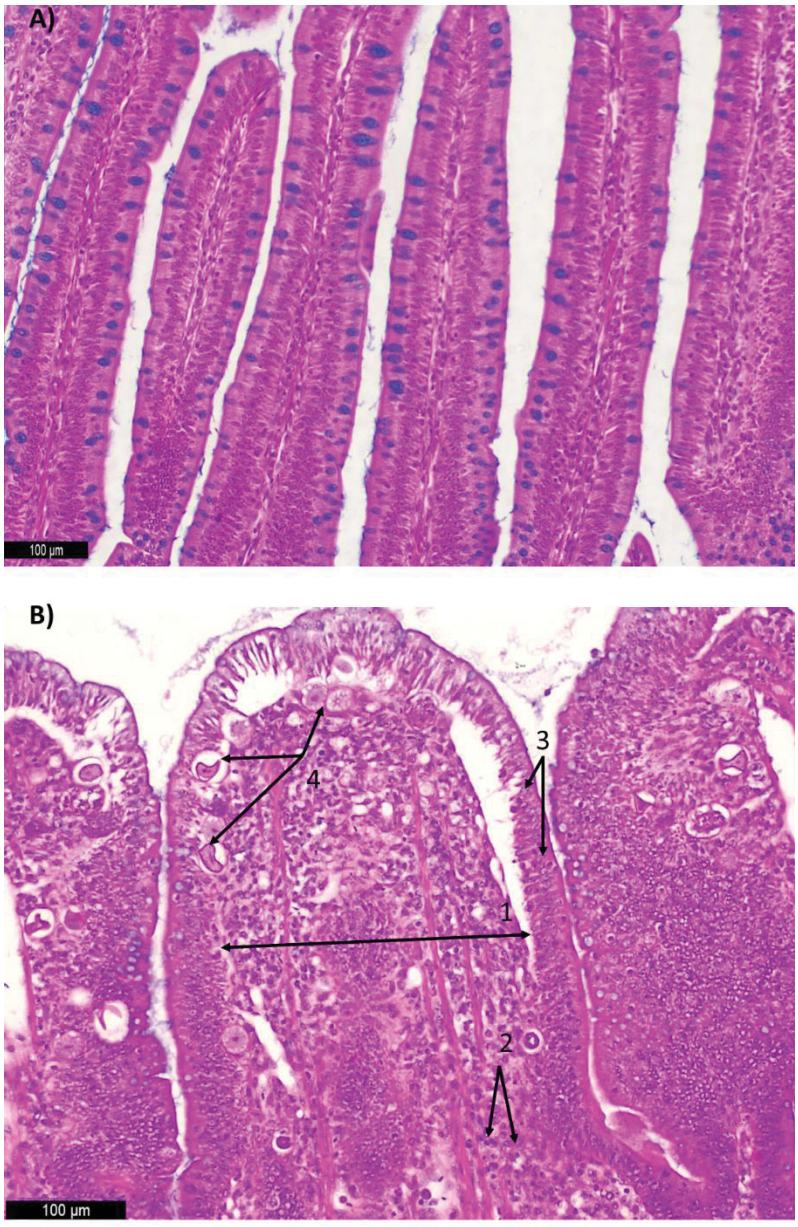

Figure 7. Photomicrographs of chicken jejunum sections stained with hematoxylin and eosin. Alcian Blue was used to stain goblet cells. A) Normal histological structure of non-challenge treatment (NC) in jejunum (200X); B) Challenged treatment (CH) (200x) 1. Increase of lamina propria thickness; 2. Lamina propria inflammatory infiltration cell; 3. Epithelium inflammatory infiltration cell and 4. Presence of oocysts; These alterations contributed to the highest ISI index (P< 0.05) in coccidiosis challenged group at 21 days in comparison to the non-challenged treatment.

