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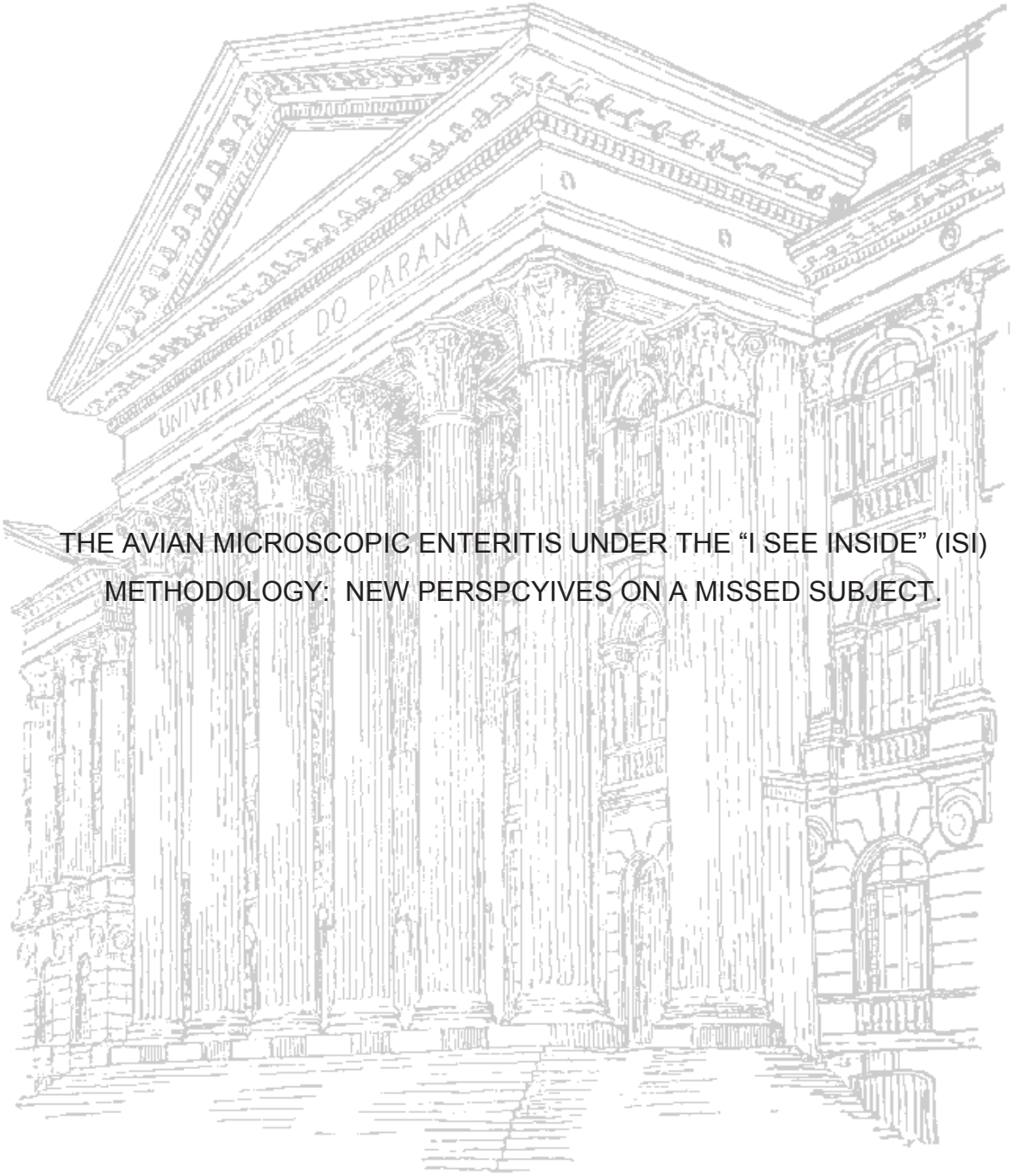
ADRIEN WILHELM DILGER SANCHES



THE AVIAN MICROSCOPIC ENTERITIS UNDER THE “I SEE INSIDE” (ISI) METHODOLOGY: NEW PERSPCYIVES ON A MISSED SUBJECT.

