UNIVERSIDADE FEDERAL DO PARANÁ

BRUNA LUIZA BELOTE

I SEE INSIDE METHOD: THE APPLIED TOOL FROM EXPERIMENTAL TRIALS TO THE REAL FIELD BROILER PRODUCTION

CURITIBA, 2022

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(MÉTODO I SEE INSIDE: A FERRAMENTA APLICADA DESDE TESTES EXPERIMENTAIS ATÉ A PRODUÇÃO REAL DE FRANGOS DE CORTE EM CAMPO)

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RESUMO

O conceito "Pecuária de precisão" é parcialmente aplicado na produção avícola, sendo a coleta de dados um dos componentes mais utilizado. Contudo, ainda nem todos estes dados são coletados de forma correta e precisa, e, na maioria das vezes ainda são armazenados fisicamente, carecendo de uma análise adequada e holística, que considere integrar toda a informação dos diferentes setores de produção (desde incubatório, fábrica de ração até o abatedouro), dificultando ou limitando sua utilização para a tomada de decisões. Especificamente dados de saúde animal, são difíceis de serem integrados com dados de desempenho zootécnico. Para colaborar na solução deste problema, nesta Tese, busca-se comprovar a eficiência do método I See Inside (ISI) para correlacionar informações de necropsias de monitoria de frangos de corte com desempenho zootécnico utilizando modelos de ensaio experimentais e estudos a campo. Dessa maneira, são apresentados aqui três estudos, divididos em três capítulos. O objetivo do primeiro capítulo foi comparar a metodologia mais difundida de avaliação de morfometria intestinal, utilizando a métrica das vilosidades intestinais/cripta com o método de escores do ISI, e a correlação destes métodos com o desempenho dos frangos. Desse estudo concluímos que uma vilosidade maior não está necessariamente relacionada com uma melhor função intestinal, uma vez que pode estar comprometida por inflamação. O ISI histológico é importante para detectar diferenças biológicas nos grupos, fornecendo mais informação sobre a saúde intestinal do animal, e, portanto, maior correlação com desempenho zootécnico. O segundo capítulo tem como objetivo a aplicação do método ISI macroscópico e histológico em frangos de corte com diferentes modelos de inflamação crônica para avaliação de biomarcadores de inflamação intestinal. Como conclusão, a calprotectina, um biomarcador já utilizado em enteropatias de seres humanos, foi detectado também no soro e nas fezes de frangos em resposta à inflamação intestinal, enquanto outros marcadores como HIF-1α e a lipocalina apresentaram menos sensibilidade como marcadores fecais de inflamação nesta espécie. E o método ISI, foi utilizado como "gold teste" para avaliar esses marcadores, uma vez que, por meio histológico, descreve a inflamação causada experimentalmente. Em adição neste estudo, também foi observado pelo método ISI que a inflamação intestinal evolui num padrão espacial e temporal ao longo do intestino delgado das aves. No terceiro capítulo foi avaliado a hipótese de que a adição de plasma seco por spray (SDP) em dietas de frangos poderia melhorar o desempenho das aves nas idades de abate comercial, e aplicou-se a metodologia ISI como a ferramenta de gestão a campo para avaliar o impacto deste produto aditivo alimentar na saúde das aves. Como conclusão desse último capítulo, o SDP melhorou o desempenho e a saúde das aves no período inicial, tanto sob circunstâncias adequadas ou não de manejo e biosseguridade, independentemente do tipo de ventilação utilizada nos aviários. Desta maneira, comprovou-se que o método ISI traduziu as melhorias de desempenho observadas no campo. Como conclusão geral se verifica que o método ISI e uma excelente ferramenta que pode ser aplicado tanto em ensaios controlados como a campo para traduzir saúde intestinal e correlacionar com desempenho zootécnico em frangos de corte.

Palavras-chave: Histologia, Lesões macroscópicas, Intestino, Biomarcadores, Frangos de corte.

ABSTRACT

The "Precision farming" concept is partially applied in poultry production, with data collection being one of the most used components. However, not all data is collected in a correct and precise way, and most of the times it is still stored physically, lacking an adequate and holistic analysis, which considers integrating all the information from different production sectors (from hatchery, feed mill to slaughterhouse), making it difficult or limiting its use for decision making. Specifically, animal health data are difficult to be integrated with zootechnical performance data. To collaborate in solving this problem, this thesis aims to prove the efficiency of the I See Inside (ISI) method to correlate information from broiler necropsy monitoring with zootechnical performance using experimental test models and field studies. Thus, three studies, divided into three chapters, are presented. The objective of the first chapter was to compare the most prevalent methodology for evaluating intestinal morphometry, using the intestinal villus/crypt metric with the ISI score method, and the correlation of these methods with broiler performance. From this study we conclude that a larger villus is not necessarily related to better intestinal function, as it may be compromised by inflammation. The histological ISI is important to detect biological differences in the groups, providing more information about the intestinal health of the animal, and therefore greater correlation with zootechnical performance. The second chapter aims to apply the ISI macroscopic and histological method in broilers with different models of chronic inflammation to evaluate biomarkers of intestinal inflammation. As a conclusion, calprotectin, a biomarker already used in enteropathies of humans, was also detected in the serum and feces of chickens in response to intestinal inflammation, while other markers such as HIF-1α and lipocalin showed less sensitivity as fecal markers of inflammation in this species. And the ISI method was used as a "gold test" to evaluate these markers, since it histologically describes the inflammation caused experimentally. In addition, in this study, it was also observed by the ISI method that the intestinal inflammation evolves in a spatial and temporal pattern along the small intestine of the birds. In the third chapter the hypothesis that the addition of spray dried plasma (SDP) in broiler diets could improve the performance of birds at commercial slaughter ages was evaluated, and the ISI methodology was applied as the field management tool to evaluate the impact of this feed additive product on bird health. As a conclusion of this last chapter, SDP improved the performance and health of the birds in the early period, both under adequate and non-adequate management and biosecurity circumstances, regardless of the type of ventilation used in the houses. Thus, the ISI method was proven to translate the performance improvements observed in the field. The general conclusion is that the ISI method is an excellent tool that can be applied both in controlled trials and in the field, to translate intestinal health and correlate it with zootechnical performance in broilers.

Key words: Histology, Macroscopic lesions, Intestine, Biomarkers, Broilers.

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CHAPTER 1

GENERAL INTRODUCTION

Development of a health monitoring system in broilers

Precision farming

There is a growing concern regarding food safety, animal welfare, product quality, environmental, social, and economic issues associated with industrialized poultry production systems. All these points, which are the principle of sustainability in animal production, can no longer be observed individually since they are all related (LEINONEN; KYRIAZAKIS, 2016).

The development and application of precision farming has already been a practice for a few years in poultry production, and now, the animal agroindustry is one step away from the digital revolution. Big data is a term used to describe technologies that are embedded in everyday matters and that are interconnected via the internet to produce large data (NEETHIRAJAN, 2020).

For poultry production, this will result in more automated farms, with sensors, and field health monitoring assessments. Collecting this data is perhaps one of the easiest components of big data for poultry production. However, not all this data is collected correctly and accurately and can be analyzed deeply, as most of the data is collected manually and available only on paper sheets, or collected in software designed for individual stage evaluations, making it an insufficient practice to deal with these data and for decision making. Importantly, big data management also refers to the use of predictive analytics that can bring greater certainty regarding the data expected from future flocks (NASIRAHMADI et al., 2019; NEETHIRAJAN, 2020; VANDERWAAL et al., 2017).

Another concept that can also be applied to agricultural food production is the connections between the microbiome, body weight, and nutrition holds significant promise for host health. Shortening the time required to raise animals to their harvest weight and enhancing their feed efficiency offers advantages in terms of both cost for producers (PYM, 2008). The future's challenge for poultry farming includes in the development of a comprehensive health monitoring system for broilers. This system will involve a digital tool that integrates with inflammatory biomarker indicators and correlates them with zootechnical performance. This integrated approach is crucial for predictive data analysis in the field, enabling better-informed decision-making and

ultimately ensuring the health and productivity of broilers. In fact, this thesis has several examples of multifactorial correlation in broiler production, especially in Chapter 4 (page 77).

Inflammation Impact on Animal Production

Inflammation is the typical, well-controlled physiological response of the immune system to an injury, with the goal of facilitating the recovery and repair of injured tissues. It stands as the primary expression of host defense when responding to disruptions in tissue balance and is triggered by innate immune receptors that identify and signal the presence of infection, host damage, or danger. In reaction to these signals, cells of the innate immune system generate signaling molecules that set off a carefully orchestrated network of immunological and physiological processes, all designed to maintain equilibrium and restore functionality (KOGUT et al., 2018, BROOM; KOGUT, 2018; LAURIDSEN, 2019).

The effectiveness, duration, and outcomes of an inflammatory reaction rely on the nature of the trigger detected by the innate immune receptors. Maintaining inflammation in the intestinal tract is a particularly intricate task, as the standard physiological inflammation represents a state of equilibrium between the tolerance of the beneficial gut microbiota and the response to potential pathogen intrusion. When this equilibrium is disrupted, an abrupt inflammatory response, known as pathological inflammation, may occur, potentially leading to unintended damage and heightened metabolic/nutritional cost. (LOCHMILLER; DEERENBERG, 2000; KOGUT et al., 2018)

Gut inflammation results in significant changes to the structure of the intestine, as evidenced by increased intestinal permeability (ROSETH et al., 1997), reduced nutrient absorption and digestibility, and the occurrence of fluid loss and diarrhea (TABLER et al 2020; RYCHLIK et al 2014; HE et al 2019). The heightened permeability of the intestinal lining, often termed "leaky gut," leads to the translocation of gut bacteria, microbial compounds, and antigens into the bloodstream, triggering a systemic immune response (TABLER et al., 2020; ROSETH et al., 1997; BENTO et al., 2012). Furthermore, an excessive or persistently activated inflammatory response also contributes to the decline in tissue function and the inefficient use of metabolic energy, ultimately causing poor performance and reduced animal welfare (BROOM; KOGUT, 2018).

Currently, commercial swine and broiler operations routinely encounter various non-infectious environmental factors that can serve as triggers for inflammation. These

factors encompass animal density, subpar quality of feed ingredients, nutrient-rich diets (high in protein or non-starch polysaccharides), alterations in feed composition, mycotoxins, diets containing substantial amounts of antinutritional elements, and exposure to heat stress. Prolonged exposure to any of these triggers, spanning from days to weeks, can result in the chronic intestinal inflammatory condition mentioned earlier. This condition leads to disrupted digestive processes, heightened oxidative stress, imbalances in the gut microbiota, compromised barrier function, and immune system dysfunctions, as discussed in our paper on the model (DAL PONT et al., 2021).

Biomarkers for chronic intestinal inflammation

Sterile inflammation generally refers to a persistent, mild inflammatory condition arising from the activation of the innate immune system by non-infectious cellular components and metabolites. Ideally, the best-case scenario involves preserving the normal physiological inflammation while minimizing exposure to other sources of inflammation or inflammatory conditions. (KOGUT et al., 2018; REISINGER et al., 2020).

Some components of the inflammatory process will naturally reach the lumen of a diseased gut, making it possible to define some biomarkers of gut inflammation in the feces as an alternative for the plasmatic targets (YI; WU, 2014).

The study outlined in Chapter 3 particularly highlights two potential biomarkers for poultry, calprotectin, lipocalin (LCN2) and HIF-1α, when compared to other less promising options for this species described in the chapter 3 (page 46).

The human calprotectin, for example, is a calcium-bound protein highly present in their neutrophils and, in a lesser quantity, in monocytes and macrophages (AYLING; KOK, 2018). This protein is known to sequestrate zinc and manganese, mechanism through which it disrupts bacterial and fungal defense pathways and acts as a component of the innate immunity. Calprotectin antimicrobial activity has been observed to interrupt the manganese-dependent defense of *Staphylococcus aureus* against the superoxide, making the bacteria more sensible to the oxidative stress (GOOSSENS et al., 2018; LEE et al., 2007). Many studies in humans have attempted to discriminate gut inflammatory diseases through their relation to certain biomarker levels in the feces, determining their sensitivity and specificity. In this context, calprotectin has been a highly accurate marker for IBD cases (TAYLOR et al., 2007; OVIEDO-RONDÓN et al., 2019; TABLER et al., 2020; KOGUT et al., 2018). Besides varying in sensitivity and specificity, fecal

calprotectin is even more applicable to rule out IBD in patients with irritable bowel syndrome symptoms (BROOM; KOGUT, 2019; LAURIDSEN et al., 2019).

A similarity of 66 and 72% between human and swine calprotectin has been reported by Barbosa et al. (2021) for the protein subunits S100-A9 and S100-A8, respectively. Applying ELISA and immunoturbidimetry tests, the authors statistically distinguished ($p<0.05$) the fecal calprotectin levels among pigs with varied severity of colitis. The ELISA test successfully pointed a higher level of the biomarker in samples of bloody diarrhea (score 4) in comparison to fecal samples ranging from normal to mucoid (scores 0 to 3). Through the immunoturbidimetry assay, animals presenting both mucoid and bloody feces had significantly increased concentrations of calprotectin compared to pigs excreting softly wet shedding. Although no effect on the fecal calprotectin concentration was seen when submitting pigs to a dietary yeast-enriched protein concentrate, Slinger at al. (2019) successfully verified a significant reduction in the biomarker from 14 to 28 days of age. This points that the age may affect the calprotectin response to enteric conditions.

The Lipocalin-2 or Neutrophil-gelatinase associated lipocalin (NGAL) is a glycoprotein majorly expressed by neutrophils and whose antimicrobial activity relies on binding to bacterial siderophores, preventing them from capturing iron for their metabolism. This ability of lipocalin provides it substantial effect on controlling bacterial infection and on the composition of the host microbiota (GOETZ et al., 2002; XIAO et al., 2017). As the calprotectin, the human fecal lipocalin has shown an important diagnostic value for inflammatory activity in IBD cases (MOSCHEN et al., 2017). Lipocalin has been used as a biomarker in pigs to evaluate enteric inflammatory conditions, however, most studies measure the protein level or expression in tissue samples. Guo et al. (2017) observed a strong up-regulation of lipocalin production in jejunum by *E. coli* K88 infection in weaned piglets, indicating the importance of this protein on controlling bacterial growth. Wang et al. (2022) applied the fecal quantification of lipocalin to assess the effects of different amino acid and carvacrol-thymol blend levels on gut integrity of post-weaned pigs. The same concept has been applied in swine, where fecal lipocalin has been targeted to evaluate the intestinal integrity response to variables such as dietary supplementation (WANG et al., 2022; CHENG et al., 2018; XU et al., 2020), pregnancy, lactation, and obesity in sows (CHENG et al., 2020; CHENG et al., 2018).

In addition, as a transcription factors as metabolic biomarkers, the hypoxia-inducible factor-1α (HIF1α) plays an important role in cellular metabolism and adaptation to cellular stress caused by hypoxia and fundamental reprogrammer of inflammatory cell metabolism that promotes inflammatory gene expression. HIF-1α regulates the expression of a set of "signature" anabolic genes/proteins involved in the control of cellular metabolism including all enzymes in the glycolysis pathway, glucose transporters, transferrin receptor, and the induction of pro-inflammatory genes. Further, HIF-1α play a role in effector functions of macrophages and T cells during inflammation. (MASSON; RATCLIFFE, 2014; CORCORAN; O'NEILL, 2016)

If a biomarker has such a potential for differential diagnosis in humans, it's natural to expect studies assessing this protein equivalent in swine and poultry to measure gut damage. It's important to note that the potential biomarkers discussed are intended as functional quantitative measurements of intestinal inflammation and are not exclusive to specific diseases. Fecal biomarkers are more precise in indicating intestinal inflammation, while plasma biomarkers provide insights into broader systemic conditions.

I See Inside (ISI) Method

The I See Inside (ISI) method, was firstly described by KRAIESKI et al., (2017) and replicated and enhanced by other studies in the following years (BELOTE et al., 2018, 2019, 2021; SANCHES et al., 2020). This methodology is a patent (BR102015003601-9) of Federal University of Parana (UFPR) – Brazil, developed by the Microbiology and ornithopathology laboratory at UFPR, under the supervision of Professor Dr. Elizabeth Santin since 2015. This method evaluates lesions based on a numeric score of alteration in macroscopic (ISI macro) and microscopic (ISI histology) analysis.

An impact factor (IF) which defined each alteration in macro and microscopic analysis according to the reduction of organ functional, based on previous knowledge of literature and background research. The IF ranges from 1 to 3, where 3 is the most impactful in reducing the organ function (e.g., necrosis, has the highest IF because the functional capacity of affected cells is totally lost). 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.

This method, when applied to macroscopic alteration in organ, can help to standardize the vision/score of different professionals through the necropsy evaluation. For ISI macro analysis, broilers were systematically evaluated following a division into

5 system groups: locomotor, respiratory system, gastrointestinal organs, intestine, and coccidiosis typical lesion.

The methodology brings the idea of all the data evaluated in the field would be linked to allow a holistic evaluation of different factors that could impact animal health and production. In the method all the morphometric alterations observed present different relations among other organs, the intestinal health, animal performance and meat quality. For example, compromised gut health in case of dysbiosis, can affect litter quality, making it more humid due to diarrhea, which may affect the locomotor system, such as pododermatitis (MONTAGNE; PLUSKE; HAMPSON, 2003; WIDEMAN; PRISBY, 2013). This last alteration, reduce animal locomotion and the long-time contact of breast in the litter may cause lesion which affect meat quality and obviously, indirectly affect the bird's access to food and drink, affection all systems and animal performance. Since the litter quality worsens, ammonia levels in the environment increase (WEI et al., 2015), and it affect respiratory tract as well (KRAIESKI et al., 2017).

For microscopic analysis, is evaluated by histological alterations in intestine and liver, and the same calculation of macro is applied. The parameters evaluated for intestine are lamina propria thickness, epithelial thickness, enterocytes proliferation, inflammatory cell infiltration in the epithelium, inflammatory cell infiltration in the lamina propria, goblet cells proliferation, congestion, presence of *Eimeria sp.* Oocysts. And for liver are lymphoid aggregates, inflammatory cells infiltration, necrosis, pericholangitis, congestion, hydropic degeneration, bile duct proliferation, vacuolization. The final ISI score is calculated as sum of all parameters evaluated. Therefore, the highest the final ISI total score the worse is the intestinal health and performance of the animal. The reason for having these specific histological parameters evaluated was described by Sanches (2019), in his doctoral thesis.

It is proved the ISI histology analysis is correlated with animal's performance (BELOTE et al., 2018 and CHAPTER 2) and in other trials and real field facilities with ISI Macro (data not published yet). The fact that ISI methodology can translate this morphologic alteration in numbers and allow it to be correlate with animal performance make it very apply to the poultry production field and is the great differential of this systems from other necropsy score described in the literature.

Since the ISI method has been proven effective in several study models in laboratory and field (BELOTE et al., 2018, 2019, 2021; CARDINAL et al., 2019; DAL PONT et al., 2021; KRAIESKI et al., 2017; SANCHES et al., 2020; STEFANELLO et

al., 2020), we predict that apply this methodology would evolve for better digital systems that created services and tools, through research and innovation, to develop applicable scientific concepts for the improvement of poultry health management.

The aim of this thesis is to evaluate the efficiency of the ISI method in different situation (experimental controlled and field trial) and divided in three chapters.

The aim of the second chapter was to compare the most applied morphometry method that use the intestinal villi/crypt measure with ISI scoring method, and their correlation with broiler performance. It was concluded that a larger villus area is not necessarily related to better intestinal function, since it might be compromised by inflammation and an afunctional absorptive surface. The ISI histopathologic scoring is important to detect biologic differences groups, providing more information about gut health and showing better correlation with animal performance.

The third chapter had as its objective was applying the ISI macro and histological method in broilers with different chronic inflammation models for evaluation of gut inflammation biomarkers. And as conclusion, calprotectin, a biomarker already applied in humans, was successfully detected in serum and feces of broilers in response to inflammation, while HIF-1α and lipocalin are less sensitive fecal markers in this specie. The ISI method was able to prove all the inflammation caused and demonstrated that the chicken intestinal inflammation evolves in a spatial and temporal pattern through the small intestine.

Finally, at fourth chapter it was applied the ISI methodology as a tool to evaluate the effect of spray-dried plasma (SDP) supplementation to broiler diets on bird performance at commercial slaughter ages. As a conclusion of this last chapter, the product showed improved performance and health in the early period under both good and poor management and biosecurity circumstances, regardless of ventilation barn type. Overall, there was a good association between the ISI method and the performance improvements observed in the field.

With the results of these main studies, we were able to prove the flexibility of using the ISI method in several situations.

CHAPTER 2:

APPLYING MORPHOMETRIC GUT MUCOSA METHODS

Applying different morphometric intestinal mucosa methods and the correlation with broilers performance under *Eimeria* challenge

Not published

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ABSTRACT

The intestinal wall has on its surface, protrusions called villi that are responsible for the absorption of nutrients. Commonly, these structures have their dimensions measured to related more area surface with better absorption. However, the measurement of these villi neglects the inflammation and the presence of immature cells that increase the surface area but affect negatively the absorption and compromise the animal performance. The measurements of villi/crypt are traditional tools in animal research; however, they may overlook alterations that impact the mucosal functionality. This study aimed to compare the morphometry of the intestinal villi/crypt with the I See Inside (ISI) scoring methodology, exploring their correlation with zootechnical performance. Therefore, broilers were grouped as non–challenged (NC) and challenged with *Eimeria* (CH) and jejunum samples were collected at 22d for histological analysis. The same villi were submitted to the ISI methodology, which is based on the scoring of 8 parameters related to the inflammatory process, and the measurements of villus height (VH), villus width (VW), crypt depth (CD), crypt width (CW), VH:CD ratio and villi absorptive surface (VAS). The CH group presented higher ISI total score, VW, CD, CW and lower VH, VH:CD and VAS in comparison to the NC group. While the villi/crypt morphometry did not exhibit correlations with performance, the presence of *Eimeria* oocysts and the ISI total score was positively correlated ($P<0.05$) with the feed conversion ratio (FCR), demonstrating a statistical inter-action between high ISI scores and worse performance. In conclusion, a larger villus is not related to better intestinal functionality when this enlargement is unleashed by the immune processes occurring inside. The scoring system that evaluates the type of alteration observed has a direct impact on the animal's zootechnical performance which is not observed with the single metric surface evaluation.