In the ileum, the CH group presented higher ISI total score when compared with the NC group (P<0.05) (Figure 8) at times 2d, 7d, 14d and 21d. No significant difference was observed between groups at 28d. At 2d, the higher ISI total score in the CH group was due to inflammatory cell infiltration in the lamina propria. At 7d, the CH group showed higher ISI total score when compared to the NC group due to higher scores (P<0.05) of inflammatory cell infiltration in the epithelium. At 14d and 21d, the CH group presented higher ISI total score when compared to the NC group due to higher scores(P<0.05) of lamina propria thickness, inflammatory cell infiltration in the lamina propria and presence of oocysts (Figure 9).

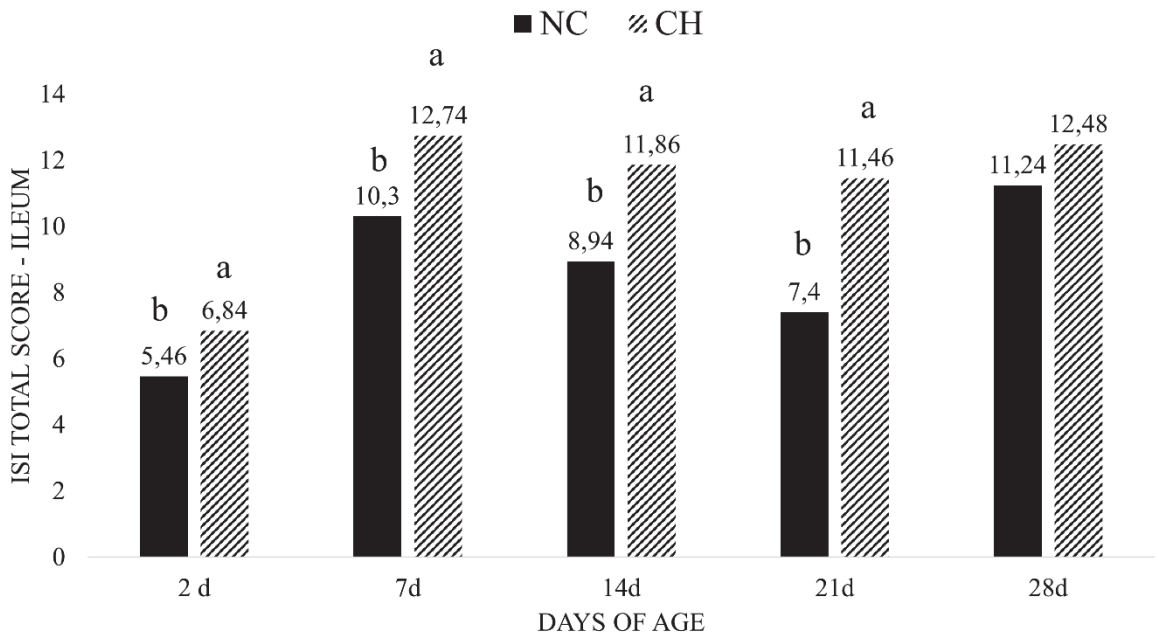

Figure 8. ISI total score of ileum at all periods. Data is the mean of treatments in all time intervals. Treatments are negative control (NC) and uninfected, untreated and Challenge (CH) – where animals were infected with 10x the manufactured recommended dose. Different superscript letters indicate a significant difference with Tukey test (P < 0.05).