CURITIBA

2019

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THE AVIAN MICROSCOPIC ENTERITIS UNDER THE “I SEE INSIDE” (ISI) METHODOLOGY: NEW PERSPCYIVES ON A MISSED SUBJECT.

Tese apresentada ao Curso de Pós-Graduação em Ciências Veterinárias, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências Veterinárias, na área de concentração em Patologia e Imunologia Aviárias.

Advisor: Profª Drª Elizabeth Santin

Co-advisor: Prof Dr Fabiano Montiani-Ferreira

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TERMO DE APROVAÇÃO

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ELIZABETH SANTIN
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Avaliador Interno (UFPR)

RICARDO MITSUO HAYASHI
Avaliador Externo (BRF/SA)

LARISSA PICKLER
Avaliador Externo (BRF/SA)

JUAN CARLOS DUQUE MORENO
Avaliador Interno (UFPR)

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RESUMO

A resposta intestinal proliferativa inata ocasiona diferentes morfologias nas vilosidades mesmo em animais em ambientes controlados alojados em gaiolas com cama de maravalha de pinus esterilizada e alimentados com ração comercial também esterilizada. Todavia, os métodos correntemente utilizados para descrever a histopatologia intestinal aviária avaliam apenas as medidas lineares de vilosidades e criptas sem considerar o tipo de lesão microscópica.

A metodologia “I See Inside” (ISI) descreve as lesões como parâmetros matemáticos a possui analogias com o sistema de classificação de Marsh usado em medicina para a Enterite Microscópica (EM). O conceito de EM e sua classificação está ligado com a mal absorção de ferro e fosfato e suas diretrizes poderiam ser usadas para explicar os achados microscópicos intestinais e alguma perda de performance em frangos de corte comerciais.

A comparação entre as alterações intestinais em frangos de corte e galinhas ”Feral” demonstrou como o maio ambiente pode alterar o perfil histopatológico entérico. Condições experimentais permitiram descrever a Enterite Microscópica Proliferativa Aviária como possuidora de um porção basal denominada Enterite Basal (EB) e de uma porção inflamatória chamada Enterite Patogênica (EP) sendo ambas formas de enterite microscópica.

Os escores totais dos parâmetros usados nos animais desafiados foram o produto dos escores encontrados na EB adicionados com os parâmetros encontrados na EP adicionados das interações entre as enterites. Removendo-se os escores dos animais não desafiados dos escores dos animais desafiados resultaria no valor real da EB causada pelo ambiente de criação. Adicionalmente, o método ISI demostrou-se flexível o suficiente para ser utilizado em amostras e outras espécies como a bovina.

Palavras chave: bovino, enterite basal, enterite microscópica, ISI, inflamação regenerativa

ABSTRACT

The innate proliferative gut response lead to many different villi morphology, even in experimental negative controlled animals housed in cages with sterilized pine litter and feed with sterilized commercial ration. However, current methods to describe avian intestinal histopathology deals only with linear measures and do not consider the type of microscopic lesions. The “I See Inside” (ISI) methodology can describes the lesions as mathematical parameters and has analogies with the Marsh classification system used in human medicine for the Microscopic Enteritis (ME). ME concept and classification is linked to malabsorption of iron and phosphate and its directives could be used to explain the microscopic findings and some deranged performance data in commercial broilers. The comparison between the tissue changes in the industrial broilers and feral chickens demonstrated how the environment can change the histopathological signature in the intestinal tissue. Experimental conditions allow the “Avian Proliferative Microscopic Enteritis” to be separated in a “Basal Enteritis” (BE) and a “Pathogenic Enteritis” (PE), both forms of ME. The scores of the parameters in challenged birds were the sum of the BE plus the PE added to interactions between them. Removing the scores of the non-challenged birds from the final scores of the challenged expressed the BE values linked to the environment. In addition, the ISI method was demonstrated to be flexible enough to be adapted to another species’ gastro intestinal tissues such as feedlot cattle.