Key words: histology, gut health, index, coccidiosis, intestinal lesion

1. INTRODUCTION

The intestinal mucosa develops through two primary associated cytological events: (1) the cell renewal (proliferation and differentiation) resulting from the mitotic divisions of totipotent cells located in the crypt and along the villi (Uni et al., 1998), and (2) the loss of cells due to desquamation, which occurs naturally at the apex of the villi. Moreover, the renewal rate of the villi (turnover), which is the balance between these two cytological processes, directly affects the digestive and absorption capacities of the intestine (Maiorka et al., 2000).

Histomorphometry analysis of these structures are often used in animal research to understand the gastrointestinal pathophysiology. These tools provide quantitative data upon morphological alterations that may occur on the intestinal mucosa under various experimental conditions such as special diets, use of drugs, and exposure to etiologic agents such coccidiosis, mycotoxins, *Clostridium*, etc. (Maiorka et al., 2000; Santin et al., 2002; Marchini et al., 2009).

Gibson-Corley et al., (2013) suggest that scoring methodologies can be successfully used to obtain data from biologic systems (e.g., tissues) in studies aiming group comparisons. These scoring tools can be applied at different levels of tissues examination, including antemortem imaging techniques, postmortem macroscopic examination, and microscopic histopathologic assessments (Eaton et al., 2007; Chapman, 2014; Kraieski et al., 2017; Belote et al., 2018, 2019, 2021; Dal Pont et al., 2021). Finally, a scoring system should exhibit 3 fundamental features: (1) to be definable, (2) to be reproducible, and (3) to provide meaningful results (Crissman et al., 2004).

Belote et al., (2018) demonstrated that the *I See Inside* (ISI®) scoring system for histological analyses of the intestine can provide high statistical correlation between its results (quality of the mucosa) and the bird zootechnical performance at several ages. Therefore, the objective of this study is to compare the ISI scoring methods, qualitative and quantitative parameters, with the measurements of villi and crypts in avian gut, as well as to assess the correlation of both methods with the zootechnical performance of the birds.

2. MATERIAL AND METHODS

2.1 Animals, Experimental Design, Diet, and Housing

The present study was conducted at the International Animal Nutrition Research Center in Quebec, Canada. A total of 720 Ross 308 male broilers were housed from one to 35 days and randomly divided into two groups of 24 replicates with 30 birds each: a non–challenged negative control group (NC) and a positive control group challenged with *Eimeria* spp (CH).

This trial was performed in pens and the birds were raised on new wood chips litter and maintained in controlled temperature according to their age. The animals had access to feed and water *ad libitum* and the diet was divided in 2 phases: a starter diet fed from 0 to 21 d and a grower formulation provided from 21 to 35 d, as described in table 1. During the first two days of age, birds were given 23 h of light (100 lux) and 1 h of darkness. From the 3 to 35 d of age, the animals were raised under 18 h of light (reducing from 60 to 10 lux from 3 to 5 d) and 6 h of darkness.

Table 1. Description of diet (g), calculated for 1000g.

Ingredients	Starter 0-21d	Grower 21-35d
Corn	319,15	357,55
Wheat	320	358
Soybean meal	276,38	200,37
Limestone	20,08	18,83
Phosphate	15,48	9,65
Fat	33,04	43,19
DL-methionine	3,13	2,47
salt	2,57	2,55
Lysine	2,5	2,46
Threonine	0,96	0,39
Sodium bicarbonat	1,5	1,5
Chicken premix	1,5	1,5
Choline	1	0,75
Valine	2,51	0,63
Tryptophane	0,21	0,17

2.2 Performance

At 1 d of age, birds were separated into the treatments (NC and CH) in a way the initial average body weight was similar in all pens selected for each treatment. Birds and feed were weekly weighed (at zero, 7, 14, 21, 28 and 35 d) to evaluate average daily feed

intake (ADFI), body weight gain (BWG), average daily gain (ADG) and feed conversion ratio (FCR).

2.3 Challenge

At 14 d, all the birds in the CH group were submitted to a one-hour fasting and then inoculated with *Eimeria* field oocysts mixed with the feed.

The inoculum was produced through oocyst propagation and purification according to Conway and McKenzie (2007). Briefly, oocysts of mixed species of *Eimeria* were isolated from samples of droppings from unvaccinated birds at the Deschambault Animal Science Research Center (CRSAD, Deschambault, Quebec, Canada) poultry house in 2018. The oocysts were propagated in healthy chickens, sporulated at 30°C in 2% potassium dichromate for 48 hours and washed 3 times with water.

For each pen 700 ml water was added to 1kg feed and then 2ml per bird (2×10^5 oocysts per bird) of the oocyst suspension (at 1×10^5 oocysts/mL) was added to this feed. After the one-hour fasting, the birds only had access to this challenge feed until it was completely consumed (approximately 4 hours later). The same procedure was done for control group with water without oocysts. Dilutions of coccidia inoculum were prepared to achieve the following oocyst levels per bird: 1×10^5 oocysts of *Eimeria acervulina*, 6×10^4 oocysts of *Eimeria maxima* and 4×10^4 oocysts of *Eimeria tenella*. Oocyst concentration was determined by microscopy using McMaster slides and *Eimeria* species characterization was realized by qPCR analyzes according to Vrba et al. (2010) and Peek et al. (2017). Standard curves using dilutions of linearized double-stranded DNA corresponding to the amplicons of each species (gBlocks Gene Fragments, Integrated DNA Technologies, Mississauga, ON) were used to determine DNA copy numbers and % for each *Eimeria* species.

2.4 Eimeria spp counting

Feces samples were collected at 14, 21 and 24 d for oocyst counting. For that, farrowing papers were placed on the floor of the pens and 2 hours later, feces are scooped with a spoon, put in a plastic pot, and lightly mixed. These samples were stored at 4°C and then sent to the Faculty of Veterinary Medicine of Montreal University, where they were submitted to the count of oocysts per gram of feces (OPG) through the McMaster technic of Conway and McKenzie (2007).

2.5 Necropsy and sampling

At 22 d of age, a total of 12 birds per treatment or one per replication, were randomly selected and euthanized by cervical dislocation for necropsy and collection of jejunum samples.

2.6 Histology Evaluation

For the histological evaluation, the samples of jejunum were collected and immediately fixed in formaldehyde 10% for at least 24 h. These samples were placed in a circulator and treated with different concentrations of alcohol and toluene. The blocks were then embedded in paraffin and cut in 3 μm slices which were stained with hematoxylin and eosin (HE). All the slides were scanned (Aperio AT2 from Leica Biosystems) and digitally analyzed (Aperio ImageScope v12.4.0.5043 from Leica Biosystems).

A total of 10 intestinal villi per bird (120 villi per treatment) were analyzed in 10X magnification (using 20X and 40X magnification to confirm alterations) using the ISI® scoring system and the method of measurement of villus and crypt. Both methods were performed on each at same villus.

2.6.1 ISI® methodology

This is a methodology first described by Kraieski et al 2017, 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. The IF ranges from 1 to 3, with 3 being the most impacting to organ function. The parameters evaluated by the ISI methodology are: lamina propria thickness, epithelial thickness, proliferation of enterocytes, inflammatory cell infiltration on the epithelial, inflammatory cell infiltration in the lamina propria, increase of goblet cell, congestion and presence of *Eimeria* oocysts. In addition, the extent of each lesion (intensity) compared to non-affected organ is evaluated in each organ/tissue with score (S) ranging from 0 to 3: score 0 (absence of lesion), score 1 (alteration up to 25% of the area), score 2 (alteration ranges from 25 to 50% of the area), and score 3 (alteration extends to more than 50% of the area). 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 sum of all parameters already mentioned, will give the total ISI value for this specific bird (each bird is a replicate for statistical analysis).

2.6.2 Villus and crypt measurements

The method of measurement of villus height (VH - from the tip of the villi to the villus crypt junction), villus width (VW- from one side to other in the midpoint of the villus), crypt depth (CD - defined as the depth of the invagination between adjacent villi) and crypt width (CW – from one side to other of the crypt. Also, VH:CD ratio (Uni et al., 1995) and absorptive surface was calculated (Kisielinski et al., 2002).

2.6.3 Absorption surface area

To calculate the absorption surface area, the method of Kisielinski et al. (2002) was applied. In this method, it was considered to calculate the size of the mucosa to serosa, as a geometric unit of the mucosa consisting of cylindrically shaped villi and at the base by cylindrical crypts. It was assumed that the entire mucosa is an interaction of this unit and the absorption surface (M) can be calculated with average values of the structures that define the mucosal villus unit: VH, VW, CW.

These authors considered the CD is important to overall function of mucosa, but for the calculation of absorptive surface it is not necessary, because there is only secretion within crypts.

$$M = \frac{(\text{villus width} \,.\, \text{villus length}) + \left(\frac{villus\ width}{2} + \frac{crypt\ width}{2}\right)^2 - \left(\frac{villus\ width}{2}\right)^2}{\left(\frac{villus\ width}{2} + \frac{crypt\ width}{2}\right)^2}$$

2.7 Statistical analysis

Data were evaluated using the statistical software R and analyzed by the Shapiro-Wilk normality test. Parametric data were submitted to analysis of variance (ANOVA) and Tukey's test for the means with a significant difference (P<0.05). Nonparametric data were submitted to the Kruskal-Wallis's and Dunn test (P<0.05). Non parametric data was performed in Spearman's correlation test coefficient (r) was used for correlation analysis,

and the software provided the P values. Only coefficient higher than r=0.50 or r=-0.50 were considered.

3. RESULTS

3.1 Performance

As expected, the *Eimeria* challenge at 14 d caused significant reductions on the ADFI, ADG and BWG and worse on the FCR (P < 0.05) in the CH group at the 28 and 35 d (Table 2).

Table 2 . BWG, FCR, ADFI and ADG from 0 to 35 d.

Treatment	Days of age	ADFI	BWG (g)	ADG	FCR
NC	0		39,7		
CH			39,8		
NC	7	15,86	124	12,08	1,313
CH		15,73	121	11,64	1,35
NC	14*	32,11	373	23,8	1,349
CH		31,51	368	23,5	1,341
NC	21	49,52	792	35,86	1,381
CH		48,72	781	35,33	1,379
NC	28	**72,31 [a]**	**1417 [a]**	**49,2 [a]**	**1,469 [b]**
CH		**69,15 [b]**	**1295 [b]**	**44,84 [b]**	**1,542 [a]**
NC	35	**93,61 [a]**	**2141 [a]**	**60,03 [a]**	**1,559 [b]**
CH		**89,75 [b]**	**1956 [b]**	**54,75 [b]**	**1,639 [a]**

Different superscript letters indicate a significant difference between groups with Tukey test (P < 0.05).

NC - Non–challenged; CH- Challenged with *Eimeria* spp

ADFI - Average daily feed intake; BWG - Body weight gain; ADG - Average daily gain; FCR - Feed conversion ratio.

*Day of challenge with *Eimeria* spp.

3.2 Oocysts Eimeria spp counting

The CH showed a high score of oocyst presence, as well as in the oocyst count with 200 oocysts per gram feces. No oocysts were observed at NC group.

3.3 Histology Evaluation

3.3.1 Measurement of villus/crypt

The *Eimeria* challenge turned the villi shorter and wider, reducing their absorptive surface. Moreover, the infection increased the depth of crypts and reduced the VH:CD ratio (P<0.05) (Table 3 and Figure 1).

Table 3. Results of measurement of villus height (VH), villus width (VW), crypt depth (CD), crypt width (CW), VH:CD, and absorptive surface between NC and CH groups (mean).

Parameter	Treatment	Mean	SE Mean	C.V.	Minimum (µm)	Maximum (µm)	P value
VH	NC	**976.53** [a]	13.26	14.875	651	1356	<0.001
	CH	**885.50** [b]	9.12	11.137	606	1163	
VW	NC	**224.02** [b]	7.56	36.973	82	453	<0.001
	CH	**259.10** [a]	9.09	38.723	112	607	
CD	NC	**152.45** [b]	4.51	32.392	47	328	<0.001
	CH	**192.32** [a]	5.17	30.908	91	315	
CW	NC	**57.00** [b]	1.30	24.951	28	116	<0.001
	CH	**72.93** [a]	3.99	62.955	36	523	
VH:CD	NC	**6.40** [a]	0.255	39.4	3.12	23.9	<0.001
	CH	**4.60** [b]	0.172	34.9	2.53	13.9	
ABSORPTIVE SURFACE	NC	**12.2** [a]	0.335	30.2	6.41	22.6	<0.001
	CH	**9.69** [b]	0.252	28.4	2.39	17.5	

Different superscript letters indicate a significant difference with Tukey test ($P < 0.05$).

NC - Non–challenged; CH- Challenged with *Eimeria* spp

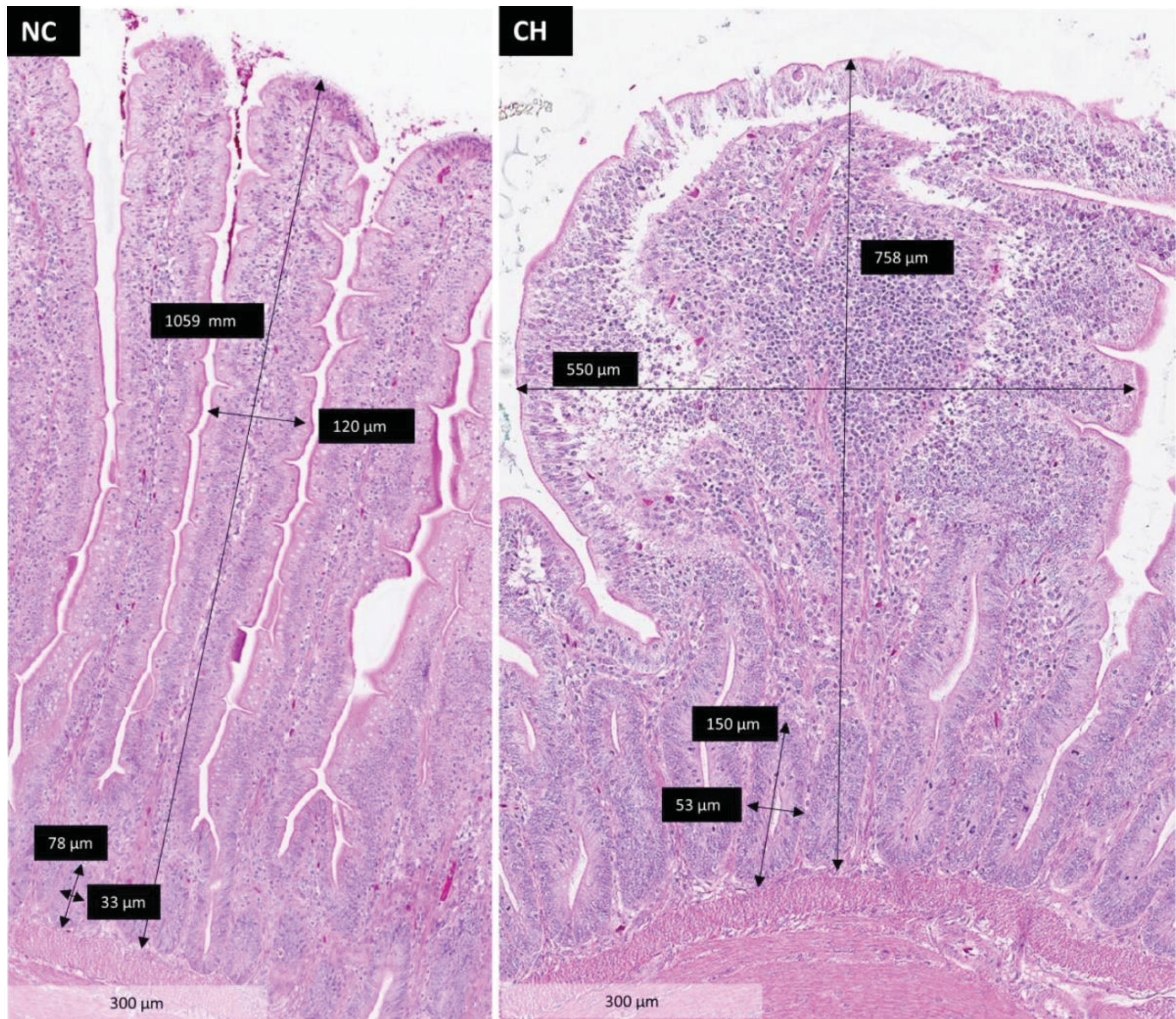

Figure 1. Photomicrographs of broiler jejunum sections stained with hematoxylin and eosin (80X). Difference of measurement of villi between the non–challenged (NC) and challenged with *Eimeria* spp (CH) groups. The NC group with villus height (VH): 1059 mm; villus width (VW): 120 µm; crypt depth (CD): 78 µm and crypt width (CW): 33 µm, and the CH group with VH: 758 µm; VW: 550 µm; CD: 150 µm and CW: 53 µm.

3.3.2 ISI® methodology

The CH group presented higher (P<0.001) ISI scores in all the evaluated parameters, and in the ISI Total Score. Contrastingly, these challenged birds displayed significant reduction (P<0.001) in the presence of goblet cells when compared to the NC group (Table 4 and figure 2).

Table 4. ISI score between NC and CH groups (mean).

Treatment	Lamina propria thickness	Epithelial thickness	Proliferation of enterocytes	Inflammatory cell infiltration on the epithelium	Inflammatory cell infiltration in the lamina propria	Increase of goblet cells	Congestion	Presence of oocysts	Total score
NC	1.35 [b]	0.86 [b]	0.81 [b]	0.51 [b]	0.83 [b]	1.21 [a]	0.30	0.00 [b]	5.90 [b]
CH	2.41 [a]	1.12 [a]	1.11 [a]	1.36 [a]	2.50 [a]	0.51 [b]	0.28	6.67 [a]	16.00 [a]
SE	0,1	0,05	0,05	0,05	0,09	0,1	0,06	0,1	0,4
P Value	<0.001	0,002	<0.001	<0.001	<0.001	<0.001	0,728	<0.001	<0.001

Different superscript letters indicate a significant difference with Dunn's test ($P < 0.001$).

NC - Non–challenged; CH- Challenged with *Eimeria* spp

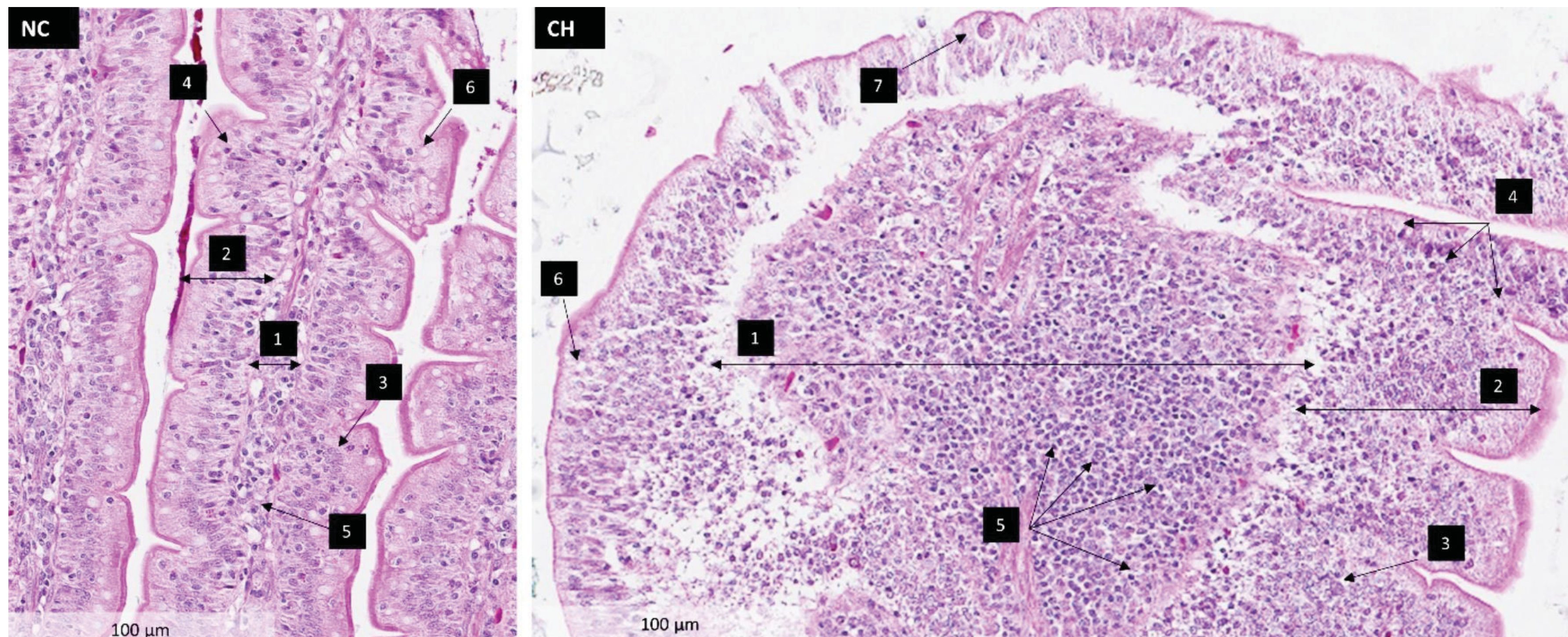

Figure 2. Photomicrographs of chicken jejunum sections stained with hematoxylin and eosin (200X). Different between the non-challenged (NC) group, with normal histological jejunum structure, and challenged group (CH) with some alteration in ISI parameters. 1. Lamina propria thickness, 2. Epithelial thickness, 3. Proliferation of enterocytes, 4. Inflammatory cell infiltration on the epithelium, 5. Inflammatory cell infiltration in the lamina propria 6. Goblet cells, 7. Presence of oocysts. These alterations contributed to the highest ISI total score ($P < 0.05$) in coccidiosis challenged group at 22 days in comparison to the non-challenged group.