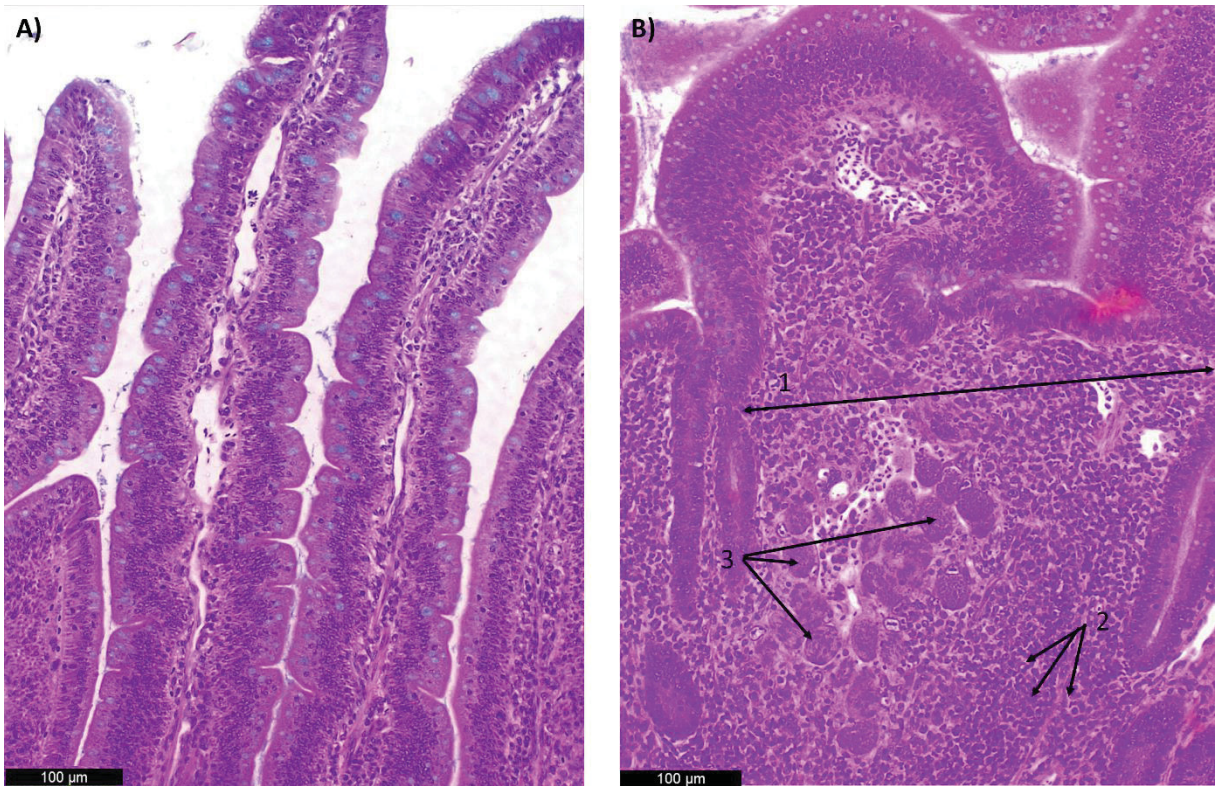

Figure 9. Photomicrographs of chicken ileum sections stained with hematoxylin and eosin. Alcian Blue was used to stain the goblet cells. A) Normal ileum histological structure of non-challenge treatment (NC) (200X); B) Challenged treatment (CH) (200x) 1. Higher lamina propria thickness; 2. Lamina propria inflammatory infiltration cell; 3. Presence of oocysts; These alterations contributed to the highest ISI index ($P < 0.05$) in coccidiosis challenged group at 21 days in comparison to the non-challenged group.

3.3 PEARSON'S CORRELATION

The results obtained with Pearson's correlation demonstrates how the damages to gut, evaluated by ISI methodology, could negatively affect the performance of broilers ($P<0.05$). It was observed that the higher the ISI total score, the worse the zootechnical performance results of broilers.

In the duodenum, the inflammatory cell infiltration in the lamina propria observed at 21d negatively affected the BWG ($r= -0.91$) and FI ($r=-0.83$) at the next time interval (28d). The ISI total score measured in the duodenum at 28d negatively influenced the BWG ($r=-0.93$) and FI ($r= -0.97$) at the same time.

The presence of oocysts observed in the microscopic analysis of the jejunum at 7d negatively affected the BWG ($r=-0.88$), FI ($r=-0.70$) and FCR ($r=0.75$) at 14d and the BWG ($r=-0.80$), FI ($r=-0.69$) and FCR ($r=0.81$) at 21d. At 14d, the presence of oocysts in the jejunum negatively affected the BWG ($r=-0.85$), FI ($r=-0.73$) and FCR

(r=0.70) at 28d. The presence of oocysts observed in the jejunum at 21d negatively affected the BWG (r=-0.81) and FI (r=-0.84) at the same time.