Key words: bovine, basal enteritis, microscopic enteritis, ISI, regenerative inflammation.

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SUMMARY

CHAPTER 1: GENERAL INTRODUCTION

1) Introduction

The current literature in broiler pathology focus mainly in the diagnostic textbook lesions. This concept is the standard routine in the pathology diagnostic centers were the major goal is the name of the disease and/or naming the causative agent. This background is correct but minor lesions that affect the performance of the flocks are neglected and labeled as “lesions without importance”. Located in physiological key sites of the enteric tissues, the sum of all these minor lesions correlated with their physiological impact factors can be linked with some amount of negative performance and health status along the lifespan of the flock. Daily samples from experimental and even backyard chickens units demonstrate the inflammatory and proliferative innate response of the avian intestine, mainly of the epithelial lineage, leading to villi and areas in them that do not contain the traditional well-differentiated enterocyte attached to a healthy basal membrane in a morphological textbook villus. In addition, inflammation in the lamina propria is over expressed in most of the villi.

Given the above, these changes when presented in the same villus lead to a very different morphology not considered in the experiments and evaluations found in the literature. In addition, the data of two previous experiments (KRAISESKY et al., 2016; BELOTE et al., 2018) demonstrated the link between the minor changes and some deranged performance. It is important to mention that these lesions are not diagnostic for any specific etiology and represent the general responses of the tissue facing present environmental challenges.

1.1) The Intestinal Normal Mucosa (avian and mammal)

When observed by light microscopy, the normal villus has an epithelial outer monolayer made of epithelial cells; most are enterocytes disposed in a single layer of elongated and closely attached to each other cells. A mucous layer over the enterocytes can also seen. The central frame of the villi is represented by the Lamina Propria that keeps the shape of the villi and holds the blood vessels, the lymphatic vessels, nerve endings, collagen tissue and smooth muscle (Figure 1A).

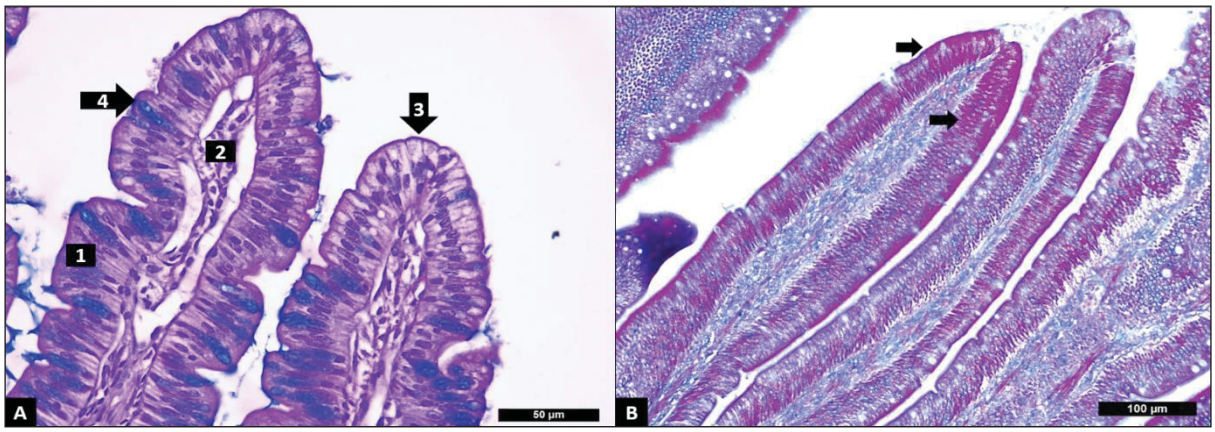
The cytoplasm of the enterocyte and its shape changed while in their migration from the Crypts of Lieberkühn to the tip of the villi (Bacha and Wood, 1990; Nasrin et al, 2012).