3.4 Spearman's test correlation

3.4.1 Measurement of villus/crypt

The villus width and absorptive surface were the only measurements that presented correlation (up to r= 0.50) with ISI parameters such as the lamina propria and epithelial thickness, proliferation of enterocytes, and inflammatory cell infiltration in the lamina propria and epithelium. Additionally, VW and absorptive surface were statistically correlated with the ISI Total Score, which is the sum of all the evaluated parameters (Figure 3 and 4). No measurement of the villi presented correlation with any performance data.

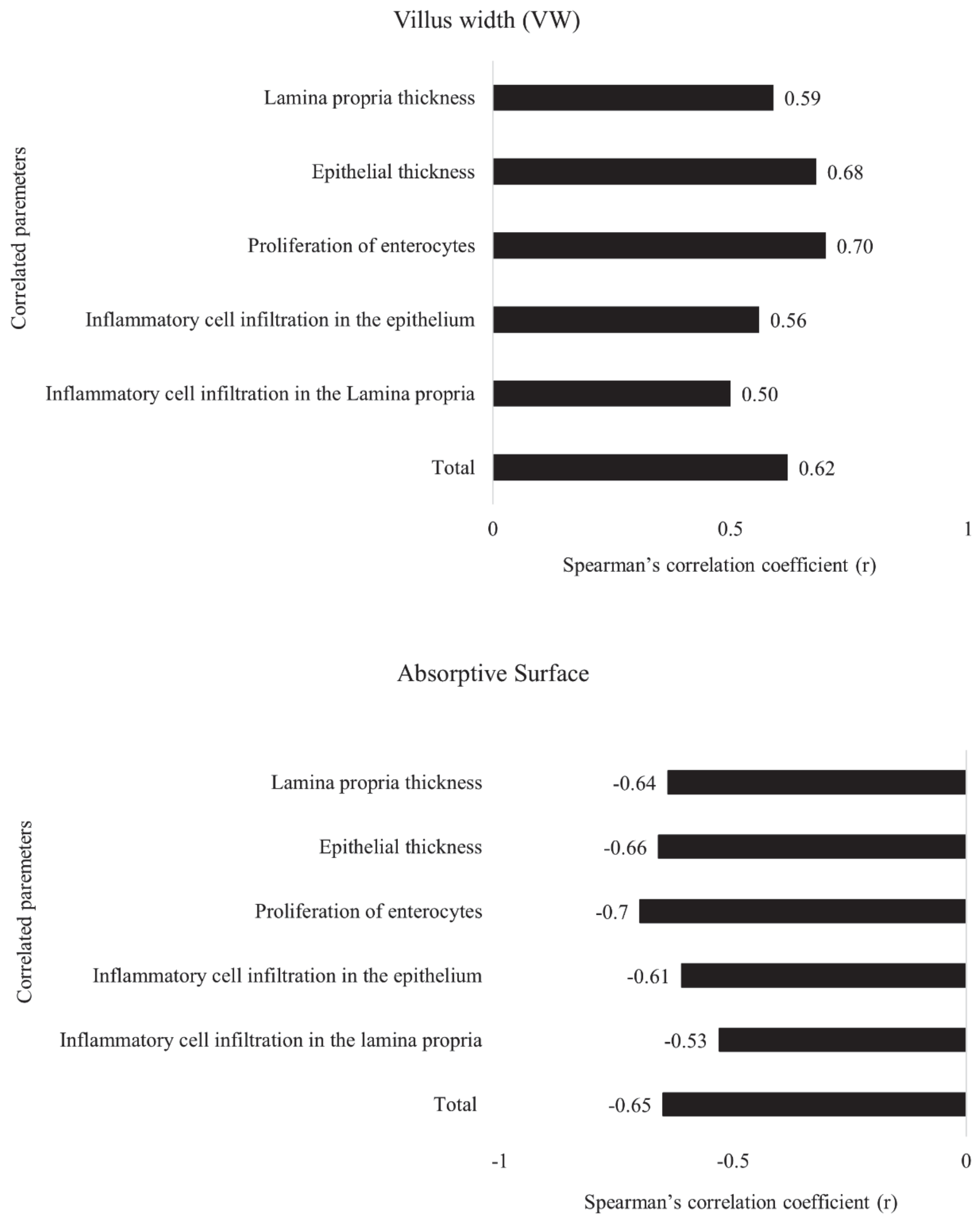

Figure 3. Correlation between measurement of villus, and ISI parameters. Spearman's test coefficient (r) ($P<0.01$).

3.4.2 ISI® methodology

Besides being statistically correlated with villi measurements, the ISI results were also internally correlated among its own histological parameters and with the zootechnical performance (Figure 4).

The ISI parameters correlated with each other (Figure 4) were the lamina propria thickness with inflammatory cell infiltration in lamina propria (r=0.72); epithelial thickness with proliferation of enterocytes (r=0.91) and inflammatory cell infiltration on the epithelium (r=0.57); and inflammatory cell infiltration in the lamina propria with inflammatory cell infiltration on the epithelium (r=0.50). The presence of oocysts and the ISI Total Score were correlated ($P<0.001$) with lamina propria and epithelial thickness, proliferation of enterocytes, inflammatory cell infiltration in lamina propria and the epithelium and with each other (r=0.77).

For performance correlation, the presence of oocysts showed correlation with FCR 21 -28d (r= 0.57), FCR 28 - 35d (r= 0.58) and FCR 35 d (r= 0.60). And the ISI total score presented correlation with FCR 28-35d (r= 0.50).

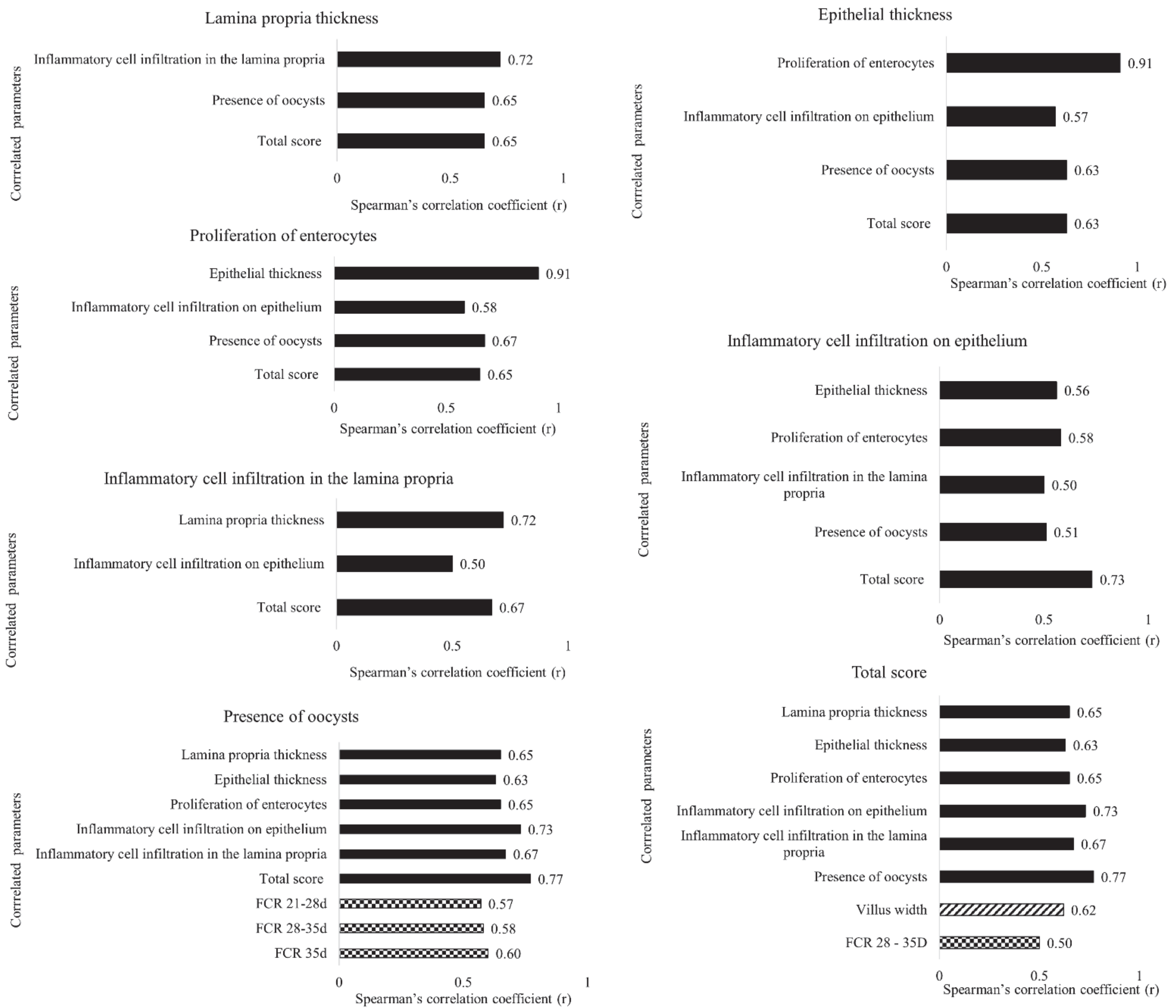

Figure 4. Correlation between all ISI parameters, villus width and feed conversion ratio (FCR) in different periods. The black bar is the correlation between the ISI method with itself. The bar in the cross-section is the correlation between ISI method with the villi and crypt measurement method. And, the cross bar is the correlation between ISI method and zootechnical performance. Spearman's correlation coefficient (r) ($P<0.001$).

4. DISCUSSION

The damage on the intestinal mucosa and the worse zootechnical results were expected in the CH group. The coccidian lifecycle takes approximately one week to get complete (Shirley et al., 2005) and the time to build an immune response is related to significant impacts on the animal production performance observed at the two following weeks (28 and 35 d) (Table 2). The inflammatory process aiming to eliminate the parasite and results in reduced feed intake, and increases the energy demands on the affected sites, disrupting the zootechnical performance of the flock (Klasing, 2007; Kogut and Klasing, 2009). According to Belote et al., (2019), lesions observed since the second week of life caused by *Eimeria* spp. impact negatively ($P<0.05$) the broilers performance until 28 days of age. Likewise, we also observed the impacts of the intestinal lesions up to 35 days.

Eimeria spp has affinity for lateral and apical areas of the villi, causing destruction of the structure (Lillehoj and Okamura, 2003). Theorical, villus height reflects a balance between the mitotic activity of the crypt enteric cells (Cera et al., 1988) and its replacement by mitotic activity from the crypts expressed as villi turnover. As observed in the present study, any damage in the villi is associated with a reduction of the villi height and of the VH:CD ratio (Luquetti et al., 2016; Mustafa et al., 2021; Teng et al., 2021) whereas it increases the crypt depth due to proliferative activity. It is suggested that the proliferative demand of the crypt is greater due the presence of the inflammatory process (Haschek et al., 2010).

Additionally, all these massive villi destruction altered the biochemical and hematological parameters, which it cannot be quantified in the method of measurement of villus/crypt (Woo et al., 2019; Sanches et al., 2020).

Applying the ISI methodology allowed us to observe higher proliferation of enterocytes, infiltration of inflammatory cells and increase of the lamina propria and epithelium thicknesses in the CH group when compared to the unchallenged group as noted in previous studies with the same challenge mode (Kraieski et al., 2017; Belote et al., 2018, 2019; Moraes et al., 2019; Stefanello et al., 2020; Sanches et al., 2020). Increase of goblet cells and congestion did not showing correlation with the final data in this model of study. In fact, the goblet cells were the only parameter increased in NC group ($P<0.05$) compared to CH group one week after challenge.

Lourenço et al (2011) observed an inverse relationship between the number of CD3+ cells infiltration and the expression of goblet cells in the intestinal mucosa, since the increase in CD3+ cells reduce the number of goblet cells after inoculation. In the present study, the reduced goblet cell in CH group compared to NC group is also related to an increase of immune cell infiltration in lamina propria and epithelium of the villi, suggesting that the presence of more specific immune cell reduces, the relative number of goblet cells in the tissue. However, more studies should be done to determine the temporal observation of this process during the immune response in intestinal mucosa.

Correlations ($P<0.001$) between the VW and the ISI scores (Figure 4) were observed, suggesting that the inflammatory process (expressed through the ISI methodology) turns the villus larger. Although, the large villi surface does not mean that the absorptive capacity will be increased. In fact, the increase in ISI parameters and the VW are both related to increase in inflammatory process, which negatively affect animal absorption and FCR. This can be better understood in the figure 5, that showed two villi at same cut and almost the same height (A - 1085 mm vs B - 1090 mm), but, the villus A present normal and healthy structure, while the villus B present higher proliferation of enterocytes and inflammatory cell infiltration, may impair the functionality of the villus. In fact, the proliferation of immature enterocytes along the villi was also observed in other studies (Belote et al., 2018, 2019; Kraieski et al., 2017; Sanches et al., 2020). Sanches et al., (2020) characterizes this process with the destruction of the lamina propria by inflammation and its invasion by the proliferated immature enterocytes, which stimulates the chronicity of the process. The authors suggest that an inflammation with regeneration could be a mechanism of the microscopic basal enteritis with the permanent induction of the host defense (minor enteritis).

For many years, absorption area has been used to understand the absorptive capacity of the poultry intestine, considering an area space of the histological field, evaluating number of villi per area in a horizontal section through the base of the villi from 10 adjacent villi or 1 cm 2 areas (Uni et al., 1998). However, for this study, we applied the method developed by Kisielinski et al., (2002) in rat, which is evaluate the absorptive surface of the villi, which has the closest similarity to the ISI and measurement villi and crypt methods, since we evaluate the villi as units. The formulation of the Kisielinski method considers an analysis of the villus and not the area space, to better understand the response of each villus as a unit in

relation to its absorptive capacity. This calculation disregards the proliferated villus base, which is considered as afunctional without absorptive capacity, and may influence villus width. But in this method, they do not consider the afunctional immature enterocyte proliferation are in the villi as ISI does.

It is interesting to notice in this study, that the calculated absorptive surface showed negative correlation (up to r=-0.50) with ISI method (figure 3). This means, that the lower the absorptive surface, the higher the ISI scores of some parameter and the ISI total, which support the idea of the healthier, the higher is the villus (Uni et al., 1995, 1998). Also, it demonstrates how the cellular immaturity of the proliferate enterocytes can negatively impact the absorptive surface since the monolayer of matured well differentiated enterocytes is absent in the proliferated sites. However, the absorptive surface showed no correlation (P<0.001) with performance data, while the sum of all parameters evaluated in the ISI method (ISI total) presented correlation with FCR at 28d to 35d period, which means, higher the ISI total, worse is the FCR (P<0.001). This correlation was observed in previous studies with the ISI method (Belote et al., 2018). The capacity of the ISI method to translate the inflammatory process as well the effect on animal performance results, is the main differential between the methods evaluated, and shows great promise for ISI to be applied in real field situation to evaluate intestinal health and predict production performance results in broilers.

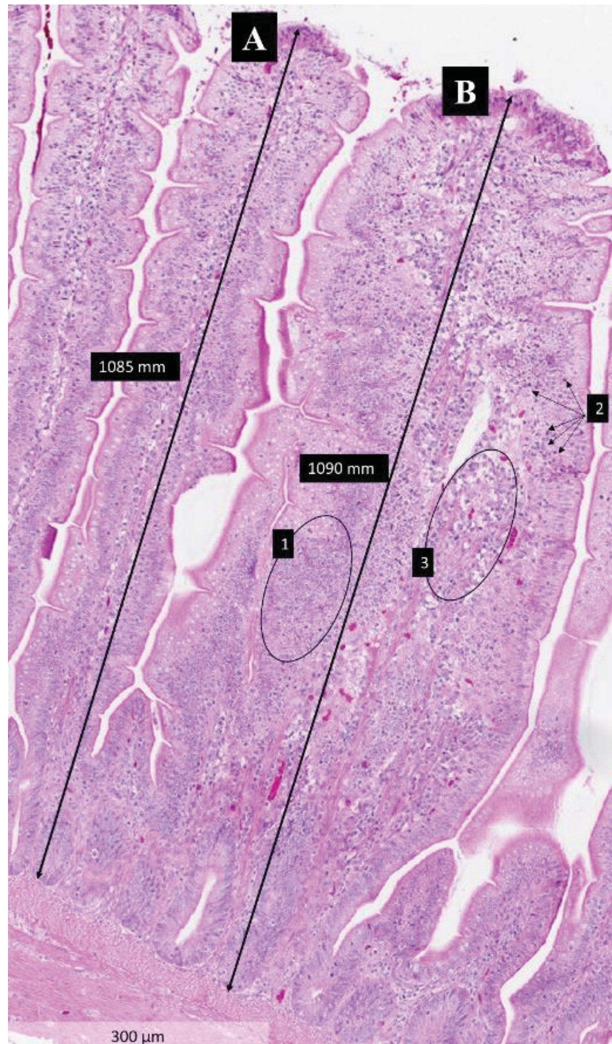

Figure 5. Photomicrographs of broiler jejunum sections of challenged group (CH) stained with hematoxylin and eosin (80X). Villus A – Present normal and healthy structure with 1085 mm of villus height (VH); and villus B – Present high ISI scores in circle 1. Proliferation of enterocytes and circle 2. inflammatory cell infiltration in the lamina propria with 1090 mm of VH.

5. CONCLUSION

In conclusion, a larger villi surface area is not necessarily related to better intestine function, since it might be compromised by inflammatory process. The ISI histopathologic scoring showed interesting results to detect biologic differences in treatment groups, providing more information about gut health, and correlation with animal's performance.

SUPPLEMENTAL DATA

Applying different morphometric intestinal mucosa methods and the correlation with broilers performance under *Eimeria* challenge

Performance

Table 1. Results of average daily feed intake (ADFI) at all periods (P<0.05)

Treatment	Variable	ADFI7d	ADFI14d	ADFI21d	ADFI28d	ADFI35d
	Mean	15.86	32.11	49.52	**72.31 a**	**93.61 a**
	SD	0.62	0.87	1.55	2.37	3.09
NC	C.V.	3.93	2.70	3.14	3.28	3.30
	Minimum	14.58	30.33	46.10	67.98	88.11
	Maximum	16.58	33.25	51.08	75.27	98.70
	Mean	15.73	31.51	48.72	**69.15 b**	**89.75 b**
	SD	0.66	0.98	1.39	2.00	3.01
CH	C.V.	4.22	3.10	2.85	2.89	3.36
	Minimum	15.09	30.41	47.26	66.65	84.97
	Maximum	17.37	33.59	51.79	72.73	95.29
P Value NC vs CH		0.62	0.13	0.19	<0.001	<0.001

Different superscript letters indicate a significant difference with Tukey test (P < 0.05).

Table 2. Results of average daily feed intake (ADG) at all periods (P<0.05).

Treatment	Variable	ADG7d	ADG14d	ADG21d	ADG28d	ADG35d
	Mean	12.08	23.80	35.86	**49.20 a**	**60.03 a**
	SD	0.66	0.50	1.07	1.47	1.81
NC	C.V.	5.48	2.11	2.99	2.99	3.02
	Minimum	11.00	22.88	33.57	46.94	56.86
	Maximum	12.95	24.60	36.93	51.13	63.01
	Mean	11.64	23.50	35.33	**44.84 b**	**54.75 a**
	SD	0.53	0.69	0.93	13.81	20.37 b
CH	C.V.	4.57	2.94	2.64	3.08	3.72
	Minimum	10.97	22.69	34.36	42.79	51.80
	Maximum	12.61	24.94	37.41	47.22	57.40
P Value NC vs CH		0.08	0.237	0.207	<0.001	<0.001

Different superscript letters indicate a significant difference with Tukey test (P < 0.05).

Table 3. Results of feed conversion ratio (FCR) at all periods (P<0.05)

Treatment	Variable	FCR7d	FCR14d	FCR21d	FCR28d	FCR35d
NC	Mean	1.313	1.349	1.381	**1.469 b**	**1.559 b**
	SD	0.027	0.017	0.014	0.011	0.010
	C.V.	2.014	1.235	1.016	0.743	0.650
	Minimum	1.267	1.326	1.363	1.448	1.546
	Maximum	1.350	1.379	1.404	1.488	1.574
CH	Mean	1.350	1.341	1.379	**1.542 a**	**1.639 a**
	SD	0.035	0.023	0.016	0.014	0.022
	C.V.	2.615	1.705	1.169	0.878	1.311
	Minimum	1.301	1.297	1.358	1.522	1.606
	Maximum	1.422	1.373	1.407	1.568	1.692
P Value NC vs CH		0.060	0.3270	0.7490	<0.001	<0.001

Different superscript letters indicate a significant difference with Tukey test (P < 0.05).

CHAPTER 3:

Novel Models for Chronic Intestinal Inflammation in Chickens: Intestinal Inflammation Pattern and Biomarkers

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ABSTRACT

For poultry producers, chronic low-grade intestinal inflammation has a negative impact on productivity by impairing nutrient absorption and allocation of nutrients for growth. Understanding the triggers of chronic intestinal inflammation and developing a non-invasive measurement is crucial to managing gut health in poultry. In this study, we developed two novel models of low-grade chronic intestinal inflammation in broiler chickens: a chemical model using dextran sodium sulfate (DSS) and a dietary model using a high non-starch polysaccharide diet (NSP). Further, we evaluated the potential of several proteins as biomarkers of gut inflammation. For these experiments, the chemical induction of inflammation consisted of two 5-day cycles of oral gavage of either 0.25mg DSS/ml or 0.35mg DSS/ml; whereas the NSP diet (30% rice bran) was fed throughout the experiment. At four times (14, 22, 27 and 36-d post-hatch), necropsies were performed to collect intestinal samples for histology, and feces and serum for biomarkers quantification. Neither DSS nor NSP treatments affected feed intake or livability. NSP-fed birds exhibited intestinal inflammation through 14-d, which stabilized by 36-d. On the other hand, the cyclic DSS-treatment produced inflammation throughout the entire experimental period. Histological examination of the intestine revealed that the inflammation induced by both models exhibited similar spatial and temporal patterns with the duodenum and jejunum affected early (at 14-d) whereas the ileum was compromised by 28-d. Calprotectin (CALP) was the only serum protein found to be increased due to inflammation. However, fecal CALP and Lipocalin-2 (LCN-2) concentrations were significantly greater in the induced inflammation groups at 28d. This experiment demonstrated for the first time, two *in vivo* models of chronic gut inflammation in chickens, a DSS and a nutritional NSP protocols. Based on these models we observed that intestinal inflammation begins in the upper segments of small intestine and moved to the lower region over time. In the searching for a fecal biomarker for intestinal inflammation, LCN-2 showed promising results. More importantly, calprotectin has a great potential as a novel biomarker for poultry measured both in serum and feces.