The increase of lamina propria thickness observed in the jejunum at 2d had a negative correlation with the FI at 7d (r=- 0.68), 14d (r=-0.86), 21d (r=-0.81) and 28d (r=-0.88) and the BWG at 21d (r=-0.73) and 28d (r=-0.76). The increase of lamina propria thickness observed in the jejunum at 21d was negatively correlated with BWG (r=-0.74) at 28d.

The inflammatory cell infiltration in the epithelium of the jejunum at 14d presented a negative correlation with BWG (r=-0.88), FI (r=-0.65) and FCR (r=0.92) at 28d. In the same intestinal portion, the inflammatory cell infiltration in the lamina propria at 21d showed a negative influence over the BWG (r=-0.87), FI(r=-0.75) and FCR (r=0.69) at 28d. The ISI total score measured in the jejunum at 21d negatively affected the BWG (r=-0.92), FI (r=-0.89) and FCR (r=0.63) at 28d.

The inflammatory cell infiltration in the epithelium of the ileum at 2d negatively affected the BWG (r=-0.72) and FI (r=-0.70) at 21d and BWG (r=-0.71) at 28d. The inflammatory cell infiltration in the lamina propria observed at 14d showed a negative influence over the BWG (r=-0.90), FI (r=-0.77) and FCR (r=0.66) at 14d, BWG (r=-0.86), FI (r=-0.80) and FCR (r=0.72) at 21d and BWG (r=-0.81) and FI (r=-0.81) at 28d.

The presence of oocysts in the ileum at 7d negatively influenced the BWG and FI at 14d (r=-0.74 and r=-0.68, respectively) and at 21d (r=-0.74 and r=-0.78, respectively). At 14d, the presence of oocysts in the ileum presented a negative influence over the BWG (r=-0.76), FI(r=-0.70) and FCR(r=0.76) at 21d.

The increase of lamina propria thickness in the ileum at 14d showed a negative effect over the BWG (r=-0.85), FCR (r=0.87) at 14d, BWG (r=-0.66) and FCR (r=0.68) at 21d, and BWG (r=-0.62), FI (r=-0.61) at 28d.

The ISI total score was measure in the ileum at 14d could strongly affect negatively the performance at other periods: BWG (r=-0.95), FI (=-0.84) and FCR (r=0.71) at 14d, BWG (r=-0.93), FI (r=-0.83) and FCR (r=0.85) at 21d, and BWG (r=-0.83) and FI (r=-0.87) at 28d. The ISI total score measured in the ileum at 21d was observed to affect negatively the BWG (r=-0.85), FI (r=-0.81) and FCR (r=0.79) at 21d and, BWG (r=-0.75) and FI (r=-0.79) at 28d.

4. DISCUSSION

The consequences of self-maintenance include a decrease in animal productivity (KLASING, 2007). The damage in the intestinal mucosa caused by *Eimeria* leads to a decrease in digestion and absorption of feed (HOFACRE et al, 2003) and it is also associated with inflammation, which reduces FI and increases energy demands (KOGUT; KLASING, 2009). In the present study, it was observed a reduction of FI of 13% at 14d, 21d and 28d and a reduction of BWG of 18% at 7d and of 19% at 21d and 28d. The biggest reduction was observed for BWG at 14d, with a decrease of 24% in CH group when compared with NC group. We also observed a decrease of 13% and 7% on FCR between groups at 14d and 21d, respectively. According to Jiang et al. (2010), the induction of an acute inflammatory reaction reduces the BWG in 22%. From this decrease, 59% comes from the reduction on FI and the other 41% would be consequence of the immune response. The histological parameters of increase of lamina propria thickness and of inflammatory cell infiltration in the lamina propria observed at 14d in the ileum presented a strong negative influence over the performance at 14d, 21d and 28d. Both these parameters were described by Belote et al (2018) as good parameters to compare intestinal health between different treatments.