On its beginning, the enterocyte had a round or oval shape with scanty cytoplasm and a big nucleus (high nucleus/cytoplasm ratio). As the migration progress, the cytoplasm starts to mature and grow in size and becomes more stained as the cell moves to the tip. Different intensities of eosinophilia can be seen in the cytoplasmic portion over the nucleus and the portion below the nucleus. In some well-matured cells in the top of the villus this difference in the staining cannot be seen, only the contrast between these cells and the cells more side way from the top were noticed. Enzymes presented in the brush border, cytoplasm and nuclei of the well-matured cells are well stained in dark red with the Mallory's Trichrome (Figure 2A) (Cheles, 2004).

Moreover, a strong red stain denotes a well mature cell. This situation was well demonstrated in the top of the villi and in some cells in the mid lateral part of the villus (Figure 1B). Goblet cells had their big vacuoles well stained with Alcian Blue (Figure 1A).

They were positioned between the nucleus and the apical portion of the cell. This stain is very helpful to demonstrate the amount of mucus produced and stored. For them the nuclei is located more basally under the big vacuole retaining the mucous (Stanforth, 2004). Attaching the epithelial cells to the lamina propria, the Basement Membrane is well portrayed as a thick dark blue line when stained with the Mallory's Trichrome (Figure 2B). Under the basement membrane lays the Lamina Propria (Figure 1A). It its composition made of vertical structures of smooth muscle, fibroblasts, collagen, blood vessels and lacteous vessels was easily demonstrated (Cheles, 2004). The Lamina Propria also hold the crypts of Lieberkühn in the bottom of the mucosa surrounded by their own basement membrane.

Figure 1: Ileum, chicken 28 days old. A Parts of a normal villi villi 1- Epithelium, 2- Lamina Propria, 3- Enterocyte and 4-Goblet cell (blue), HE, 400X. B - Enzymatic staining in matured enterocytes at the apex of a villus, Mallory's Trichrome, 200X. Fonte: Source: The author (2019).



The initial formation of the crypts are observed as small multifocal aggregations of epithelial cells inside the lamina propria, below the villi **(**Figure 3A). As their cells multiplied, a lumen could be observed. These niches fused their basement membranes aggregating the epithelial cells and by this creating a new bigger crypt and even a new villus that glowed upward the mucosa. Fusing of crypts with the epithelium of growing villi was observed. Mitotic figure were seen in the crypts cells and alongside the villi (Figure 3B). Since the enzymatic amount of the smooth muscle is high, it dyed in deep red too.

Figure 2: Ileum chicken 28 days old. A-Brush border enzymes stained in red, Mallory's Trichrome 1000X. B- Basement membrane stained (blue) and matured enterocytes (red/orange), Mallory's Trichrome, 100X. Source: The author (2019).