Keywords: Calprotectin, DSS, rice bran, gut health, lipocalin, models of intestinal inflammation, ISI index

1. INTRODUCTION

Antibiotics used as growth promoters (AGP) have successfully controlled dysbiosis and enteropathogens for the past 50 years (1). However, the recent increase in worldwide non-AGP poultry production is challenging the industry in management, health, and animal welfare due to the increase of enteric and systemic diseases (2, 3). One of the most accepted theories of AGP mechanism is its role in reducing low-level inflammation (4) and immunologic stress (5) that can be caused by environmental factors, pathogens, and feed ingredients (6). Therefore, removing AGP may increase the inflammatory level of the non-AGP flocks and reduce their performance, which raises the importance of understanding intestinal health and applied practices to its promotion (7).

Despite the importance of studying chronic gut inflammation (8), there is a lack of a practical model to induce a low-grade chronic response in broilers that would mimic field situations. Among the developed models in poultry species, most produce strong negative effects causing clinical signs such as the reduction in body weight (9-13). Other models are difficult to replicate or apply in large experiments due to complex methodology (14), including chemicals furnished via drinking water (15), injections (11, 13), or pathogen-specific challenge. Therefore, there is a need for an easily replicated research model that will best mimic the intestinal response in the field.

A nutritional model to produce gut inflammation would be advantageous because the diet would induce a non-specific immune response, also it is easier to apply and might resembles industry situations. Rice bran is an alternative ingredient for energy used in animal nutrition. Compared to other alternatives, such as wheat, rice has higher metabolizable energy and lower fiber content (16). Although rice bran is not considered a soluble ingredient (17), it still has a considerable percentage of fiber, one of the highest phytate content in vegetable ingredients (1.37%), and a large amount of lipids (14.2%) that may suffer peroxidation (16); (18). The negative effects of rancid lipids and phytate on the intestinal mucosa are well known (6, 19-22). Thus, diets with rice bran inclusion may provoke an intestinal challenge in which the degree will vary with the inclusion rate of the ingredient. Alternatively, chemical models of intestinal inflammation also produce a non-specific response that can be used as a positive control in certain situations, although they may not be identical to industry situations

and should be used with caution. Dextran sodium sulfate (DSS) is a polymer widely used in rodent studies to produce colitis. DSS has previously been shown to induce intestinal inflammation in chickens. Oral gavage of DSS had been shown to induce loose stools, histology alterations and weight loss (9, 10, 23). However, most of the rodents and all the DSS chicken protocols produced an acute inflammatory response in the intestine. To study chronic intestinal disease, such as Crohn´s and inflammatory bowel diseases, Bento and collaborators (2012) developed a protocol that produced chronic intestinal inflammation after subjecting mice through to two cycles of DSS via oral gavage.

In addition to establishing a chronic inflammation model, the poultry industry seeks reliable non-invasive biomarker(s) of intestinal inflammation. Several potential biomarkers have already been evaluated in the chicken, such as lipocalin (LCN2), alpha-1 antitrypsin, intestinal alkaline phosphatase, superoxide dismutase, fibronectin (13), and ovotransferrin (24, 25). However, only a few (ovotransferrin, lipocalin and fibronectin) have shown promising but inconsistent results in previous research. Studies in human medicine have proven that fecal concentration of calprotectin, a protein present in neutrophils, monocytes, and macrophages, is a non-invasive method to evaluate the gut inflammation process (26, 27). Recently, calprotectin has been used to aid diagnosis of colonic diseases, for monitoring disease and cancer, and to evaluate efficacy of treatments (28-31). To our knowledge, there have been no studies quantifying levels of calprotectin in poultry excreta or fluids. However, increased gene expression of calprotectin was identified in the intestine of broilers exposed to *Salmonella enterica* (32), *Eimeria maxima* and *Clostridium perfringens* (33), and heat stress (34). Thus, we believe calprotectin has the potential to serve as a biomarker of inflammation in the small intestine of broilers.

Therefore, for this study, we hypothesized that a diet with high inclusion of rice bran or a challenge with DSS based on the Bento et al. (33) protocol have the potential to induce a low-grade chronic inflammation in the intestine of chickens. In the current study, we utilized a) a diet with 30% of rice bran and b) two cycles of DSS oral administration at two different levels, to produce a model of low-grade intestinal inflammation in broilers. Moreover, we evaluated the effect of the challenges overtime in the small intestine and analyzed several biomarkers in the blood and excreta of chickens. This allowed us to: (1) identify two simplistic low-grade inflammation models without inducing major clinical signs

and/or a pathogenic infection, (2) evaluate calprotectin as a novel biomarker, and (3) quantify in low-grade chronic inflammation model potential biomarkers previously reported.

2. MATERIAL AND METHODS

The experiment was conducted in accordance with guidelines set by the United States Department of Agriculture Animal Care and Use Committee (USDA IACUC #2019012). The trial was conducted at the Agricultural Research Service Facility of the United States Department of Agriculture (ARS-USDA), College Station, Texas, US.

To evaluate the two different intestinal inflammation models, a total of 180 Cobb male by-product day-of-hatch chickens were randomly assigned to four experimental treatment groups with three repetitions of 15 birds each. The treatments were: (1) Control (CNT) with a corn/soybean meal standard diet; the second and third groups received control diet and were submitted to two cycles of (2) 0.25mg/ml of DSS (25DSS), or (3) 0.35mg/ml of DSS (35DSS), respectively; and (4) the fourth group had a high non-starch polysaccharide diet (NSP) (30% of rice bran) (Table 1). DSS was used as a chemical inducer of intestinal inflammation. The birds in 25DSS- and 35DSS-treated groups received a DSS solution administrated via oral gavage in two cycles of 5 consecutive days, interrupted by a recovery period of 9 days as described for a murine model of intestinal chronic inflammation (35). The first cycle lasted from day 9 to day 14 of age; the second cycle was applied from day 23 to day 27 of age (Figure 1). Birds in the NSP treatment received feed with 30% of rice bran inclusion during the entire experimental period (1 to 36-d of age).

The different diets offered were iso-energetic, iso-nitrogenous, and formulated to meet or exceed the broiler's requirements (Cobb manual, 2018) (Table 2). Two feeding phases were used: starter (1-21days) and grower (21-36 days). Dextran sodium sulfate solutions were produced by solubilizing DSS (MW ca 40,000; Sigma Aldrich) in distilled water to the desired concentrations.

Chickens were placed from day 1 to 36 of age on 0.91x1.21 m^2 pens and a minimum of 0.074m^2/bird was maintained during the whole experimental period; the floor was covered with new pine shavings. Water and feed were offered *ad libitum* and environmental conditions were maintained for each growing phase according to the Cobb manual recommendations (2018).

Table 1. Experimental treatments.

Treatment	Abbreviation	Diet	Intestinal Challenge
Control	CNT	Corn/soybean meal standard diet	No
0.25mg/ml of DSS	25DSS	Corn/soybean meal standard diet	0.25mg/ml of DSS oral gavage daily from 9 to14-d and 23 to 27-d
0.35mg/ml of DSS	35DSS	Corn/soybean meal standard diet	0.35mg/ml of DSS oral gavage daily from 9 to14-d and 23 to 27-d
High NSP	NSP	30% of rice bran inclusion	High NSP diet

NSP: non-starch polyssacharide; CNT: Control; DSS: Dextran sodium sulfate

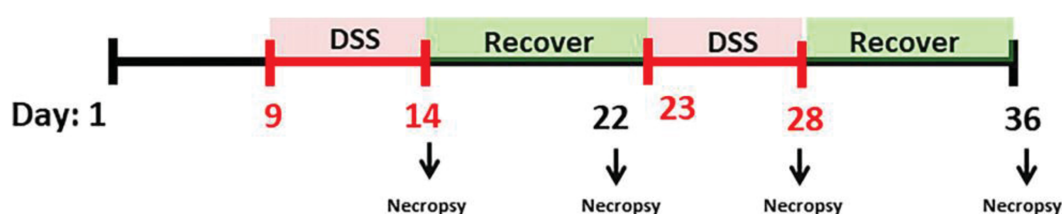

Figure 1. Experimental timeline. Male by-product day-of-hatch chickens were divided in four experimental treatments and raised up to 36 days. The broilers assigned to treatments with DSS challenge received 0.25mg/ml or 0.35mg/ml of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d (induction phase period represented with red). After each DSS cycle animals were allowed to a 9 days recovery period (from 14 to 23-d and 27 to 36-d, marked in green). Birds in the NSP treatment received a diet with 30% of rice bran from 1 to 36-d. Animals in the control group were not submitted to any challenge. Necropsies were performed on days 14, 22, 27 and 36 for collection of intestinal tissue, blood, and fecal samples.

Table 2. Ingredients and calculated nutritional composition of experimental diets.

Ingredients (%)	Starter (1-21 d)		Grower (22-36 d)	
	Control	High NSP	Control	High NSP
Corn	58.87	31.08	65.97	38.185
Soybean meal[1]	34.75	29.93	27.87	22.515
Rice bran	0	30	0	30
Soybean oil	2.40	5.62	2.74	5.95
Monocalcium phosphate	1.72	1.333	1.42	1.03
Limestone	1.08	1.255	0.93	1.10
NaCl	0.37	0.35	0.37	0.35
DL-Methionine	0.35	0.35	0.27	0.30
L-Lysine HCl	0.22	0.29	0.18	0.259
L-Threonine	0.098	0.165	0.012	0.079
Choline chloride	0.05	0.05	0.05	0.05
Vitamin premix[2]	0.05	0.05	0.1	0.1
Mineral premix[3]	0.03	0.03	0.05	0.05

Calculated composition

Metabolizable energy (kcal/kg)	2990	2990	3100	3100
Crude protein (%)	22	22	19	19
Lysine dig. (%)	1.22	1.22	1.02	1.02
Methionine (%)	0.61	0.63	0.53	0.55
Meth. + cysteine dig. (%)	0.91	0.91	0.80	0.80
Threonine dig. (%)	0.83	0.83	0.66	0.66
Av. Phosphorus (%)	0.45	0.45	0.38	0.38
Calcium (%)	0.90	0.90	0.76	0.76
Potassium (%)	0.92	1.14	0.79	1.02
Sodium (%)	0.16	0.16	0.16	0.16

[1] Soybean meal 49% of crude protein

[2] Composition of minimum (per kg of feed): Vit A 8,818,342 IU; Vit D3 3,086,420 IU; Vit E 3,674 IU; Vit B12 130 mg; Vit K 1,177mg; Vit B2 4,775 mg; pantothenic acid 16,168 mg; Vit B1 2,350 mg; Vit B3 36,742 mg; Vit B6 5,732 mg; folic acid 1.1,398 mg; choline 104,460 mg; biotin 441mg.

[3] Minimum of Fe 12%; Cu 1.4%; I 800ppm; Zn 12%; Mn 173.0 mg; Mg 12%
Av.: available; dig.: digestible.

2.1 Necropsy and sampling

Necropsies were performed on days 14, 22, 27 and 36 of age and intestinal tissue, blood, and fecal samples were collected to evaluate the gastrointestinal tract (GIT) inflammation (Figure 1). At necropsy, two birds from each pen (6 birds from each treatment) were randomly selected for sample collection. Initially, blood was collected in serum tubes from the jugular vein, and finally the bird was euthanized by cervical dislocation. The collected blood tubs were laid flat for clotting (at least for 30 min) and the serum was separated and collected after centrifugation (15 min on 3000 rpm) and stored at -20ºC for further analyses.

Following euthanasia, each carcass was systematically evaluated to identify the health status and physical condition of the gastrointestinal tract based on the “I See Inside” (ISI) methodology (36). The intestine (duodenum, jejunum, ileum and cecum) and GIT-associated organs (liver, yolk, proventriculus, gizzard, pancreas) were evaluated grossly as

described (36). Several macroscopic characteristics were scored on a 0-3 scale and the scores were then multiplied by impact factors (1-3), depending on their importance (in detail in Table 1 of "Supplemental data"). The ISI macro total score (sum of all observed lesions) in the study can reach 78, stemming from a total intestinal score of 54 and a total GIT-associated organs of 24. The higher the ISI score, the worse the GIT health status.

During the necropsy, samples of duodenum, jejunum and ileum were collected for posterior histological analysis. For the tissue collection, intestinal anatomy was used to ensure sampling of a similar segment in all birds. Duodenum samples (5cm) were taken 1cm caudal the duodenal loop. Meckel´s diverticulum was used to separate jejunum and ileum and the sampling was made in the middle of each segment (5cm). The intestinal samples were washed with 0.9% NaCl solution and fixed in 10% buffered formalin.

2.2 Fecal sampling

Fresh excreta samples were collected at 27 and 36-d from 2 birds per repetition (6 samples for each treatment), immediately frozen in liquid nitrogen and stored at -80ºC. For downstream ELISA array, 0.4 g of excreta was vortexed with 4 ml of PBS and centrifuged for 20 min at 4ºC at 3000 rpm. The supernatant was removed and stored at -20ºC for posterior assays.

2.3 ELISA analysis

For the biomarker searching concentration of calprotectin (avian MRP-126), lipocalin, S100 protein, hypoxia inducible factor- 1 subunit alpha (HIF1α), lipopolysaccharides (LPS) and ovotransferrin (OVT) were quantified using the fecal homogenate and/or serum at different time points (Table 3). All biomarkers were quantified with ELISA kits from MyBiosource Inc. (San Diego, CA-USA).

Table 3. Proteins quantified in serum and/or feces of broilers at 14, 22, 28 or 36 days post-hatch by ELISA.

	14-d	22-d	28-d	36-d	ELISA catalog #
Calprotectin (avian MRP126)	Serum	Serum	Feces	Feces	MBS1601938
Lipocalin	Serum	Serum	Serum and Feces	-	MBS005459
S100A	-	-	Feces	Feces	MBS2504874
HIF1α	-	-	Feces	Feces	MBS287105
Ovotransferrin	-	-	Feces	-	MBS2533639
LPS	Serum		Serum	Serum	MBS268415

S100A: S100A protein; HIF1α: hypoxia inducible factor-1 subunit alpha; LPS: lipopolysaccharides

2.4 Histology and I See Inside methodology

The intestinal samples stored in formalin were dehydrated, infiltrated, and embedded in paraffin following standard histological practices. Paraffin blocks were cut into 5 μm sections and stained with hematoxylin and eosin.

For evaluation of microscopic alterations, 20 villi sections per bird were evaluated at 10X magnification (using 40X magnification to confirm alterations) by a treatment blinded person using an optical microscope (Nikon Eclipse E200, Sao Paulo, Brazil). The “I See Inside” (ISI) microscopy methodology (patent INPI BR 1020150036019), was used to measure histologic alterations on the intestine (37), and a final intestinal health index was produced. A score (0-3) was given to each alteration depending on the extent of the lesion: the greater the lesion, the higher the score. The score was then multiplied by an impact factor (IF; 1-3) which is based on the consequence of alteration on the organ functionality. The final ISI index was then calculated as the sum of all alteration index:

$$ISI\ index = \sum score * IF$$

The alterations evaluated, and their impact factor were: lamina propria thickness*(2), epithelial thickness*(1), enterocytes proliferation*(1), inflammatory cell infiltration in the epithelium*(1), inflammatory cell infiltration in the lamina propria*(3), goblet cells proliferation*(2), congestion*(2), and the presence of *Eimeria sp.* oocysts*(3). Therefore, animals with a high final ISI index had worse intestinal health. For statistical analysis each bird was considered as a replicate.

2.5 Statistical analysis

Data were analyzed accordingly to a complete randomized design. At first, normality of all the data was verified through Shapiro-Wilk's test and variables with non-normal distribution were analyzed by Kruskal Wallis test, as all data generated by the microscopic and macroscopic ISI evaluation. Then, data with normal distribution were analyzed using ANOVA (P-value<0.05), and means were compared by Tukey. Dunnet's test was also used in the biomarkers data to compare challenged groups with the CNT group. For feed intake, each pen was used as an experimental unit, and for the remaining analyses, each bird was used as an experimental unit. Due to the low number of experimental units (3 pens per treatment), feed intake was only used as a clinical sign and not to compare performance between groups. Software JMP® Pro 15.0.0 (SAS Institute Inc.) was used, and for all tests P-value<0.05 was considered statistically significant.

3. RESULTS

3.1 Chronic Inflammation model

3.1.1 Macroscopic gut observations

It was observed that neither the DSS treatment nor the rice bran diet had an effect on feed intake ("Supplemental data"- Table 2) or mortality. However, necropsy revealed intestinal lesions in birds treated with DSS or fed the rice bran diet (Table 4). By the macroscopic "I See Inside" method, NSP exhibited a more consistent pattern of inflammation which negatively affected the intestinal health as seen by the significantly higher macroscopic lesion scores on days 14 and 36 with a similar trend on days 22 and 28 (Table 4). Both DSS cycles and the high NSP diet induced localized intestinal lesions, but no gross inflammatory lesions in extra-intestinal organs (pancreas, yolk-sac, liver, gizzard and proventriculus) were observed. Moreover, the macroscopic ISI score lesions of each section of the intestine showed that the duodenum was affected more in the early days of the treatments (Table 5) whereas, the posterior parts of the intestine showed compromised intestinal health at a later stage (36 days), suggesting a spatial and temporal impact on the intestinal lumen.

3.1.2 Microscopic histological gut observations and alterations

The histological examination of the intestine provided us more detailed and sensitive information about the gut status of the broilers. The microscopic ISI revealed an clear spatial and temporal impact of the treatments in the intestinal lumen. Histologically, the ileum of the birds was affected later, after 28 days, while duodenum and jejunum presented inflammation already in the first necropsy at 14 days of age (Figures 2 to 4).

The NSP diet induced higher microscopic ISI scores than the CNT. This difference started to be seeing at 22-d in the duodenum and at 14-d in the jejunum, followed by a stabilization ISI score in these segments by 36-d (Figure 2 and 3). However, histological alterations in ileal tissue did not appear until 28-d of NSP diet consumption (Figure 4). The main alteration observed in broilers fed the high NSP diet was increased infiltration of inflammatory cells in the lamina propria and epithelium which also seemed to shift in a temporal (from 14-d to 26-d) and spatial fashion (from duodenum towards ileum; Supplemental data- Table 3, 4 and 5). At later stages of growth (22-d to 36-d), an increase in epithelial and lamina propria thickness was also observed (Supplemental data- Table 3, 4 and 5). Broilers fed the NSP diet also had a variation in the goblet cells probably as a response to the mucus production by the intestine. Goblet cells were initially (14-d) reduced in the duodenum in this treatment compared to control ($P < 0.0001$), but at 22 and 28-d of age broilers showed an increase in goblet cells at duodenum and jejunum ($P < 0.01$; Supplemental data- Table 3 and 4) (Supplemental data- Table 5). This goblet cell response was stabilized at 36 days of age ($P < 0.0001$; Supplemental data- Table 6). Moreover, birds fed with the high NSP diet presented an increased abnormal proliferation of enterocytes which was constant in the jejunum throughout the experiment and affected the whole small intestine by 28 days (Supplemental data- Table 3, 4, 5 and 6).

The chemical model of low-grade inflammation (two cycles of DSS) also was able to reduce intestinal health as indicated by the increase in the ISI histological scores. The duodenal and jejunal tissue were already affected by the first DSS cycle (Figures 2 and 3), but the ileum showed a significant increase in ISI only after the second DSS challenge. Even after 9 days of recovery, the duodenum and jejunum had higher ISI scores than the control (Figure 2 and 3) and after the second DSS cycle (28-d), the birds presented several histological alterations diffused in the small intestine showing an additive effect of the cycles

over the time. The observations of the 28-d are noteworth because all evaluated parameters were elevated. The duodenum, jejunum and ileum presented higher scores for lamina propria and epithelial thickness, proliferation of enterocytes, and infiltration of inflammatory cells in epithelium and lamina propria than CNT (Supplemental data- Table 5). Moreover, an increase in goblet cells is observed in duodenum and jejunum at that time point.

Lastly, the ISI scores of the 35DSS treatment were higher across all intestinal segments at 36 days showing a decreased intestinal health even after 9-d of recovery from the second DSS cycle. At the end of the experiment, animals in the 35DSS group had higher inflammatory cell infiltration in lamina propria and epithelium as well as a consistent increase in globet cells in all intestine segments (P-value<0.05), higher lamina propria and epithelial thickness in jejunum and ileum (P-value<0.01), and increased enterocytes proliferation in the jejunum (P-value=0.0003; Supplemental data). Thus, the histological alteration indicates that even following 9 days of recovery, two cycles of 0.35mg/ml of DSS were able to induce a long-lasting inflammation.

Therefore, both the high inclusion of rice bran and the two DSS models proved successful in producing chronic intestinal inflammation. The NSP diet induced a marked inflammatory response through the first 28 days of broiler age whereas, the birds showed a partial reduction of the intestinal inflammation when they aged (observed at 36-d). Additionally, the NSP diet produced a lower-grade inflammation when compared to the DSS treatments, the latter presenting consistently a higher ISI score (Figure 2 to 4). The two DSS cycles of 0.35mg/ml produced a long-lasting inflammation persistent even after 9 days of recovery and presented a more consistent microscopic pattern than the 0.25mg/ml DSS challenge. However, independent of the trigger, the inflammation response of the broilers showed a similar pattern and the intestinal inflammation progressed from the cranial segments of the small intestine to the caudal areas through time (Figure 5).

Table 4. Macroscopically I See Inside (ISI) lesions scores of related organs, intestine and total score of broilers submitted to different intestinal challenges at 14, 22, 28 and 36 days of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge.