The infection by *Eimeria* sp. changes the structure of the intestinal villi, decreasing the absorption capacity due to destruction of intestine epithelial cells (SHIRLEY; LILLEHOJ, 2012). The *Eimeria* sp. biological cycle is very complex and comprises intracellular, extracellular, asexual and sexual stages. The species *E. acervulina, E. maxima* and *E. tenella* reach the crypt epithelium of the host (SHIRLEY; LILLEHOJ, 2012). These protozoans induce a local inflammatory response due to intracellular development (HONG et al., 2006) and their replication leads to cellular damage in the epithelium (SHIRLEY; LILLEHOJ, 2012). The highest number of oocysts of *E. acervulina* and *E. maxima* in the litter of commercial flocks are found on the period of 4-5 weeks after the infection (LONG; TOMPKINS; MILLARD, 1975). In the duodenum, the highest ISI total scores of lesions were observed on the 4th week post-challenge, what could be related to oocysts release from the villi

During the 2nd and 3rd phase of the asexual replication, gut damage becomes more evident because of the high number of merozoites infecting enterocytes. This asexual life phase results in an explosion in parasite number (CHAPMAN, 2003).

Sporulated oocysts must excyst in the intestine after the ingestion, but not all of them really excyst. Up to 20% of ingested sporulated oocysts can pass through undamaged and still sporulate later (WILLIAN, 1995). This means that if a small proportion of the sporulated oocysts in an inoculum of a live vaccine fail to infect a chick, they can be re-ingested within a day of vaccination and infect a chick on a subsequent occasion (WILLIAN, 1998). About 1 week after vaccination, a small number of the attenuated vaccine oocysts are observed multiplying with a small peak of production at 2-4 weeks, during which time, protective immunity is built up as the parasites recycle, and another peak appears at 4–6 weeks, decreasing thereafter (CHAPMAN et al., 2016; WILLIAN, 1998). The presence of oocysts in histological analysis of the jejunum and ileum at 7d indicate a negative effect on performance in the next 2 weeks (14d and 21d) ($P<0.05$). The *E. maxima* oocyst sporulation in litter containing 5% moisture increasing to 16% during 106h and in litter containing 60% moisture, increasing to 62% (WILLIAN, 1998). It is noteworthy that specific *E. maxima* variants may be more frequent in broiler farms experiencing low performance of animals (SCHWARZ et al., 2009). When the ISI total score was measured in the ileum at 14d, we observed a strong negative effect on performance from that period on (14d, 21d and 28d) ($P<0.05$), which suggests the use of this parameters as a good tool to control the *Eimeria* infection in broilers.

5 CONCLUSION

We observed that the challenge of birds with 10X dose of *Eimeria* vaccine leads to mild macroscopic lesion at the intestine and significant histological alterations as well as reduction on zootechnical performance;

The application of the ISI methodology allows a numeric translation of histologic lesion and its correlation with losses in animal performance;

The most expressive correlation was the total ISI score of the ileum at 14d that shows a strong negatively effect on performance at following periods (14d, 21d and 28d) ($P<0.05$), which suggests the use of this methodology could be a good tool to control *Eimeria* infection in broilers.

FINAL CONSIDERATIONS

It was observed in both studies that when gut health (higher ISI score) is compromised, the zootechnical performance is directly affect. In the study presented in the first chapter, although it was not observed statistical difference on feed intake, it was observed that the challenged group presented lower BWG from 1-14d, 1-21 and 1-28d as well as worst FCR at 1 to 7 and 1 to 21 days periods when compared to the non-challenged group. The challenge group treated with 10 ppm of enramycin in diet did not show statistical difference between non-challenge and challenge groups at zootechnical performance, however, applying the ISI methodology, it was observed that the use of this product significantly minimized the total ISI score in the ileum at 21 and 28 days when compared to challenge group.

In the study presented in the second chapter, shows the application of the ISI methodology allows a numeric translation of histologic lesion and its correlation with losses in animal performance. The lesions observed in the first weeks of animal's life, may interfere in the performance over the next weeks.

The results of this both studies demonstrate the validity of the ISI methodology for better evaluation of the intestinal health.

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