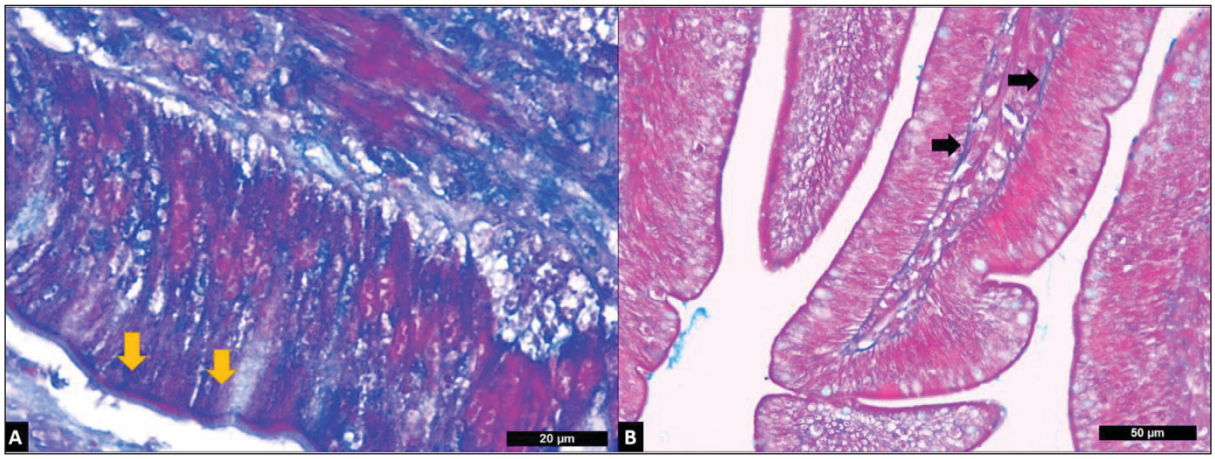


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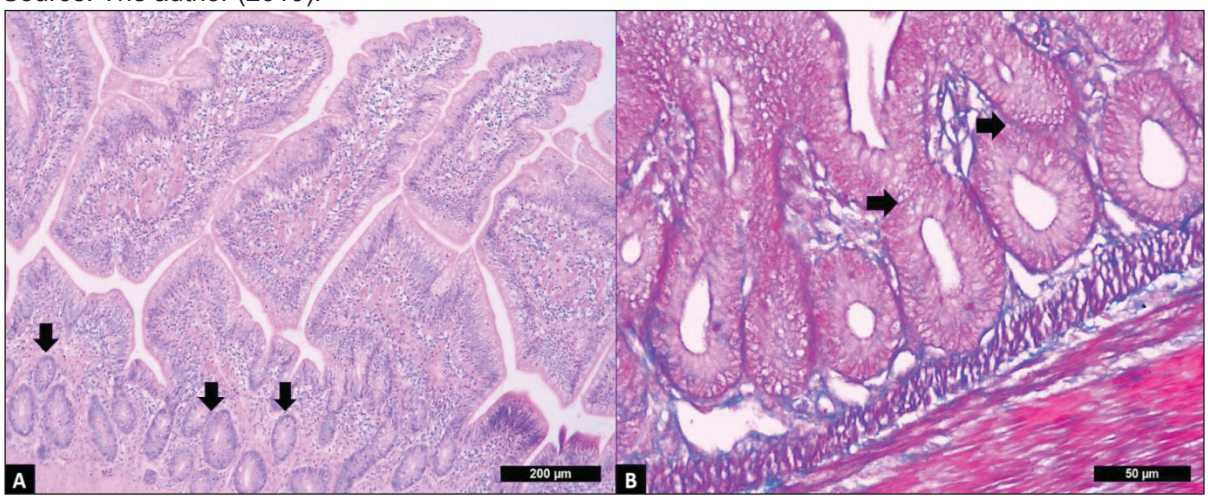
Figure 3: Ileum, chicken 28 days old. A- Formation of crypts seen as multifocal niches of epithelial cells. B-Fusion of basement membranes among crypts and upper epithelium, Mallory's Trichrome, 1000X. Source: The author (2019).



1.2) Current concepts in enteropathology: Microscopic Enteritis, Epithelial Hyperplasia and Intestinal Stem cells.

The occurrence of chronic inflammatory lesions in animals lead to comparisons with some ileocolic conditions in humans, such as **Crohn's disease and Coeliac Disease (**CD**)** (MCORIST,1994). Microscopic inflammatory conditions affecting the human gastrointestinal tract also include eosinophilic/ and lymphocytic esophagitis, gastritis or enteritis, lymphocytic and collagenous gastroenteritis (RUSTAGI and SCHOLES, 2011) and other autoimmune disorders (VERRES, 2004). A group of microscopic lesions had been the subject of study in an attempt to classify the lesions found in human Celiac Disease. The concept of Microscopic Enteritis (ME) has arisen from the mucosal changes associated with CD, as originally described in detail by as categories defined as Marsh 0, Marsh I, Marsh and Marsh III (MARSH, 1992).