Treatment	14 days			22 days			28 days			36 days		
	Related organs	intestine	Total	Related organs	Intestine	Total	Related organs	Intestine	Total	Related organs	Intestine	Total
Control	4.83	5.66 b	10.50 b	2.66	5.00	7.66	2.16	5.50	7.66	1.83	1.50 b	3.33
25DSS	4.50	9.33 ab	14.16 ab	3.33	5.50	8.83	2.33	9.33	11.66	1.00	4.83 ab	5.83
35DSS	5.16	10.66 ab	15.83 ab	1.83	7.16	9.00	2.66	6.16	8.83	1.83	3.50 b	5.33
NSP	4.83	13.66 a	18.50 a	3.66	6.33	10.00	2.50	9.66	12.16	0.83	7.83 a	8.66
SEM[1]	1.224	1.686	1.804	0.850	1.139	1.285	0.677	1.381	1.770	0.7216	10.672	14.912
P-value	0.985	0.024	0.036	0.456	0.563	0.651	0.958	0.098	0.241	0.656	0.003	0.122

Related organs evaluated: liver, yolk sac, proventriculus, gizzard, pancreas.
[ab] Different superscript letters indicate significant difference with Tukey test.
[1] Pooled standard error of the mean
Maximum macroscopic ISI score: Related organs 24; Intestine 54; Total 78.
n= 6 animals/ treatment; 2 animals/ pen

Table 5. Macroscopically I See Inside lesions scores of duodenum (Duod), jejunum (Jejun), ileum and ceca of broilers submitted to different intestinal challenges at 14, 22, 28 and 36 days of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge.

	14 days				22 days				28 days				36 days			
Treatment	Duod	Jejun	Ileum	Ceca	Duod	Jejun	Ileum	Ceca	Duod	Jejun	Ileum	Ceca	Duod	Jejun	Ileum	Ceca
Control	1.5^{b}	2.00	1.33	0.83	1.5	0.66	1.50	1.33	1.66	1.50^{bc}	1.5	0.83	0.66	0.00^{b}	0.166^{c}	0.66
25DSS	2.66^{b}	2.83	1.50	2.33	2.33	0.83	0.66	1.66	1.83	3.50^{b}	1.5	2.5	2.00	1.83^{ab}	0.5^{bc}	0.50
35DSS	3.83^{ab}	3.16	1.83	1.83	1.66	2.00	1.33	2.16	1.66	0.66^{c}	1.66	2.16	0.66	0.83^{b}	1.00^{ab}	1.00
NSP	5.66^{a}	3.16	2.66	2.16	0.33	3.00	1.50	1.50	1.66	4.00^{a}	3.00	1.00	0.83	3.66^{a}	2.00^{a}	1.33
SEM[1]	0.8113	0.5809	0.540	0.631	0.667	0.606	0.383	0.524	0.554	0.709	0.621	0.509	0.602	0.747	0.263	0.318
P-value	0.0157	0.4338	0.4932	0.3789	0.1891	0.0907	0.3314	0.714	0.9955	0.0164	0.3016	0.0535	0.3481	0.0240	0.0042	0.2881

[ab] Different superscript letters indicate significant difference with Tukey test.
[1] Pooled standard error of the mean
n= 6 animals/ treatment; 2 animals/ pen

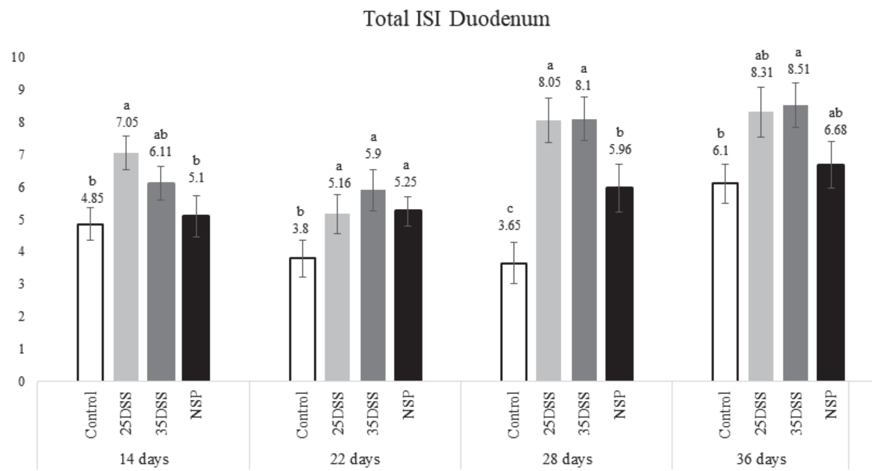

Figure 2. I See Inside (ISI) total microscopically lesions scores of duodenum of broilers submitted to different intestinal challenges at 14, 22, 28 and 36 days of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge. [abc] Different superscript letters indicate significant difference with Tukey test. n= 6 animals/ treatment; 2 animals/ pen

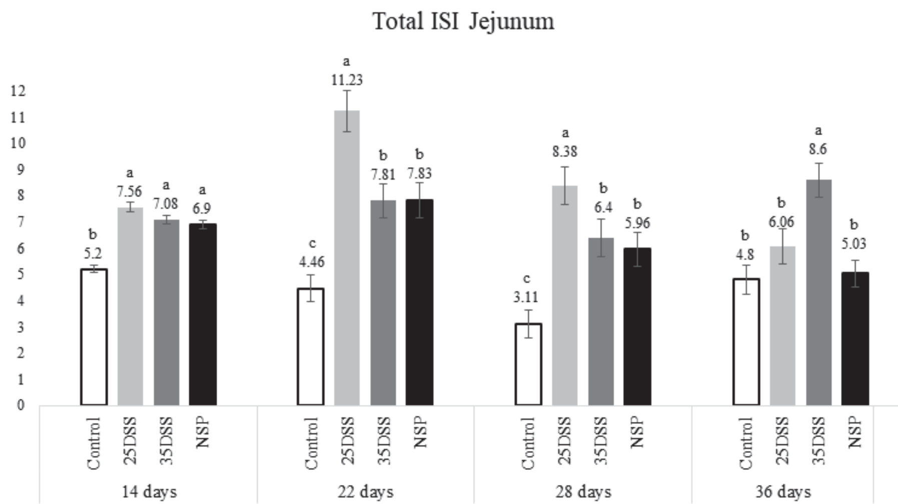

Figure 3. I See Inside (ISI) total microscopically lesions scores of jejunum of broilers submitted to different intestinal challenges at 14, 22, 28 and 36 days of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge. [abc] Different superscript letters indicate significant difference with Tukey test ($P<0.05$). n= 6 animals/ treatment; 2 animals/ pen

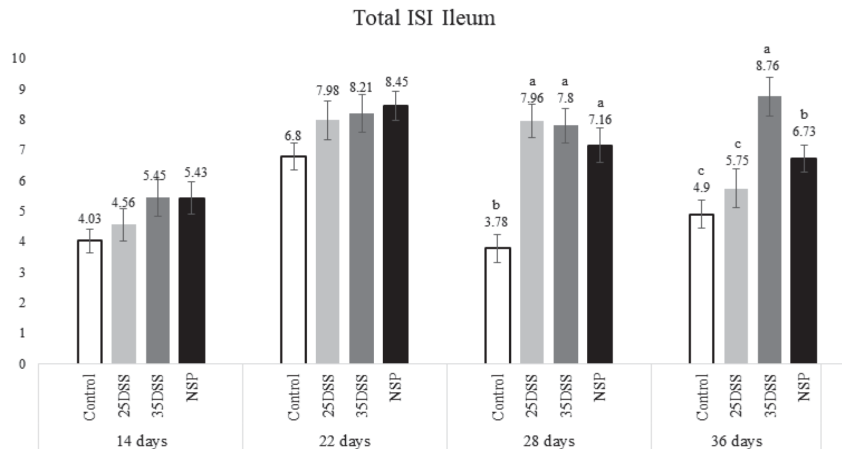

Figure 4. I See Inside (ISI) total microscopically lesions scores of ilea of broilers submitted to different intestinal challenges at 14, 22, 28 and 36 days of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge. [abc] Different superscript letters indicate significant difference with Tukey test (P<0.05). n= 6 animals/ treatment; 2 animals/ pen

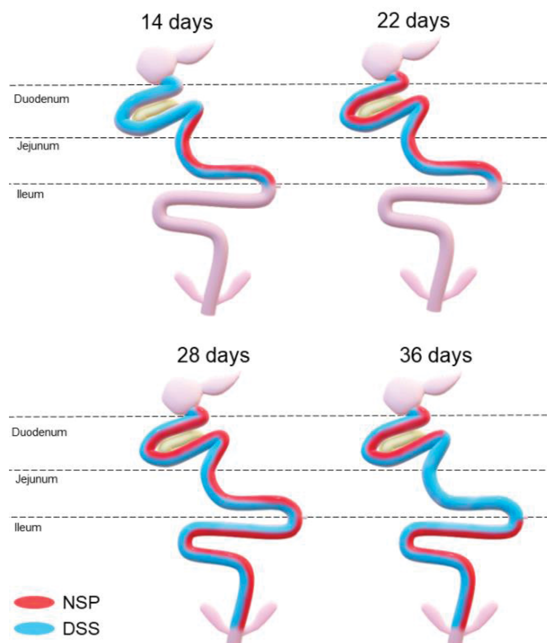

Figure 5. Temporal and spatial evolution of the intestinal inflammation response triggered by a high non-starch pollisacharyde diet (NSP) diet or a DSS challenge in broilers. Areas painted with red and/or blue signalizing the affected* areas in birds fed NSP diet (red) or in birds challenged with the DSS protocol (blue). *affected areas: areas with poor intestinal health (higher microscopic ISI scores).

3.2 Biomarkers

Calprotectin, lipocalin and LPS were analyzed in the serum of broilers but the only serum biomarker that showed a statistical difference in the challenged groups was calprotectin (CALP) (Table 6). The concentration of CALP in the blood was higher in the presence of intestinal inflammation (P-value=0.0365) at 14 days of age. Calprotectin concentration in the serum was 135% higher in animals from the 35DSS group when compared with control birds. However, after the recovery period (at 22-d) of the first DSS cycle, the difference in calprotectin concentration of both groups disappeared. The rice bran presented a CALP elevation of 60.9% but it was not sufficient to be different than the CNT at 14-d. In addition, calprotectin concentration in the serum might be age dependent. A 10-fold increase of calprotectin concentration in the serum was found in all treatments between days 14 and 22 (Table 6). The serum concentrations of lipocalin and LPS were not statistically different among the treatments at any of the timepoints measured (Table 7 and Supplemental Data-Table 7, respectively).

Among the analyzed fecal biomarkers, calprotectin and lipocalin showed statistical differences among treatments at day 28 (P-value=0.0395 and P-value=0.0075, respectively; Table 8). After the second cycle of DSS, the 35DSS-treated birds showed an increase in fecal calprotectin concentration when compared to the control by 24.99%. DSS (25DSS) and NSP treatments had a numerical increase in fecal calprotectin concentration, but no statistical difference compared to the control. Broilers fed the high NSP diet also showed a statistical increase in fecal lipocalin concentration (27.09%) (P-value= 0.0075). However, by 36 days of age, no differences were observed in either fecal calprotectin or lipocalin concentrations, suggesting there is an optimal time to analyze the markers or that the numbers of samples were not enough to detect differences among treatments in older birds. Interestingly, fecal HIF-1α presented a tendency (P-value=0.062) at 36 days of age with 35DSS showing the highest concentration. No differences were observed in the concentration of fecal S100 at any day analyzed or fecal ovotransferrin at 28-d (Table 8 and Supplemental data Table 8, respectively)

Table 6. Calprotectin concentration in the serum of broilers submitted to different intestinal challenges at 14 and 22 days of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge.

	14 days		22 days	
Treatment	Calprotectin (ng/ml)	±S.E.M.	Calprotectin (ng/ml)	±S.E.M.
Control	16.86 ^{b}	1.74	221.34	36.61
25DSS	21.14 ^{ab}	2.86	188.49	19.33
35DSS	39.68 ^{a*}	8.29	232.47	21.17
NSP	27.13 ^{ab}	4.81	203.79	18.83
p-value	0.0365		0.6562	

* Treatments differ from Control by Dunnet's test (P-value<0.05)
^{abc} Different superscript letters indicate significant difference with Tukey test in the same column. n= 6 animals/ treatment; 2 animals/ pen
DSS: Dextran sodium sulfate; NSP: non-starch polyssacharide

Table 7. Lipocalin concentration in the serum of broilers submitted to different intestinal challenges at 14, 22 and 28 days of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge.

Treatment	14 days Lipocalin (ng/ml)	±S.E.M.	22 days Lipocalin (ng/ml)	±S.E.M.	28 days Lipocalin (ng/ml)	±S.E.M.
Control	85.60	3.66	90.06	7.83	50.21	6.28
25DSS	95.49	3.39	93.53	8.58	53.85	3.99
35DSS	86.67	4.70	94.85	7.83	48.8	5.46
NSP	86.86	2.97	84.72	7.83	60.36	4.64
p-value	0.2463		0.8061		0.4188	

n= 6 animals/ treatment; 2 animals/ pen
DSS: Dextran sodium sulfate; NSP: non-starch polyssacharide

Table 8. Biomarker concentration on excreta of broilers submitted to different intestinal challenges at 28 and 36 days of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge.

Collection day	Treatment	Calprotectin (ng/g)	±S.E.M.	Lipocalin (ng/g)	±S.E.M.	S100A (ng/g)	±S.E.M.	HIF-1α (pg/g)	±S.E.M.
28 Days	Control	5168.6 ^{b}	238	3557.44^{b}	264.8	118.7	5.49	1195.2	53.6
	25DSS	5705.1 ^{ab}	246.9	3383.49^{b}	186.1	103.9	3.6	1158.3	38.72
	35DSS	6460.7 ^{a*}	88.53	3693.66^{ab}	253.5	108.1	8.9	1063.9	104.6
	NSP	5986.8 ^{ab}	413.7	4521.49^{a*}	96.9	93.9	7.98	990.5	91.86
	P-value	0.0395		0.0075		0.1281		0.28	
36 Days		Calprotectin	±S.E.M.	Lipocalin (ng/g)	±S.E.M.	S100A (ng/g)	±S.E.M.	HIF-1α (pg/g)	±S.E.M.
	Control	6176.4	488.3	1039.6	133.6	166.39	17.67	143.3	28.15
	25DSS	6321.7	725.4	1252.2	131.2	181.4	22.36	244.7	20.73
	35DSS	6171.7	485.6	1156	154.3	160.6	16.32	249.6	24.93
	NSP	6365.7	480.4	1360.6	141.7	135.7	8.64	225	39.6
	P-value	0.992		0.4364		0.3174		0.0627	

*Treatments differ from Control by Dunnet's test (P-value<0.05)
^{abc} Different superscript letters indicate significant difference with Tukey test in the same column.
n= 6 animals/ treatment; 2 animals/ pen
DSS: Dextran sodium sulfate; NSP: non-starch polyssacharide

4. DISCUSSION

4.1 Inflammation model: DSS and NSP

The present data revealed that the intestinal mucosal reaction (i.e. inflammation) to stressors followed a spatial and temporal pattern in broilers, from the duodenum to the ileum from 14 to 36 days post-hatch (Figure 5). The histological assessment indicated that the duodenum and jejunum were affected earlier than the ileum. Thus, it seems that the lower part of the gut was affected only after the upper gut was compromised in terms of intestinal health (Figures 2 to 4). Duodenum and jejunum had signs of injury at the first necropsy (14-d). However, indications of acute inflammation, such as the presence of inflammatory cells in the lamina propria and epithelium were observed in the ileum from day 22 and were maintained until the end of the experiment in 35DSS and NSP groups. Evidence of a higher upper gut sensitivity has already been described in broilers exposed to heat stress with greater histo-morphological and morphometric

alteration observed in the duodenum and jejunum (38). Therefore, the stronger inflammatory response of the upper gut might be not related to the stressor source, since it happened with NSP rich diet, chemical (DSS) and even heat challenge. However, the spatial characteristics of intestinal inflammation verified in the present study contrasted with findings in the murine model. Previous research showed that the distal colon of mice is more affected by DSS challenge than its proximal colon (Shastri (39-42).

Overall, NSP exhibited a more consistent pattern of inflammation during the trial although it was milder when compared with the DSS-treated birds (Figures 2 to 3). NSP negatively affected intestinal health, as seen by the significantly higher macroscopic ISI score as well as the microscopic lesion scores (Table 5 and Figures 2 to 3).

DSS challenge promoted higher levels of micro-ISI scores in the duodenum than control ($P<0.001$) throughout the entire experiment (Figure 2). And at the last day of sampling (36-d), the 35DSS group presented the worst (highest) ISI scores throughout the small intestine. The higher ISI scores were mainly due to the high inflammatory cell infiltration in the lamina propria and epithelium. DSS groups showed a consistent increase in the number of goblet cells through the end of the trial (28 and 36-d). The DSS model proposed in this paper was adapted to chickens based on a mice model for chronic inflammation (35). These authors observed that mice recovered faster from the first DSS cycle (9-d) but, after the second round of chemical oral gavage 9-d was not enough to have a full recovery from the inflammation caused by DSS. Our study demonstrated that broilers exposed to a similar protocol presented a major intestinal inflammation in the second DSS cycle as was observed in the murine model (35). However, for broilers, 9 days was not sufficient for full recovery from the first challenge cycle. Since chickens appear to be more sensitive to oral DSS (15), the current observation is not surprising. Moreover, in mice the inflammatory response after the second recovery period is different from the response after de first recovery. After two cycles of DSS and the full recover period, mice presented a chronic inflammation identified by the infiltration of mononuclear cells, and imbalance of pro and anti-inflammatory response. Although in the present study, we did observe a prolonged inflammatory response in the three intestinal segments evaluated even after the second recovery, we did not categorize and quantify the inflammatory cells and cytokines present in the intestine at each time point.

In the current experiment, all the challenged groups presented a high level of enterocyte proliferation and inflammatory cell infiltration in the epithelium of the small intestine at 28 days. Sanches and colleagues (8) observed that during inflammation the avian intestine responds to the lesions in the lamina propria mostly with a strong proliferation of enterocytes that further stimulates the inflammatory response since these new enterocytes are not mature. They also described a microscopic proliferative enteritis in broilers with *Eimeria spp*. and *Clostridium perfringens*, confirming this pattern.

Furthermore, the current data suggest that histologic methodologies are superior to the evaluation of gross lesions for small intestine inflammation in research settings. Other than its precision and ability to provide details, a histologic evaluation may be crucial to detect Microscopic Enteritis (ME). Human pathology uses ME as a classification for an inflammatory condition of the small intestine which presents microscopic or sub-microscopic abnormalities but not gross lesions (43). The histological observations in ME include preserved villous structure, crypt hyperplasia (deeper crypt), infiltration of small lymphocytes in the epithelium, sub-microscopic abnormalities, increase in plasma cells and inflammatory cells in the lamina propria also may be present. Even if the lesions appear minor, ME is associated with gastrointestinal symptoms, and malabsorption (44). Microscopic enteritis was already identified in broilers through microscopic ISI evaluation (8), and Belote et al. (33) have shown that the increase of inflammatory cells in the epithelium has a negative impact on animal performance by reducing growth performance.

4.2 Biomarkers

The poultry industry is constantly looking for tools to measure gut health in order to evaluate flocks and decide when to use feed additives or medications. Moreover, these tools should be: (1) able to provide a rapid answer to allow intervention during the production period; (2) minimally invasive; and (3) easy to collect and store the required samples. Several ways to identify gut inflammation and/or gut health are available for research settings; however, most require tissue sampling and euthanasia of animals. Thus, blood and excreta were chosen in the current experiment to fulfill the 2nd and 3rd requirements. Moreover, ELISA assays were selected for their relative simplicity in methodology, low requirement in lab equipment, quick generation of results, high specificity and easy commercial access. It is believed that the relevant findings from this

research will facilitate further research in different labs including widespread industry application. ELISA assays offer an additional advantage over molecular methods (i.e. mRNA gene expression) in the sense that the targeted biomarker refers to finally expressed (protein) levels, which is not the case in the latter.

From the biomarkers evaluated in the current experiment, calprotectin was observed to be the most promising. Calprotectin, also known as S100A8/S100A9 heterocomplex, is a protein involved in the innate immune response to infection. It activates pro-inflammatory signaling pathways and has chemokine and bacteriostatic activity (45-48). Avian and reptile species express a protein homologue to calprotectin, named MRP126. This protein has already been found in abundance in chicken heterophils and in lower quantities in macrophages (49, 50). Recently, the chicken MRP126 has been biophysically described by Bozzi and Nolan with important observation of its metal binding (Ca^{+2}-dependent Zn^{+2} sequestration) and antimicrobial property activities (51). Moreover, recombinant chicken MRP126 had been shown to stimulate chicken TLR4 to activate NF-κB (52). Therefore, it appears that the cytokine-like and bacteriostatic activity of MRP126 protein was maintained during evolution. A previous study has shown that neutrophils release calprotectin with activation or death and monocytes release the protein after adhesion (53). Thus, calprotectin can be detected in fluids of inflamed tissue, such as serum, urine, cerebrospinal fluid and feces (54). Sekelova and collaborators (50) observed that the abundance of MRP126 protein increase in avian macrophages after intravenous infection with *Salmonella* Enteritidis. Therefore, the increased calprotectin from blood and feces found in the current study might be correlated to the immune response of the challenged groups. Although fluctuations were observed throughout the experimental period, calprotectin was increased only right after the DSS cycles. Two factors can play a role in this finding: first, calprotectin is more abundant in heterophils in blood, and second, the number of animals sampled may not be sufficient to detect small variations in the concentration of proteins after the recovery period. Heterophils are the first immune cells to respond and migrate to a site of infection (55) and are found in great quantity in acute inflammation. However, with the evolution of acute to chronic inflammation more mononuclear cells are identified with macrophages being the predominant cell type in chronically inflamed tissues (56). Thus, the change in cell type predominance at the moment of the sampling may have influenced the calprotectin concentration, suggesting the protein as an indicator of more robust

inflammation. Calprotectin may be a promising biomarker for inflammation in broiler measured both in serum and excreta.