Despite some contradictions in this model, it leaded to the first description of the ME as a standard descriptive name or classification to be used in the histological descriptions of human chronic enteropathies (ROSTAMI, 2009). It is considered an emergent diagnostic category first described in 2009 (ROSTAMI and VILLANACCI, 2009).

In humans ME is an early stage of mucosal abnormalities found in several inflammatory conditions. It represents a common finding in patients with intestinal inflammation from different etiologies (SHARBATI et al, 2003) also represents the stage of microscopic and sub-microscopic changes (ultrastructure) associated with the symptoms of micronutrient deficiencies (ROSTAMI, 2009). It may be associated with symptomatic malabsorption, or more subtle micronutrient deficiencies. At the histological level, villous structure is largely preserved, but the epithelium is variably infiltrated by small lymphocytes, and there may be increases in crypt depth (crypt hyperplasia). These mucosal abnormalities may extend to subtle microvillous changes at sub-microscopic level, an increase in plasma cells, eosinophils and other inflammatory cells is likely to be present in the lamina propria. (ROSTAMI et al., 2015). Notably, the constant contact that the gastrointestinal system has with microbes has allowed it to acquire important immune functions, including prevention of bacterial invasion and maintenance of tolerance.

In addition, liver parenchymal cells, and to lesser extent intestinal epithelial cells (IECs), metabolize and detoxify foodborne, waterborne and microbiota-generated toxic compounds. These protective functions are associated with a certain degree of collateral damage, which causes cell loss through physical attrition, chemical injury and immune destruction — processes that are especially pronounced in the mucosal lining of the gut. To prevent tissue loss and dysfunction and maintain homeostasis, the mammalian gastrointestinal system manifests strong regenerative capacity throughout its life (KARIN and CLEVERS., 2016). In aviary samples, the immature proliferated epithelial cells were found too. This increased proliferation causes a “lamination” of cells making the epithelium thicker than one layer. The cell types here are formed by the enterocyte and goblet cell lineages. Both types can be seen in a thicker epithelium. This multiplication is a hyperplasia. In addition, hypertrophic cells are found among the major cells. This proliferative trend in the avian gut can be found, associated with inflammation, at least in one type of reptile the leopard geckos (*Eublepharis macularius*) representing the birds ancestors (TERRELL et al., 2003).

Those cells in the case of birds proliferate much more that those found in mammals where the multiplication remains most inside the lamina propria and less over the villi. This process is a form of defense too and as such the role of the Intestinal Stem cells (ISCs) had to be prominent once without them there is no gut regeneration

(LU and LI, 2015). In adult mammalian intestine, stem cells are located near the base of each crypt. These ISCs divide to form progenitor transient amplifying cells (TA), not enteroblasts, in the transit amplifying zone, providing larger number of precursor cells that can replenish cells of various lineages (CROSNIER et al., 2006; WALKER and STAPPENBECK, 2008; YEN and WRIGHT, 2006). The avian samples are characterized by a large amount of undifferentiated epithelial cells that invade and destroy the villi. Such a process could be achieved by pilling up cells that do not divide anymore (enteroblasts). As occurs in tumors, a population of mitotic active cells multiplies and invade the basement membrane and the lamina propria (stroma) of the tissue around.

The presence of mitotic activity not just in the crypt but also along the axis of the villus (UNI et al, 1997) points the mitotic capabilities of the avian epithelial cells. By these facts transient amplifying cells (TA), are better suited to name the proliferated observed cells.

1.3) Inflammation and the Concept of Regenerative Inflammation

The animal production industries and the veterinary community are familiar with enteritis as a pathological process associated with a decrease in weight gain, feed intake, feed efficiency, survivability, and uniformity (ROURA *et al*, 1992; GASKINS, 2008; KOGUT, 2013; ISERI and KLASING, 2014). However, recent research has shown that inflammation, although tightly regulated, displays phenotypic plasticity that is dependent upon the “trigger” that activates the response (MEDZHITOV, 2008; ASHLEY *et al*, 2012). Inflammation is controlled and mediated through the cytokines that are low-molecular weight polypeptide proteins that are mainly known for their role in immune response and inflammation (BROUGHTON *et al*, 2012) and innate immune receptors (KOGUT *et al*, 2016; KOGUT and ARSENAULT, 2017). Cytokines are produced by a broad range of cell types of haematopoietic and non-haematopoietic origin that have suppressive or enhancive effects on cellular proliferation, differentiation, activation, and motility (BALKWILL & BURKE, 1989; ARAI et al., 1990).

Most often, cytokines have been classified based on their similar functional roles in regulating inflammation and immunity, i.e. interferons, colony stimulating factors, and pro-inflammatory cytokines.

Pro-inflammatory cytokines in response to an infection or tissue injury, starts a cascade of nonspecific events (acute phase response) is initiated that provides early protection by restricting the stimulus to the local site. The acute phase response is initiated after the activation of both local phagocytic cells and nonimmune cells (fibroblasts and epithelial) to produce pro-inflammatory cytokines: interleukin–1, –6, and –12 (IL– 1, IL–6, and IL–12, respectively) and tumor necrosis factor-alpha (TNF-a). These cytokines act synergistically to induce local and systemic responses to the stimuli (LOYD & OPPENHEIM, 1992; XING et al., 1994; JUNG et al., 1995). The local responses initiated by the production of pro-inflammatory cytokines include: (a) increased vascular permeability, (b) induced expression of adhesion molecules on vascular endothelium, and (c) induced local production of chemokines, i.e. IL–8, to attract specific leukocyte populations to the site (POBER & COTRAN, 1990; ALLISON & EUGUI, 1995).

The systemic responses mediated by the pro-inflammatory cytokines include fever, hepatic production of acute phase proteins, and secretion of CSF by endothelial cells, resulting in haematopoiesis and a transient increase in the required white blood cells to fight infections (TRACEY et al., 1987; ALLISON & EUQUI, 1995). In mammals, the regeneration (healing) process can be activated by IL-6, which is homologous to the *Drosophila* Upd cytokines. Similarly, IL-6 may impair iron transport in enterocytes by direct action at the local level or indirectly via hepcidin induction. Growth factors are polypeptides that usually induce cell growth or proliferation and may have overlapping functions with cytokines in both *Drosophila* and mammals (PANAYIDOU and APIDIANAKIS, 2013).

In broilers, was demonstrated that differences in resistance to S. enteritidis were accompanied by increases in avian heterophil mRNA expression of pro-inflammatory mediators including interleukin (IL)-6, IL-1b, and IL-8 (FERRO et al., 2004). Comparison of the mRNA expression of aviary IL-10, IL-6, TGF-b4, and granulocyte macrophage-colony stimulating factor (GM-CSF) in heterophils isolated from the Fayoumi line were higher than heterophils from either Leghorn or broiler lines (REDMOND et al., 2009); expression of IL-8, a pro-inflammatory chemokine, was higher in heterophils from Leghorns compared to those isolated the Fayoumi line (REDMOND et al., 2011b).

Chicken IL-2 also acts to prime chicken heterophils to increase the expression of IL-8 and IL- 18 during phagocytosis of Salmonella (KOGUT et al., 2003 a,b). Dietary additives, probiotic bacteria (FARNELL et al., 2006; STRINGFELLOW et al., 2011) and cationic peptides isolated from Gram-positive bacterium *Brevibacillus texasporus* (KOGUT et al., 2007, 2010, 2012a, b,c,d), also show augmentation of the functional activities of chicken heterophils). Salmonella *spp* may induce production of IL-8 by gut epithelium in mammalian models of gastroenteritis (WALLIS & GALYOV, 2000). These leads to an influx of neutrophils that damage the epithelium allowing bacteria to enter, and as a consequence cause tissue damage and fluid secretion leading to diarrhea.

The chicken chemokine IL8/CAF appears to be the equivalent of mammalian IL-8 in the chicken. Like mammalian IL-8, cCAF has angiogenic properties; these properties resides in the C-terminus of the molecule. Due to the high homology to human IL-8, this chemokine has been considered the homologue of IL-8 in chickens; instead, recent studies have shown that the gene that encodes for cCAF is the orthologue of human IL-8 (KAISER et al, 1999).