The ideal biomarker should also detect inflammation consistently in different models, such as physiological and nutritional models (13); therefore, in the current study, we also analyzed biomarkers previously described in the literature such as LPS, ovotransferrin and lipocalin-2. Lipocalin 2 is an acute phase protein highly produced by the liver (57) and neutrophils, although the majority of LCN2 found in the intestine is produced by epithelial cells which mostly secrete the protein into the lumen (58). Similar to calprotectin, lipocalin inhibits bacterial growth by sequestering nutrients from the bacteria, such as iron (57), and it also is involved in apoptosis (59). Chassaing and collaborators (60) reported that fecal LCN-2 is a sensitive marker capable of monitoring low-grade (sub-clinical) and robust intestinal inflammation in mice since the protein showed responsiveness to mucosal healing and high stability in the feces. The authors observed an increase in fecal LCN-2 after challenging mice with different dosages of DSS in the drinking water, and when the challenge was discontinued, LCN-2 dramatically decreased its concentration. However, they observed that lipocalin concentration in the serum was less responsive to the DSS challenge, being a better detector of robust intestinal inflammation (caused by high doses of DSS). Similarly, these current results show that LCN-2 appears to be a better marker of low-grade inflammation in broilers in the feces than in the serum. In the present trial, broilers fed for 28 days with the NSP showed higher fecal lipocalin at 28 days, compared to the control group. However, with the healing of the upper gut after the first recovery period, LCN-2 returned to basal levels, indicating LCN-2 as a potential fecal biomarker for the health status of upper gut segments. Previously, it has been reported that fecal lipocalin in chickens (13) had a tendency (P=0.086) to increase in a dexamethasone model of intestinal inflammation. However, the lipocalin differences probably was less proeminent due to the control treatment used which had a diet with high inclusion of wheat (65.2%). This diet may have negatively impacted the gut due to its high soluble fiber content promoting inflammation and reducing the differences between control and other treatments in the study. Therefore, the current data sustain previous findings in the literature and supports the value of lipocalin as a biomarker for intestinal inflammation in chickens.

The LPS present in the serum was quantified to detect bacterial translocation from the intestinal lumen to blood, thus evaluating intestinal barrier function. However, no difference between challenge groups and control was found at 14, 28 or 36 days. The measuring of serum

LPS in chickens is controversial in the literature and it seems to vary according to the intensity of the challenge to which birds are exposed (61-63). Ovotransferrin, an acute phase protein mainly produced during inflammation, has been suggested as a fecal biomarker of intestinal inflammation due to its increase in excreta of broilers facing necrotic enteritis and coccidiosis (64), or diet with 52% of rye (13). However, in our study, no increase of fecal ovotransferrin was detected in the groups suffering intestinal inflammation, which might occur due to the sub-clinical inflammation observed in the current study compared to the acute and more robust inflammation produced by the other models in the literature.

Lastly, HIF-1α is a transcription factor that regulates genes involved in inflammation and cell death (65). HIF-1α has been shown to be an important hypoxia-elicited barrier protection through nonclassical barrier function (non-tight junctions related) (66). Moreover, the HIF-1α pathway can be activated by NF-kB, thus initiating inflammation and vice versa (66, 67). Gene expression of HIF-1α has been measured in broilers facing intestinal inflammation due to heat stress and an up-regulation of the HIF-1α gene was detected in the ileum, but not jejunum (68). Our results followed a similar response, since the HIF-1α concentration presents a tendency to be increased in the feces of broilers with lower ileum health status (P=0.0627). Likewise, He and collaborators (69) only observed intestinal upregulation of HIF-1α expression after a chronic heat stress exposure (for 14 days), but not after 7 days of thermal challenge. Although HIF-1α levels need to be further quantified in the excreta of broilers, the HIF-1α seems to be a potential fecal biomarker for chronic inflammation or ileal health status. Further experiments are underway to confirm the use of HIF-1α as a potential inflammatory marker.

The ideal biomarker has been described with several characteristics: minimally invasive, specific, precocious, sensitive, robust, consistent among different challenge models and responsive to tissue healing (13, 60, 70). However, an important point that is usually missed is the practicality of sampling and processing, and the time to produce results. Although, authors advocate that fecal biomarkers should be more accurate in determining gastrointestinal inflammation and gut barrier (71), our data demonstrated that serum biomarker (calprotectin) presented bigger differences (135%) compared to fecal biomarkers (CALP and LCN-2) (~25% increase). A biomarker with a broader range would permit a better distinction among good gut health, low-grade and robust inflammation. The difference between the biomarkers response in serum and feces can be physiological, due to age and may be associated with the difficulty of fecal processing, variation,

and representativeness. The presence of proteases in the intestinal content can diminish some proteins with biomarker potential even before the excretion. Moreover, fecal samples are vulnerable to protein degradation and usually are flash frozen right after sampling in liquid nitrogen and stored at -80ºC (13, 64). This methodology might impair successful collections in the field. In our experiment, we observed easy handling and processing of the blood samples, minimal equipment needed, combined with the advantage of monitoring individual birds at the same time on necropsy days. Therefore, to benefit the research performed in industry settings, there should be a focus not solely on feces but also on blood biomarkers, as well as to further evaluate biomarkers stability in the samples throughout different experimental situations.

5. CONCLUSION

Both NSP diet and two cycles of 0.35mg/ml of DSS models produced an inflammatory response in the intestine without compromising other organs or producing clinical signs. Therefore, this study presents feasible models of low-grade chronic inflammation although the judgment between the models should be made based on the time response desired and the practicality. The high NSP diet is a nutritional model which is easy to replicate and should be used if an early intestinal inflammation is preferred (up to 28d). On the other hand, cyclic DSS challenges is a chemical model which continues producing inflammation later in the broiler's life (up to 36d). Interestingly, the current study demonstrated that the chicken intestinal inflammation evolves in a spatial and temporal pattern, shown by the lower small intestine being affected later than the upper parts. After a challenge, the duodenum and jejunum were affected earlier, followed by the ileum which was compromised only after 4-wk of the life span of the chicken. Moreover, this physiological response might not vary with the stressor's characteristic since it was consistent between a nutritional-continuous challenge and a cyclic-chemical model.

Lastly, we described promising biomarkers for low-grade intestinal inflammation in the current paper such as calprotectin, lipocalin and HIF-1α. Calprotectin, a novel biomarker for chickens, was successfully detected in response to inflammation from serum and feces, while HIF-1α and lipocalin may be potential fecal markers. To the best of our knowledge, this is the first time that calprotectin has been described as a biomarker of intestinal health in broiler chickens. Even more promising, is the fact that it can be easily analyzed in the blood and be correlated with

intestinal health, which may reduce the variation between birds, be less time-consuming, and reduce the risk of degradation during sample processing.

SUPPLEMENTAL DATA

Development of a Feed-Induced Chronic Intestinal Inflammation Model and Identification of Inflammatory Biomarkers for Gut Inflammation

Macroscopic I See Inside methodology

Table 1. Characteristics evaluated and their impact factors (IF) used for calculation of the macroscopic I See Inside (ISI). Each characteristic was scored based in the extend of the lesion being: (0) absence of lesion, (1) up to 25% of the organ affected, (2) 25-50% of the organ affected, (3) more than 50% of the organ affected. Then scores were then multiplied by their respective impact factors and the sum of all score*IF of an animal resulted in its macroscopic ISI.

Organ	Characteristic evaluated	Impact factor
Liver	Red color (congestion)	2
	Yellow and hypertrophic	2
	Yellow and hypotrophic	3
Yolk	Persistance	1
Proventriculus	Inflammation	1
Gizzard	Erosion	1
Pancreas	Hypertrophic	2
	Hypotrophic	2
Duodenum	Inflammation process at serosa or mucosa layer	1
	Cell debris and thick mucus at mucosa	2
	Eimeria acervulina lesion (0-4)	2
Jejunum	Necrosis	3
	Inflammation process at serosa or mucosa layer	1
	Cell debris and thick mucus at mucosa	2
	Decrease muscular tonus (thin intestine wall)	2
	Eimeria maxima lesion (0-4)	3
Ileum	Inflammation process at serosa or mucosa layer	1
	Cell debris and thick mucus at mucosa	2
	Undigested food and/or gas presence	1
Ceacum	Inflammation process at mucosa	2
	Gas presence	1
	Eimeria tenella lesion (0-4)	2

Table 2. Broiler mortality (%) in the entire experimental period and feed intake (FI) of 1 to 21 days and 1 to 36 days of broilers submitted to different intestinal challenges. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge.

Treatment	FI (kg) 1-21 days	FI (kg) 1-36 days	Mortality (%) 1-36 days
Control	1.037	2.442	0
25DSS	1.094	2.573	2.22
35DSS	1.049	2.422	2.22
NSP	1.019	2.312	2.22
SEM[1]	0.04337	0.10545	-
P-value	0.6663	0.4269	0.7904

[1] Pooled standard error of the mean

Table 3. Detailed histologic alteration on duodenum, jejunum and ileum of broilers submitted to different intestinal challenges at 14 of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge.

	Treatment	Lamina propria thickness	Epithelial thickness	Proliferation of enterocytes	Inflammatory cell epithelium	Inflammatory cell lamina propria	Increase of goblet cells	Congestion
Duodenum	**Control**	0.93^{ab}	1.08^{ab}	0.67	0.43^{b}	0.27^{b}	1.30^{b}	0.17
	25DSS	1.27^{a}	1.22^{a}	0.53	0.87^{a}	0.80^{a}	2.17^{a}	0.20
	35DSS	1.20^{a}	0.95^{b}	0.57	0.80^{a}	0.57^{a}	1.60^{b}	0.43
	NSP	0.77^{b}	0.83^{b}	0.43	0.87^{a}	0.87^{a}	0.87^{c}	0.47
Jejunum	**Control**	0.80	0.93	0.56^{d}	0.53^{b}	0.50	1.50	0.36^{a}
	25DSS	1.36	1.20	0.96^{bc}	0.90^{a}	0.76	1.90	0.46^{a}
	35DSS	1.26	1.13	0.85^{c}	0.86^{a}	0.76	1.73	0.46^{a}
	NSP	1.13	1.26	1.08^{ab}	0.91^{a}	0.80	1.63	0.06^{b}
Ileum	**Control**	0.60^{b}	0.80	0.60	0.53	0.23	1.27	0
	25DSS	1.00^{ab}	0.72	0.65	0.73	0.70	0.77	0
	35DSS	1.23^{a}	0.92	0.83	0.77	0.50	1.20	0
	NSP	1.43^{a}	0.95	0.70	0.78	0.77	0.80	0
Duodenum	SEM[1]	0.152	0.096	0.079	0.082	0.128	0.169	0.118
	P-value	0.0156	0.0279	0.2066	0.0002	0.0077	<.0001	0.1789
Jejunum	SEM[1]	0.159	0.089	0.081	0.078	0.132	0.197	0.101
	P-value	0.1005	0.0609	<.0001	0.0008	0.3497	0.4998	0.0159
Ileum	SEM[1]	0.171	0.089	0.084	0.085	0.144	0.164	0
	P-value	0.0045	0.2196	0.285	0.2438	0.0731	0.0561	1

[abc] Different superscript letters indicate significant difference with Tukey test in the same column.

[1] Pooled standard error of the mean

Table 4. Detailed histologic alteration on duodenum, jejunum and ileum of broilers submitted to different intestinal challenges at 22 of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge.

	Treatment	Lamina propria thickness	Epithelial thickness	Proliferation of enterocytes	Inflammatory cell epithelium	Inflammatory cell lamina propria	Increase of goblet cells	Congestion
Duodenum	**Control**	0.67	0.78	0.60	0.42^{b}	0.37	0.73^{b}	0.23
	25DSS	0.80	1.00	0.68	0.65^{a}	0.67	1.13^{b}	0.23
	35DSS	0.87	1.10	0.73	0.70^{a}	0.73	1.53^{a}	0.23
	NSP	0.93	1.10	0.85	0.73^{a}	0.60	0.97^{b}	0.07
Jejunum	**Control**	0.77^{b}	0.77^{c}	0.50^{b}	0.87^{c}	0.50^{b}	1.07^{c}	0.00^{b}
	25DSS	1.37^{a}	1.65^{a}	1.23^{a}	1.95^{a}	1.67^{a}	2.90^{a}	0.47^{a}
	35DSS	0.80^{b}	1.28^{b}	1.02^{a}	1.32^{b}	0.73^{b}	2.47^{ab}	0.20^{a}
	NSP	0.80^{b}	1.45^{ab}	1.07^{a}	1.58^{b}	0.67^{b}	2.07^{b}	0.20^{a}
Ileum	**Control**	1.10^{b}	1.32^{a}	0.87	1.08	0.73^{b}	1.47^{a}	0.23
	25DSS	1.47^{b}	1.25^{a}	1.02	1.22	1.00^{b}	1.77^{a}	0.27
	35DSS	2.40^{a}	0.82^{b}	0.80	1.07	2.20^{a}	0.83^{b}	0.10
	NSP	2.30^{a}	1.05^{b}	0.97	1.13	2.10^{a}	0.73^{b}	0.17
Duodenum	SEM[1]	0.148	0.085	0.082	0.088	0.135	0.160	0.080
	P-value	0.6825	0.0221	0.1159	0.0343	0.3141	0.0027	0.3369
Jejunum	SEM[1]	0.148	0.106	0.092	0.094	0.143	0.210	0.100
	P-value	0.0182	<.0001	<.0001	<.0001	<.0001	<.0001	0.0232
Ileum	SEM[1]	0.161	0.094	0.090	0.086	0.182	0.196	0.080
	P-value	<.0001	0.0008	0.2619	0.6845	<.0001	0.0011	0.4596

[abc] Different superscript letters indicate significant difference with Tukey test in the same column.

[1] Pooled standard error of the mean

Table 5. Detailed histologic alteration on duodenum, jejunum and ileum of broilers submitted to different intestinal challenges at 28 of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge.

	Treatment	Lamina propria thickness	Epithelial thickness	Proliferation of enterocytes	Inflammatory cell epithelium	Inflammatory cell lamina propria	Increase of goblet cells	Congestion
Duodenum	**Control**	0.53^{b}	0.62^{c}	0.42^{c}	0.62^{b}	0.27^{c}	0.73^{c}	0.47
	25DSS	1.07^{a}	1.30^{a}	1.03^{a}	1.18^{a}	0.77^{ab}	1.93^{a}	0.77
	35DSS	1.27^{a}	1.25^{a}	0.93^{ab}	1.12^{a}	1.07^{a}	1.87^{a}	0.60
	NSP	0.67^{b}	0.95^{b}	0.72^{bc}	1.13^{a}	0.63^{b}	1.23^{b}	0.63
Jejunum	**Control**	0.53^{b}	0.42^{b}	0.35^{b}	0.52^{b}	0.47^{b}	0.47^{c}	0.37^{b}
	25DSS	1.13^{a}	1.20^{a}	1.00^{a}	1.22^{a}	1.03^{a}	1.97^{a}	0.83^{a}
	35DSS	0.77^{ab}	1.12^{a}	0.90^{a}	1.25^{a}	0.83^{ab}	1.40^{b}	0.13^{b}
	NSP	1.07^{a}	0.92^{a}	0.80^{a}	1.12^{a}	0.70^{ab}	1.00^{b}	0.37^{b}
Ileum	**Control**	1.03^{c}	0.62^{b}	0.53^{b}	0.37^{b}	0.40^{b}	0.73	0.10
	25DSS	1.97^{ab}	1.12^{a}	0.95^{a}	1.23^{a}	1.47^{a}	1.13	0.10
	35DSS	2.27^{a}	1.12^{a}	1.10^{a}	1.12^{a}	1.17^{a}	0.87	0.17
	NSP	1.87^{b}	1.02^{a}	0.97^{a}	1.15^{a}	1.40^{a}	0.77	0.00
Duodenum	SEM[1]	0.141	0.105	0.100	0.104	0.131	0.182	0.141
	P-value	0.0007	<.0001	<.0001	0.0002	0.0007	<.0001	0.4055
Jejunum	SEM[1]	0.134	0.098	0.097	0.097	0.136	0.190	0.117
	P-value	0.0035	<.0001	<.0001	<.0001	0.0178	<.0001	0.0021
Ileum	SEM[1]	0.147	0.081	0.083	0.079	0.149	0.148	0.059
	P-value	<.0001	<.0001	<.0001	<.0001	<.0001	0.3023	0.1485

[abc] Different superscript letters indicate significant difference with Tukey test in the same column.

[1] Pooled standard error of the mean

Table 6. Detailed histologic alteration on duodenum, jejunum and ileum of broilers submitted to different intestinal challenges at 36 of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage everyday from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge.

	Treatments	Lamina propria thickness	Epithelial thickness	Proliferation of enterocytes	Inflammatory cell epithelium	Inflammatory cell lamina propria	Increase of goblet cells	Congestion
Duodenum	**Control**	0.73	1.17	0.58	1.02^{b}	0.63^{b}	1.67^{b}	0.30^{b}
	25DSS	0.97	1.20	0.87	1.18^{ab}	0.90^{b}	2.33^{a}	0.87^{a}
	35DSS	1.23	1.17	0.83	1.45^{a}	1.30^{a}	2.17^{ab}	0.37^{b}
	NSP	1.10	1.02	0.78	1.05^{b}	0.90^{b}	1.57^{b}	0.27^{b}
Jejunum	**Control**	1.00^{b}	0.52^{c}	0.38^{c}	0.80^{c}	1.17^{a}	0.63^{b}	0.30
	25DSS	0.87^{b}	0.87^{b}	0.65^{b}	1.15^{ab}	0.87^{b}	1.03^{b}	0.63
	35DSS	1.63^{a}	1.17^{a}	0.93^{a}	1.30^{a}	1.60^{a}	1.63^{a}	0.33
	NSP	1.00^{b}	0.80^{b}	0.70^{ab}	1.03^{b}	0.60^{b}	0.60^{b}	0.30
Ileum	**Control**	1.03^{b}	0.83^{b}	0.65	0.75^{b}	0.57^{c}	0.97^{b}	0.10
	25DSS	1.57^{b}	0.72^{b}	0.75	0.92^{b}	1.10^{b}	0.63^{b}	0.07
	35DSS	2.17^{a}	1.02^{a}	0.85	1.20^{a}	1.77^{a}	1.47^{a}	0.30
	NSP	1.43^{b}	1.18^{a}	0.98	1.20^{a}	0.90^{b}	0.93^{b}	0.10
Duodenum	SEM[1]	0.152	0.106	0.099	0.096	0.151	0.191	0.119
	P-value	0.0822	0.5756	0.2545	0.0159	0.0068	0.0205	0.0022
Jejunum	SEM[1]	0.142	0.097	0.089	0.085	0.148	0.168	0.110
	P-value	0.0010	0.0001	0.0003	0.0006	0.0001	<.0001	0.1336
Ileum	SEM[1]	0.171	0.100	0.096	0.083	0.173	0.172	0.070
	P-value	0.0003	0.0042	0.0535	0.0001	0.0001	0.0049	0.1136

[abc] Different superscript letters indicate significant difference with Tukey test in the same column.

[1] Pooled standard error of the mean

Table 7. Serum liposaccharide (LPS) of broilers submitted to different intestinal challenges at 14, 28 and 36 days of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage every daily from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge.

Treatment	14 days LPS (ng/ml)	±S.E.M.	28 days LPS (ng/ml)	±S.E.M.	36 days LPS (ng/ml)	±S.E.M.
Control	130.83	7.45	133.94	12.45	99.29	11.64
25DSS	136.25	8.59	84.31	7.50	86.20	23.91
35DSS	115.48	19.80	100.83	23.83	70.98	11.79
NSP	103.6	11.64	101.22	24.56	65.47	14.39
p-value	0.3199		0.3602		0.4149	

Table 8. Concentration of Fecal ovotransferrin of broilers submitted to different intestinal challenges at 14 and 22 days of age. The broilers challenged with DSS received 0.25mg/ml (25DSS) or 0.35mg/ml (35DSS) of DSS via oral gavage daily from 9 to 14-d and 23 to 27-d; birds in the NSP treatment received a diet with 30% of rice bran during the whole experiment, and animals in the control group were not submitted to any challenge.

Treatment	28 days Ovotransferrin (pg/g)	±S.E.M.
Control	1008.76	86.948
25DSS	961.93	97.211
35DSS	948.89	86.948
NSP	1001.38	86.948
p-value	0.9526	

CHAPTER 4:

Field evaluation of feeding spray-dried plasma in the starter period on final performance and overall health of broilers

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ABSTRACT

Feeding spray-dried plasma (SDP) during the starter period was evaluated with a commercial broiler integrator on performance and overall health of broilers. The I See Inside methodology (ISI®) assessing gut health in broilers was used as a tool to evaluate the impact of dietary interventions under commercial conditions. 100 farms with approximately 1.1 million broilers were used at a Brazilian broiler integrator. Two groups of farms were fed either a Control or an SDP diet containing 1% SDP, from 0 to 10 d of age. Diets were formulated to have similar nutritional density, contained zinc bacitracin and CuSO4 from 0 to 28 d. After 10 d, both groups were fed common commercial diets. Performance data were analyzed together or by type of ventilation system: positive pressured (PP) or negative pressured (NP). Birds were sent to market as they reached 3.05 kg; therefore, age at slaughter (AS) was evaluated as a dependent variable along with other performance measures. From the 100 farms in the trial, 35 (16 Control and 19 SDP farms) were selected for the assessment of broilers health, biosecurity, and local management. For that, 6 broilers per farm at 14±2 d of age were necropsied and ileum sampling for the ISI methodology evaluation. Biosecurity and management were also evaluated to obtain the influence of those parameters on animal health. SDP-fed birds improved FCR, reduced mortality and 1 d less for AS ($P<0.05$) vs Control group ($P<0.05$) regardless of type of ventilation. During the necropsy, birds fed SDP showed lower coccidiosis and locomotor system lesion as overall ISI score compared to Controls. Histologic intestinal alterations were also lower in SDP-fed broilers ($P<0.05$). In conclusion, feeding 1% SDP in the starter period to broilers resulted in improved performance and health under both good and bad management and biosecurity standards independent of type of ventilation. Overall, there was good agreement between ISI® method and performance improvements observed.

Key words: ISI methodology, histology, gut, management, biosecurity.

1. INTRODUCTION

Spray-dried plasma (SDP) is a highly digestible protein ingredient rich in functional molecules, manufactured from animal blood collected from federally-inspected slaughter facilities, and spray-dried to preserve the functionality of its components (Coffey and Cromwell, 2001). The SDP is a diverse mixture of functional molecules consisting of immunoglobulins, albumin, fibrinogen, lipids, growth factors, enzymes, hormones and other components that have biologic activity when orally-fed independently of their nutritional value (Campbell et al., 2008).

The high digestibility of SDP allows fast amino acids absorption and lower protein fermentation in the gut, being able to improve the efficiency of the immune response, consequently restoring immune system homeostasis and maintaining the structural integrity of the intestinal barrier and stabilize immune system activation. (Humphrey and Klasing, 2004; Campbell el al., 2019). Other studies have presented similar effects in broilers when added in the starter diets improving health and performance (Campbell et al., 2003); (Campbell et al., 2006); (Henn et al., 2013).

While its bioactive components have shown to modulate the immune response of the host providing improvements in body weight daily gain, feed efficiency and animal survival in pig starter diets and calf milk replacers (Torrallardona et al., 2003); (Bosi et al., 2004); (Pierce et al., 2005). Although several studies (Pérez-Bosque et al., 2010; Maijó et al., 2012; Song et al., 2015, Pérez-Bosque et al., 2016) have reported modulation of the immune response in animals supplemented with SDP in the feed in different challenges and housing, it has not been determined which component in SDP elicits immunomodulation and reduced inflammation to improve well-being of the animal. However, the diverse mixture of bioactive proteins or peptides are likely contributing to these beneficial effects (Campbell et al 2019).

Campbell et al. (2019) reviewed the current understanding of the mode of action of SDP in the context of the published literature available in poultry and suggested that its immune modulatory effects are pivotal to understand SDP's functional properties. Inflammation is reduced by feeding SDP when induced either by stress or by pathogenic challenges, and regardless of whether the primary site affected is the gastrointestinal, respiratory or the reproductive tracts. SDP reduces gut permeability and improves nutrient uptake and structural integrity during periods of stress (Pérez-Bosque et al, 2006). All these

effects are likely and partially mediated by a reduction in the expression of pro-inflammatory cytokines, and by an elevation in the expression of anti-inflammatory cytokines. Data showing a reduction in lymphocyte activation and infiltration, lessening of edema, and changes in the gut microbiota, have also been discussed by Campbell, et al (2019), suggesting SDP could be improving intestinal health through multiple modes of action including either directly influencing the immune inflammatory response and immunomodulation both locally and systemically according to these authors. All these changes may contribute to an improved overall health and performance in chickens and may explain why the beneficial effects of the SDP seem to be more pronounced in animals under challenging conditions vs those with no challenges (Campbell et al., 2008).

Under commercial broiler conditions, the presence of environmental challenges, pathogens, mycotoxins, heat or cold stress, antinutritional factors, high stocking density, etc., could compromise the intestinal barrier resulting in immune system activation, reduction of intestinal function (i.e., nutrient absorption), and lower performance (Johnson, 1997); (Spurlock et al., 1997).

The I See Inside (ISI®) methodology has been used in broilers to assess overall health, especially gut health by translating macroscopic and microscopic tissue alterations into numeric data. Due to the possible systemically effect of the SDP product, was applied the ISI macroscopic to evaluate all the animal systems. Different from villi height and crypt depth measurement, the ISI® histology encompasses components of the inflammatory process such as immune cell infiltration and proliferative response at the villi (Kraieski et al., 2016); (Belote et al., 2018a); (Belote et al., 2019a). Thus, the use of ISI in the field may be a management tool to evaluate the effect of SDP and other diet changes in commercial production.

It was hypothesized that adding SDP in starter broiler diets could improve overall performance at processing age in commercial broilers; and that the ISI® methodology could be a management tool in the field to assess the impact of SDP on gut health.

2. MATERIALS AND METHODS

2.1 Animals, Experimental Design and Housing

The study was conducted in a broiler integrator company in the State of São Paulo, Brazil. A total of 100 farms were used totaling 1,101,100 broilers housed under commercial conditions; thus, each farm was considered an epidemiological unit. The experiment followed a 2 X 2 factorial design (Table 1), considering the starter diets with or without 1% SDP and two different house ventilation systems described as negative pressure (NP) or positive pressure (PP). The NP ventilation system used herein creates a partial vacuum and pulls air into the house evenly through all inlets, improving temperature uniformity within the barn. This system operates through fans and temperature sensors, while the barn walls have plastic canvas closed all the time. The PP ventilation system is composed of sidewalls and ridge vents that allow the external condition to control the inner environment. Fans are installed to regulate the intern temperature of barn and air renewal to adjust the humidity, while the barn walls have plastic canvas opened by morning and closed by night depending on the weather and desired barn temperature.

Table 1. Description of number of farms under type of ventilation system in different groups

Type of ventilation	SDP in the feed		Total
	Yes	No	
Negative Pressure (NP)	4	13	17
Positive Pressure (PP)	50	33	83
Total	54	46	100

Cobb 500 Slow Feather broilers were raised in agreement with the commercial management and sanitary practices in place at the integrator. Peanut shell, pine, rice husk or wood shavings were used as litter material. Automatic feeders and nipple drinkers were used, and the birds were housed at density between 10 and 12 birds/m².

2.2 Diet

The standard dietary program used consisted of 5 dietary phases with estimated intakes of: 350, 700, 800, 2000 and 1700 g/bird for the pre-starter, starter, grower, finisher 1 and finisher 2 diets, respectively. Treatments consisted of starter experimental diets formulated with or without 1% SDP (AP920, APC Brazil, São Paulo, Brazil) and offered from 0 to 10 d of age. The SPD and control formulations were isonutritional to avoid effects

between treatments, and it being reformulated according to the nutritional profile of the ingredients received weekly. The nutrient profile of SDP was accounted for in formulation (Table 2). Nutrient levels were formulated to meet or exceed the bird's requirements (Cobb manual, 2018). The raw matter was analyzed weekly, and the analyzes of the feed were monitored following the method described on AOAC (2000) and if there was a nutritional deviation above 10% the feeds were reprocessed. However, in the experimental period, no nutritional deviation was identified.

Subsequent dietary phases were common diets fed to the two groups until slaughter age. All diets contained zinc bacitracin added as a growth promoter and 200 ppm of CuSO4 from 0 to 28 d.

Table 2. Description of the ingredients and calculated nutritional composition of diets.

	Starter	
Ingredients (Kg/ton)	**SDP**	**Control**
Corn	568.69	554.66
Soybean oil	21.14	26.00
Soybean meal	326.29	344.29
Meat meal	52.00	52.57
SDP	10.000	0.000
Sodium choride	4.199	4.286
Sodium Sulphate	1.171	1.714
Limestone	2.714	2.000
Methionin MHA Liq	2.342	2.571
L-Lysine 78,0%	2.128	2.400
DL-Methionine 98,0%	1.428	1.429
L-Threonine 98,0%	0.557	0.800
Choline 75% liquide	0.914	0.857
Premix**	6.701	6.701
Total	**1000**	**1000**
Crude protein (%)	22.50	22.50
Crude fat (%)	5.04	5.49
Crude fiber (%)	2.85	2.91
Moisture (%)	11.39	11.34

**PREMIX: Premix mineral vitamin (4.737 g/ton); zinc bacitracin as growth promoter (15% /370 g/ton); mycotoxin adsorbent antioxidant (1.000 g/ton); phytase 500FTU (0.065 g/ton), copper (II) sulfate (CuSO4) (35% / 0.800 g/ton).

[1] Composition of minimum (per kg): Vit A 8,818,342 IU; Vit D3 3,086,420 IU; Vit E 3,674 IU; Vit B12 130 mg; Vit K 1,177mg; Vit B2 4,775 mg; pantothenic acid 16,168 mg; Vit B1 2,350 mg; Vit B3 36,742 mg; Vit B6 5,732 mg; folic acid 1.1,398 mg; choline 104,460 mg; biotin 441mg. Minimum of Fe 12%; Cu 1.4%; I 800ppm; Zn 12%; Mn 173.0 mg; Mg 12%

2.3 Performance

The following zootechnical parameters were evaluated: feed intake (FI), average daily gain (ADG), feed conversion ratio (FCR), feed conversion ratio adjusted to 2.80 kg (FCRA), mortality (MT), age at slaughter (AS) and weight at processing (WP). As birds were sent to the market when achieving the desired weight (3.05 kg), the age at slaughter (AS) was evaluated as a dependent variable along with other performance measures in 100 farms.

2.4 I See Inside methodology

Briefly, the I See Inside (ISI®) is a health evaluation methodology based on the quantification of selected pathologic parameters present in a pre-determined list of organs and/or tissues. This methodology was first described by Kraieski et al. (2017) whereby a pre-defined and fixed impact factor (IF) value, ranging from 1 to 3, is attributed to each macroscopic (necropsy) and microscopic (histology) parameter evaluated in each organ or tissue. The IFs values reflect the level of impairment that each alteration causes to the selected organ or tissue´s functionality based on previous knowledge from the literature and research. For example, the necrosis has an IF of 3 since that alteration causes complete loss of tissue functionality. In addition, during the evaluation, the observer assigns a score value (S) ranging from 0 to 3 to describe the extent of the observed alteration (score 0 = no alteration; score 1 = alteration reaches up to 25% of extension or intensity in relation to the normal organ; score 2 = alteration reaches up to 50% of extension or intensity in relation to the normal organ and score 3 = alteration is greater than 50% of extension or intensity relative to the normal organ). The final provided value is the ISI® Total Score which is obtained by the formula ISI® = $\sum$ (IF * S), where the IF is multiplied by the assigned score and the values obtained from each parameter are summed up. The ISI® results are presented as mean scores, with a higher value indicating greater alterations observed. For example, necrosis has an IF = 3. If the observer assigns the maximum alteration score (S = 3) to this parameter, then the final score for necrosis alteration will be 9 (IF * S or 3 * 3 = 9).

A total of 35 farms (16 Controls and 19 SDP) randomly selected from the 100 farms previously mentioned and representing 526,011 broilers were used. However, the farm was considered the experimental unit and not the birds.

This farm sub-group were stratified in a random sampling model by region and date of housing. A total of 6 birds per farm aged 14±2 d was sampled and euthanized by cervical dislocation (210 birds in total) and used for both macroscopic and microscopic evaluations.

This value was based on the study by Johnson and Reid (1970), where they report that a total of 5 to10 birds from each house would be reasonable for macroscopic evaluation, with at least 5 birds being able to evaluate gross lesions per experimental unit. The birds that were euthanized must represent the uniformity of the flock. Therefore, if the flock has over 80% uniformity, per example, from 6 birds that were selected, 4 birds should be on average, one bird above average and one bird below average.

2.4.1 ISI Macroscopic evaluation (necropsy)

After euthanasia, the carcass was systematically evaluated to identify overall health status of the broiler. The ISI macroscopic is divided by systems as locomotor, respiratory, gastrointestinal organs, intestine and coccidiosis lesions. Each one of these systems were evaluated using parameters, described on table 3. The final ISI score is calculated as sum of all parameters evaluated. Therefore, highest the final ISI total score, worse is the intestinal health of the animal. This system can also be assessed by a software in www.isiinstitute.com.

Table 3 – ISI® macroscopic parameters applied in the necropsy

System								
Locomotor		Respiratory		Gastrointestinal		Intestinal		Coccidiosis
Organ	**Parameter**	**Organ**	**Parameter**	**Organ**	**Parameter**	**Organ**	**Parameter**	**Parameter**
	Pododermatitis	Trachea	Tracheitis	Oral cavity	Oral mucosa lesion	Duodenum	Inflammation process at serose or mucosa	*Eimeria acervulina*
	Foot pat pigmentation						Cell debris and thick mucus at mucosa	
				Liver	Reddish (Congestion)		Necrosis	
	Femoral head necrosis				Yellowish	Jejunum	Inflammation process at serose or mucosa	*Eimeria maxima*
	Tibial dyscondroplasia	Heart	Hydropericardium	Proventriculus	Proventriculitis		Cell debris and thick mucus at mucosa	
External and locomotor	Bone resistence			Gizzard	Erosion		Deacrease muscular tonus	
				Pancreas	Hypertrophic	Ileum	Inflammation process at serose or mucosa	*Eimeria maxima*
	Muscle hemorrhage				Hypotrophic		Cell debris and thick mucus at mucosa	
		Air Sacs	Airsacculitis	Yolk	Persistence		Undigested food and/or gas presence	
				Kidney	Hypertrophic	Cecum	Inflammation process at serose or mucosa	*Eimeria tenella*
							Gas presence	

2.4.2 ISI Microscopic evaluation (histology)

For the histological evaluation, samples of ileum were collected and immediately 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 h. Samples were then 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 cell staining (Rapp and Wurster, 1978).

For histologic ileal evaluation, one slide per bird containing 20 intestinal villi were analyzed in 10X magnification (using 20X and 40X magnification to confirm alterations) under an optical microscope (Nikon Eclipse E200, Sao Paulo-SP- Brazil).

The ISI histology parameters evaluated are lamina propria thickness, epithelial thickness, enterocytes proliferation, inflammatory cell infiltration in the epithelium, inflammatory cell infiltration in the lamina propria, goblet cells proliferation, congestion, presence of *Eimeria sp.* Oocysts. The final ISI score is calculated as sum of all parameters evaluated. Therefore, highest the final ISI total score worse is the intestinal health of the animal (Kraieski et al., 2017; Belote et al., 2018 and Belote et al., 2019)

2.5 Biosecurity and Management

Biosecurity and local management practices were evaluated to obtain the influence of those parameters on animal health. During each visit, the 35 farms used for the ISI® evaluation, farms were classified according to their biosecurity standards as good or bad based on the following observations: presence of a farm gate, a functional vehicle disinfection system at the farm´s gate, farm´s appearance (organized and clean), presence of appropriate fencing, and presence of barn seals. If more than half of these items were in place, biosecurity was regarded as good or considered bad. Management practices were also evaluated and regarded as good or bad based on the following observations: quality of drinking water (clarity/ water flow (ml/1minute/presence of leaks)), nipple height, ambience (temperature, ventilation), feed's physical appearance, feeder height and litter quality (compaction and moisture). If more than a half of this parameters were regarded as acceptable, the farm was considered to have a good management, or considered bad.

2.6 Statistical Analysis

Data on age at slaughter (AS), average daily gain (ADG), weight at processing (WP), feed conversion ratio (FCR), feed conversion ratio adjusted to 2.80 kg (FCRA) and mortality (MT) were provided by the broiler integrator. A complete descriptive statistic of all variables in general was included in supplementary material (mean, median, standard deviation, minimum, maximum and skewness calculation). Mean, median, standard deviation and interquartile range by group are presented in tables 4 and 5. The Shapiro-Wilk test was performed to evaluate the adherence of data to a normal distribution, which revealed that all the six performance variables tested were asymmetric to normal distribution (p-values in supplementary material). Because of this, a mean test (T Test, for example) cannot be used to compare groups and the Mann-Whitney U test (nonparametric approach) was used to verify differences between farms fed with or without SDP for each variable separated by type of housing. All performance parameters were analyzed using SPSS® 20.0 (IBM, 2011) software.

The Shapiro-Wilk normality test was used for the macroscopic, microscopic (histology), biosecurity, management, and factorial analyses. Parametric data were subjected to analysis of variance (ANOVA) and Tukey's test was used to establish differences among means. Conversely, the Kruskal-Wallis test at 5% probability was used for nonparametric data. All data were analyzed using the statistical software Statistix 9. Statistical significance was considered for $P<0.05$ and tendency for $P<0.10$.

3. RESULTS

The results of the interaction between the diet and performance are presented in clusters by type of ventilation in Tables 4 and 5. Independent of the ventilation system, feeding SDP reduced the AS by one day compared to the untreated group of farms ($P <0.05$; Table 4) and have a tendency to reduce FCR ($P = 0.077$; Table 4). Farms with NP presented better results of FCR than PP farms, showing a reduction of 0.07 points (mean) in farms that received SDP, and 0.08 points (mean) in control farms among NP and PP, respectively (Table 5).

Feeding SDP in the starter diet yielded improvements in FCRA and MT at the end of the production cycle in both NP and PP farms ($P<0.05$) vs the Control birds so that a

reduction of 0.08 points (mean) in FCRA and 0.8% in overall MT were observed when broilers were raised in NP farms; and lower FCRA by 0.05 points (mean) and overall MT by 0.5% were measured when housed in PP farms.

Table 4. Effect of adding Spray-Dried Plasma at 1% in the starter diet from 0 to 10 d of age on overall performance

All farms	**SDP**			**Control**			**P value**
	M	**MD**	**SD**	**M**	**MD**	**SD**	
AS (d)	53	**53 b**	2	54	**54 a**	2	**0.016**
WP (g/bird)	3081	3100	209	3115	3090	136	0.614
ADG (g/bird/d)	58.9	58.7	3.2	58.7	58.3	2.5	0.810
FCRA (g/g)	1.922	1.910	0.124	1.944	1.940	0.107	0.163
FCR (g/g)	2.002	1.978	0.102	2.033	2.050	0.093	0.077
MT (%)	3.8	3.7	1.2	4.1	3.7	1.0	0.174

M=mean; MD=median; SD=standard deviation; AS=age at slaughter; ADG=average daily gain; WP=weight at processing; FCR=feed conversion ratio; FCRA=feed conversion ratio adjusted to 2.8kg; MT=mortality

Table 5. Effect of adding Spray-Dried Plasma at 1% in the starter diet from 0 to 10 d of age on overall performance in birds raised in farms with different ventilation systems

Type of ventilation	**SDP**			**Control**			**P value**
	M	**MD**	**SD**	**M**	**MD**	**SD**	
Negative pressure farms							
AS (d)	52	**52 b**	0	53	**53 a**	1	**0.023**
WP (g/bird)	3240	3240	92	3130	3180	191	0.296
ADG (g/bird/d)	62.3	62.3	1.7	60.2	60.9	1.8	0.202
FCRA (g/g)	1.805	**1.805 b**	0.075	1.883	**1.900 a**	0.061	**0.023**
FCR (g/g)	1.932	1.932	0.054	1.977	1.994	0.058	0.130
MT (%)	2.5	**2.5 b**	0.6	3.4	**3.3 a**	0.4	**0.045**
Positive pressure farms							
AS (d)	53	**53 b**	2	54	**54 a**	2	**0.033**
WP (g/bird)	3068	3090	211	3078	3070	84	0.812
ADG (g/bird/d)	58.6	58.7	3.1	57.5	57.2	1.9	0.172
FCRA (g/g)	1.932	**1.930 b**	0.123	1.981	**2.020 a**	0.111	**0.042**
FCR (g/g)	2.008	**1.984 b**	0.103	2.059	**2.102 a**	0.101	**0.013**
MT (%)	3.9	**3.9 b**	1.2	4.5	**4.4 a**	1.1	**0.018**

M=mean; MD=median; SD=standard deviation; AS=age at slaughter; ADG=average daily gain; WP=weight at processing; FCR=feed conversion ratio; FCRA=feed conversion ratio adjusted to 2.8kg; MT=mortality

The results of the ISI® macroscopic evaluation (Figure 1) indicate that farms fed SDP had ($P < 0.05$) 45% less alterations in the locomotor systems, 70% less coccidiosis associate lesion in gut, and a reduction of 28% in the ISI® Total Score. The other parameters were not significantly different.

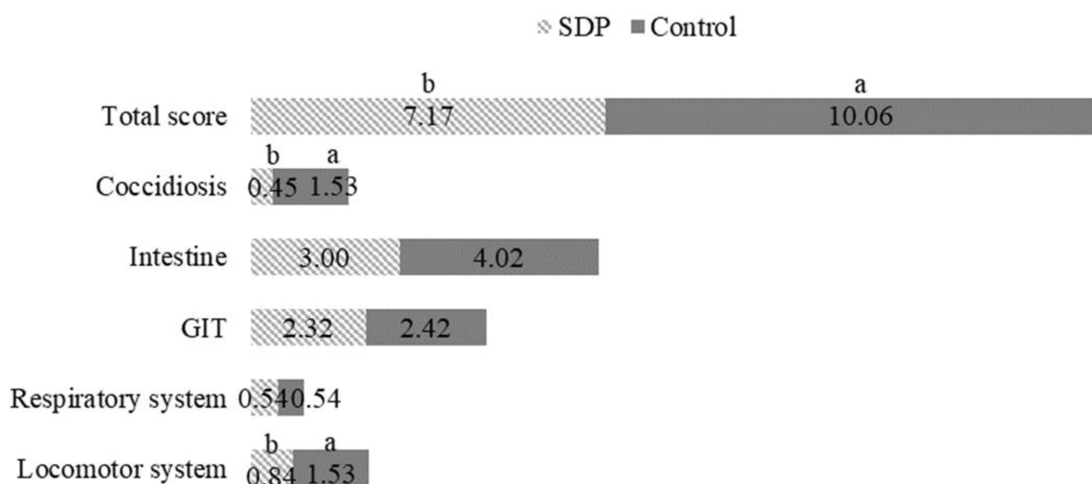

Figure 1. Necropsy findings in broilers raised in farms fed Spray-Dried Plasma (SDP) or a Control starter diet by the ISI® Methodology described in Table 3. [a,b] Different letters in the same column indicate a significant difference ($P < 0.05$).

Higher ($P<0.05$) ISI® values for intestinal scores and coccidia scores during necropsy were observed in birds housed under bad vs good biosecurity conditions, and in birds fed Control diets vs SDP (Figure 2). It was observed that birds under bad biosecurity fed with SDP, showed same ($P<0.05$) results compared with birds of control group under good biosecurity. While birds under good biosecurity and fed with SDP, presented better ($P<0.05$) results compared with birds of control group under bad biosecurity.

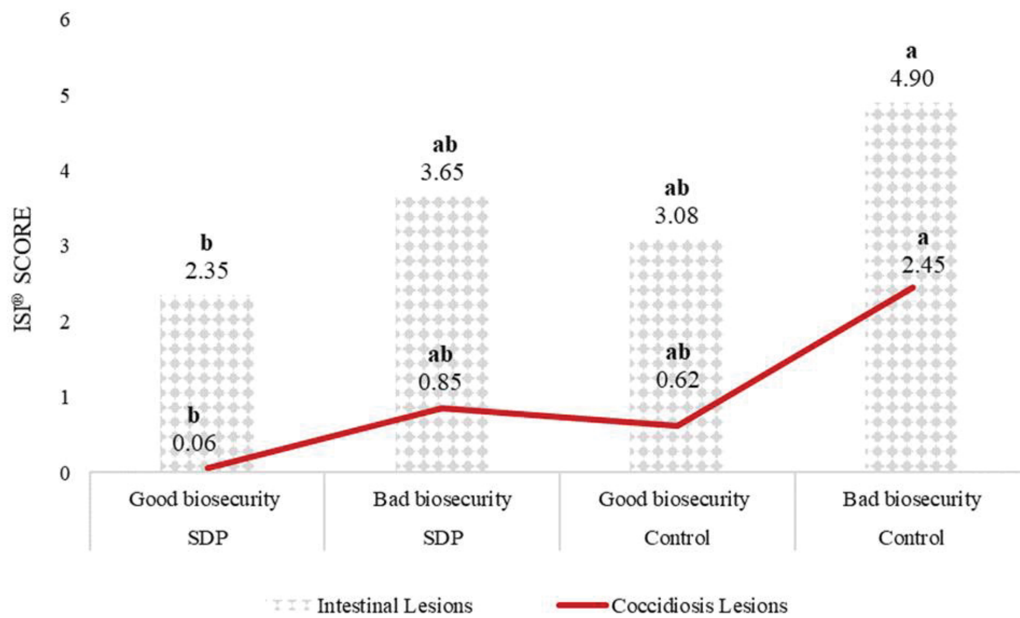

Figure 2. ISI® Scores observed during necropsy relative to intestinal lesions and coccidiosis lesions in broilers fed a starter diet with or without Spray-Dried Plasma (SDP). and housed in farms with good or bad biosecurity standards. [a,b] Different letters in the same column indicate a significant difference ($P < 0.05$) in ISI® Scores as described in Table 3.

The ileal histologic evaluation pointed out less pathologic alterations in birds fed SDP in comparison to those fed a control starter diet. The product promoted lower scores of lamina propria thickness, inflammatory cell infiltration in the epithelium and lamina propria, and congestion ($P<0.05$). Collectively, the reduced scores in these parameters resulted in the lower ISI® total score recorded in SDP-treated bird, even thought, the product has caused increased score of goblet cell proliferation in these animals (Figures 3 to 5).

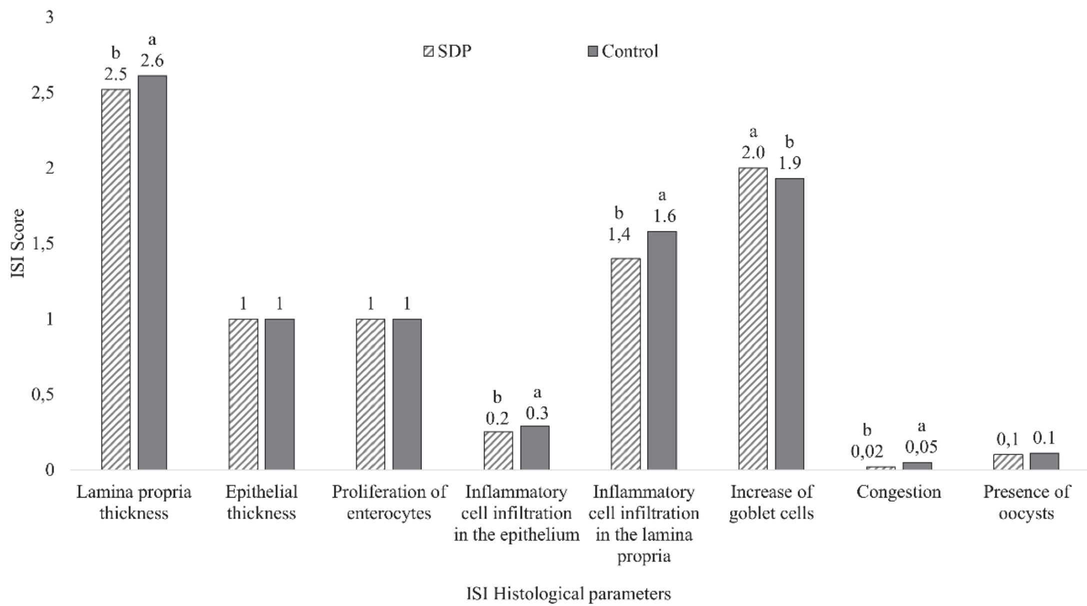

Figure 3. Parameters of ISI methodology of ileum evaluation between control farms and SDP fed farms. [a.b] Different subscript letters indicate significant difference (P<0.01).

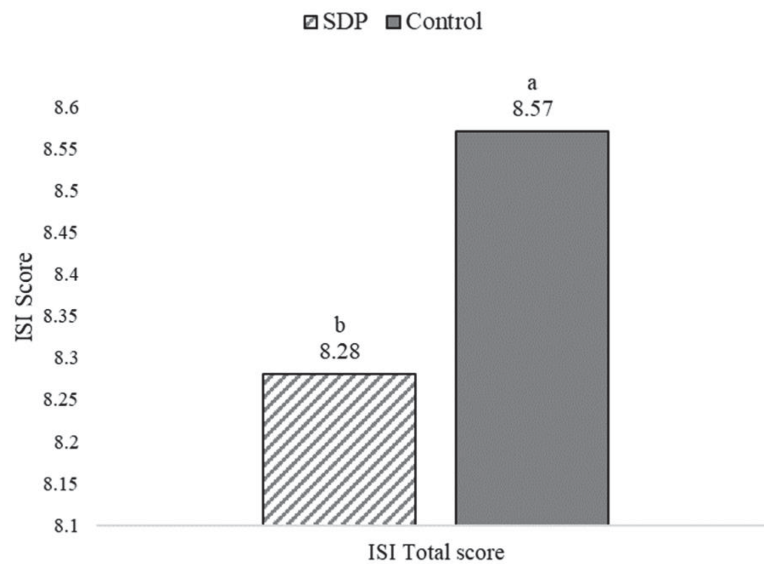

Figure 4. ISI® Total Score (Σ(IF*S)) of ileum evaluation between control farms and SDP fed farms. [a.b] Different subscript letters indicate significant difference (P<0.01)

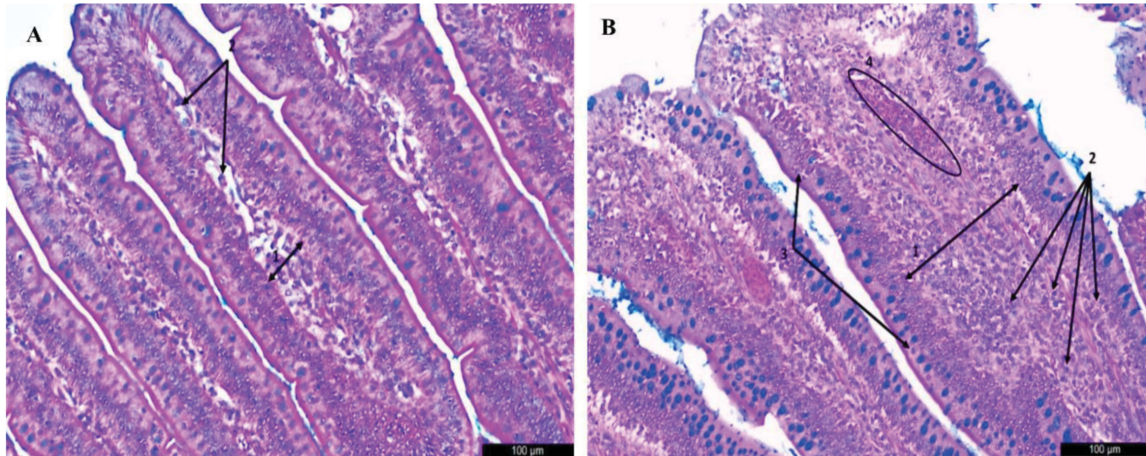

Figure 5. Photomicrography of ileum sections of broilers stained with hematoxylin and eosin. Alcian Blue was used to stain goblet cells. A) 1- Low ISI score of lamina propria thickness; 2- low ISI score of inflammatory cells infiltration in the lamina propria in the farms fed with SDP (200X); B) 1- High ISI score of lamina propria thickness; 2- High ISI score of inflammatory cells infiltration in the lamina propria; 3- shows inflammatory cells infiltration in the epithelium; 4 - High ISI score of congestion in the birds of control group (200X).

A factorial analysis of ileal histology (Table 6) showed that birds fed SDP had lower total pathologic alterations independently of whether biosecurity measures were classified as good or bad; thus, higher ISI® scores for lamina propria thickness, inflammatory cell infiltration in the epithelium and the lamina propria, greater congestion, but lower goblet cells, were observed in birds fed a starter diet without SDP (P<0.05). Birds from farms under bad biosecurity showed greater total scores and presence of oocysts vs those under good biosecurity, and lower congestion was scored in this group when fed a control diet. The birds with the lowest (P<0.05) total scores were fed SDP and kept under good biosecurity measures.

The interaction between SDP feeding and farm management on ileal histologic alteration scores is detailed in Table 7. Feeding SDP lowered total ISI® scores independently of whether the birds were under good or bad management practices (P<0.05). Feeding SDP lowered lamina propria thickness, inflammatory cell infiltration in both epithelium and lamina propria, and congestion (P<0.05) but increased goblet cell scores. Farms classified as having bad management showed lower total ISI® scores, lamina propria thickness and presence of oocysts particularly when SDP was fed.

Data analysis exploring the three-way interaction among SDP feeding, biosecurity and management practices (Table 8) for ileal histologic alteration scores shows that feeding SDP lowered scores of inflammatory cell infiltration in the lamina propria, independently of having good or bad management and biosecurity. The lowest ISI® total scores were observed in birds fed SDP and housed under bad management and biosecurity compared with other combinations without SDP in the diet.

Table 6. Ileal histologic ISI® scores as affected by feeding Spray-Dried Plasma (SDP). and biosecurity standards at the farm in broilers.

SDP in feed	Biosecurity Standards	Lamina propria thickness	Epithelial thickness	Proliferation of enterocytes	inflammatory cell infiltration in the epithelium	inflammatory cell infiltration in Lamina propria	Increase of goblet cells	Congestion	Presence of oocysts	Total score
Yes	Good	2.47 b	1.00	1.00	0.23 b	1.27 c	1.99 a	0.02 b	0.00 b	8.00 c
Yes	Bad	2.49 b	0.99	0.99	0.24 ab	1.45 b	2.00 a	0.01 b	0.13 a	8.35 b
Control	Good	2.68 a	0.98	0.98	0.29 a	1.64 a	1.85 b	0.09 a	0.00 b	8.54 ab
Control	Bad	2.80 a	1.01	1.01	0.28 ab	1.67 a	1.85 b	0.008 b	0.17 a	8.82 a
P value		**0.05**	**0.09**	**0.09**	**0.05**	**0.05**	**0.05**	**<0.001**	**0.05**	**<0.001**

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

Table 7. Ileal histologic ISI® scores as affected by feeding Spray-Dried Plasma (SDP). and management conditions at the farm in broilers.

SDP in feed	Management	Lamina propria thickness	Epithelial thickness	Proliferation of enterocytes	inflammatory cell infiltration in the epithelium	inflammatory cell infiltration in the Lamina propria	Increase of goblet cells	Congestion	Presence of oocysts	Total score
Yes	Good	2.60 b	0.99	0.99	0.25 ab	1.41 b	2.01 a	0.01 b	0.13 a	8.43 b
Yes	Bad	2.36 c	1.00	1.00	0.22 b	1.31 b	1.98 a	0.02 ab	0.00 b	7.92 c
Control	Good	2.83 a	0.98	0.98	0.30 a	1.74 a	1.85 b	0.05 ab	0.06 ab	8.82 a
Control	Bad	2.64 ab	1.01	1.01	0.28 ab	1.57 a	1.86 b	0.06 a	0.10 ab	8.54 ab
P value		**0.05**	**0.41**	**0.41**	**0.05**	**0.05**	**0.05**	**0.05**	**0.006**	**<0.001**

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

Table 8. Ileal histologic ISI® scores as affected by feeding Spray-Dried Plasma (SDP), biosecurity and management conditions at the farm in broilers.

SDP in feed	Biosecurity	Management	Lamina propria thickness	Epithelial thickness	Proliferation of enterocytes	inflammatory cell infiltration on the epithelium	inflammatory cell infiltration in the amina propria	Increase of goblet cells	Congestion	Presence of oocysts	Total score
Yes	Good	Good	2.56 cd	1.00 ab	1.00	0.21	1.20 d	2.02 a	0.01	0.00b	8.04 bc
Yes	Good	Bad	2.37 d	1.00 ab	1.00	0.24	1.34 cd	1.95 b	0.04	0.00b	7.96 c
Yes	Bad	Good	2.63 bc	0.98 ab	0.98	0.29	1.62 ab	1.99 ab	0.01	0.27 a	8.81 a
Yes	Bad	Bad	2.35 d	1.01 ab	1.01	0.20	1.28 cd	2.01 ab	0.00	0.00b	7.88 c
Control	Good	Good	2.52 cd	0.96 b	0.96	0.30	1.61 ab	2.00 ab	0.10	0.00b	8.47 ab
Control	Good	Bad	2.84 ab	1.00 ab	1.00	0.29	1.67 ab	1.71 c	0.09	0.00 b	8.62 a
Control	Bad	Good	3.15 a	1.00 ab	1.00	0.30	1.88 a	1.70 c	0.00	0.12 ab	9.16 a
Control	Bad	Bad	2.45 cd	1.02 a	1.02	0.26	1.47 bc	2.01 ab	0.01	0.21 a	8.47 a
	P Value		**0.00**	**0.05**	**0.26**	**0.24**	**0.00**	**0.00**	**0.16**	**0.006**	**0.00**

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

4. DISCUSSION

The current trial reports data from approximately 1.1 million broilers housed in 100 commercial farms located in the same region, housed at similar time, and fed a commercial starter diet with (54 farms) or without (46 farms) 1% SDP. To the best of our knowledge this is the largest field broiler trial evaluating the effect of feeding SDP in the starter diet on commercial performance and health, along with the first evaluation of the ISI® Scoring System used and validated in a large commercial broiler flock. This large number of farms (farm as experimental unit) allowed us to test the effect of SDP and to use the ISI® Scores, under a wide variety of situations such as different housing systems, good or bad management and/or biosecurity measures.

Nutritional strategies that support the immune system, promote intestinal integrity and functionality, and increase tolerance to stress and disease challenges are of interest to the poultry industry. Additionally, early nutrition has been recognized as an opportunity to further advance nutritional practices and improve overall performance and health status of commercial broiler flocks (Noy and Uni, 2010). In the current trial, 1% SDP was fed the first 10 d of life while performance benefits and a reduction in mortality were reported at the end of the production cycle at 53 and 54 d of age (Tables 4 and 5). Total intake of SDP per bird in those 10 d was calculated to be approximately 3 g per broiler.

The performance of broilers was better in NP vs PP farms indicating that NP provided more favorable conditions for growth rather than PP, and that feeding 1% SDP improved performance of birds housed in both types of ventilation at about the same magnitude (Tables 4 and 5). Feeding SDP to broilers in the first few days of life has improved performance such as weight gain and feed efficiency at the end of the production cycle in healthy flocks (Beski et al., 2015; 2016a). Walters et al (2019) fed 0% or 2% SDP to coccidia-vaccinated broilers for 10 d of life, and 0 or 50 ppm of bacitracin methylene disalicylate (BMD) from 0 to 41 d of age in a factorial 2 x 2 design. These authors reported that birds improved performance at 41 d when fed either BMD or SDP. Furthermore, the birds fed BMD improved performance when SDP was offered suggesting that the effect of SDP was additive and independent of BMD. In the current experiment, no coccidia vaccine was used but all diets contained zinc bacitracin as growth promoter and improvements in response to feeding SDP were observed

in growth as evidenced by the reduction in age at slaughter by 1 d while maintaining similar BW (Table 5) and by an improvement in feed efficiency of about 4.5 and 5% for birds under NP and PP barns, respectively.

Campbell et al. (2008) suggested that the benefits of feeding SDP could be more pronounced when fed to birds with higher pathogen exposure however, in the current study the performance improvements and reductions in morality attributed to feeding SDP were similar in PP and NP farms vs Controls. Likewise, significantly less pathologic alterations were recorded in birds fed SDP vs a Control diet after a holistic necropsy evaluation (Table 3) particularly in those associated with coccidiosis and the locomotor system ($P<0.05$; Figure 1). However, greater reductions in macroscopic intestinal lesions associated or not to coccidiosis were found in farms with bad vs good biosecurity standards in response to SDP (Figure 2). For locomotor system, the parameter that show a less frequency of alterations was bone resistance, presenting 9.6% of normal tissue (score 0), 7.1% less mild lesions (score 1), 1.6 % less of moderate lesions (score 2) and showed no serious lesion (score 3) compared to control group. Similarly with this report, Jiang et al (2000) found an increase bone density in swine, when feeding SDP.

The improvement of locomotor systems could demonstrate the importance of intestinal health in relation to the absorption of nutrients that act on bone structure and maintenance. Beyond the possibility of systemic effect related by Campbell et al (2019).

Histologic scoring of ileal samples indicates an overall reduction in alterations in observations related to inflammation such as inflammatory cell infiltration, congestion, and lamina propria thickness suggesting a healthier gut in birds fed SDP (Figure 3). During the inflammation process, innate immune cells (macrophages, granulocytes, dendritic cells) are found diffusely in the epithelium and lamina propria (Keestra et al., 2013; Kogut et al., 2018). The increase of the lamina propria thickness and the inflammatory cells infiltration was described by Belote et al. (2018) as good parameters to compare intestinal health between different treatments. These parameters have a strong correlation with zootechnical performance, which means the higher pontuation in these parameters, the worse animal's performance results.

The coordinate activation of inflammation is part of the non-specific immune response that can occurs in reaction to any type of bodily damage caused by several factors

(Ferrero-Miliani, et al, 2007). The signs of inflammation are characterized by congestion due to increased blood flow and vasodilatation, cells recruited from the blood and inflammatory mediator levels in resident tissue cells (Ferrero-Miliani et al, 2007; Chen et al., 2018).

In general, these observations were similar irrespective of the biosecurity and management standards as defined herein (Tables 6 – 8), suggesting that feeding SDP can benefit broilers in a wide variety of conditions. As discussed by Pérez-Bosque et al. (2016) and Campbell et al. (2019) gut health benefits measured as improved barrier function, mucosa permeability and integrity are frequently associated with feeding SDP to animals in many disease and stress experimental models. Likewise, greater resistance to disease has been reported in broilers under a natural outbreak of necrotic enteritis (Campbell et al., 2004a), challenged with *Salmonella sofia* (Beski et al., 2016b), with high mortality where *E coli* and *Streptococcus* were isolated (Gonzalez-Esquerra, et al., 2019), and subjected to heat stress (Ruff et al., 2020), and in turkeys challenged with *Pasteurella multocida* (Campbell et al., 2004b) when fed SDP. Collectively, these observations suggest that feeding SDP causes an unspecific and systemic effect.

Belote et al (2019) observed that the intestinal mucosal alterations scored and analyzed using the ISI® methodology correlate well with the final performance of broilers at 28 d of age. The former findings indicate the importance of gut health at that age for optimal performance later in life. In the current report, the ISI® macroscopic and histologic evaluations were performed in 14±2 d old broilers and both were able to indicate health benefits from feeding SDP in commercial broilers correlating well with performance observations at market age. Likewise, greater ISI® scores were found in farms with bad biosecurity, which is a well-known factor that impacts performance. Those observations indicate that this technique can be a useful tool to uncover factors that can impact early health, and therefore the final performance, of broilers under commercial conditions.

5. CONCLUSION

Feeding SDP in the starter diet reduced mortality and improved growth and feed efficiency at a similar rate in commercial broilers housed in either NP or PP barns, with PP being less favorable to broilers performance than NP. During necropsy procedures, irrespective of having good or bad biosecurity standards, feeding SDP to broilers resulted in

better overall health with reduced coccidia lesions, and other pathologic alterations in small intestine and cecum, and less pathologies in the locomotor system. Additionally, SDP-fed birds under good or bad management practices had lower histopathologic alterations in the ileum. Collectively, these observations suggest that feeding SDP in the first days of life can provide benefits to commercial broilers under a wide variety of circumstances. Additionally, there was good agreement between necropsy and histologic ISI® Scores obtained in commercial broilers and their final performance suggesting this method could be a useful tool to evaluate the impact of nutritional strategies, or other, in the health and performance of broilers under field conditions.

GENERAL CONCLUSION

The ISI method proved to be effective in all situations evaluated at all chapters, as an excellent tool for evaluating the intestinal health of poultry, both for scientific experiments in laboratory structures and for real situations in the field. It is also allowed to make correlation and prediction of animal performance.

FINAL CONSIDERATION

The entire trajectory of the doctorate degree was developed with communication between researchers, industry, and society was an important link to prove our scientific hypotheses. In this way, linking various areas of knowledge, we brought a system for economic evaluation of health in poultry production from the patent that we developed at the UFPR in 2015. Based on all these years of research, we developed the startup ISI Institute, where the author of the thesis is a co-developer and partner of the company, together with her supervisor Elizabeth Santin. Today, we have managed to put into practice the use of a digital tool that monitors of broilers health in several places around the world, in scientific experiments in great universities, research centers and in application in real field situations, and it has been gaining strength every day.

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