In mammals, the interleukin-10 (IL-10) is an anti-inflammatory cytokine that controls the nature and extent of inflammatory responses during infection with viruses, bacteria, fungi, protozoa and helminths (Couper et al., 2008; Moore et al., 2001) and has a particular central role in intestinal immunity and homeostasis (Manzanillo et al., 2015). IL-10 was first described as an inhibitor of cytokine synthesis in mice, a product of the TH2 subset of T cells that inhibited synthesis of pro-inflammatory cytokines by TH1 cells (Fiorentino et al., 1989). It is now recognized as a multifunctional cytokine produced by many immune cell types including macrophages, monocytes, dendritic cells, TH1, TH2, TH17 and regulatory T cell subsets and B cells and a feedback regulator of diverse immune responses to infections (COUPER et al., 2008; MOORE et al., 2001; SARAIVA and O'GARRA, 2010). Infectious diseases are a major threat to intensive poultry production. There is less evidence of the function of IL-10 in birds.

The cDNA of chicken IL-10 was isolated from cecal tonsils of *Eimeria tenella* infected chickens and the expressed protein product inhibited IFN-g synthesis by mitogen activated lymphocytes (ROTHWELL et al., 2004). IL-10 is a key immune regulator during infection with pathogens including intracellular protozoa (GAZZINELLI et al., 1996; WILSONET et al., 2005). IL-10 was undetectable in the serum of healthy uninfected birds. In infected birds the level of circulating IL-10 was substantially

increased around 5 days following either low or high dose challenge. These observations indicate that circulating IL-10 could provide a marker for infection, and that Eimeria *spp* infections could produce systemic immunosuppression (ROTHWELL et al.,2004). The pathogenicity of NE can occur in any organs but is particularly found in the small intestine. The first step of *Clostridium perfringens* (CP) in Necrotic Enteritis is colonization by adhesion to epithelial cells, and toxins produced by CP followed by killing epithelial cells by forming pores (LANCO et al., 2017). Significant amounts of cytokines are produced during coccidiosis or bacterial infection, including NE in the small intestine of poultry (SHIVARAMAIAH et al., 2014; LEE et al., 2017). Many pro-inflammatory cytokines seem to be controlled by IL-10 (ROTHWELL et al., 2004; CORNELISSEN et al., 2009; HARITOVA AND STANILOVA, 2012).

In chickens, expression of IL-6 and IL-10 mRNA was rapidly upregulated in intestinal intraepithelial lymphocytes after intestinal infection with Eimeria spp. (HONG et al., 2006). We thus monitored IL-6 expression in the jejunum to see if CP could induce these genes. As expected, both IL-6 and IL-10 were expressed in the jejunum of CP-infected chickens, indicating that NE-induced inflammation involved the expression of these cytokine genes.

In mammals, mucosal infection may accelerate the rate of regeneration and induce inflammatory cytokines and concomitant infiltration of immune cells, such as neutrophils, macrophages and T cells in the mucosa that may cause further damage and induce regeneration of the epithelium (CHRISTOFI and APIDIANAKIS., 2015). Reactive oxygen species (ROS) produced during tissue injury and infection are an important cue that couples inflammation to ISC proliferation through activation of Jun N-terminal kinases (JNKs) and the antioxidant transcription factor and NRF2 homologue CncC19.

Activated immune cells produce numerous inflammatory cytokines, including tumor necrosis factor (TNF), IL-6, IL-10 and IL-17 family members. In addition to the propagation of intestinal inflammation, these cytokines control the regenerative response, which depends on ISC proliferation. IL-6 and TNF also promote regeneration of the injured intestinal mucosa, acting directly on epithelial cells by engaging (MAKI et al, 2013).

Another important regenerative cytokine is IL-22, a member of the IL-10 family 66. IL-22 is produced by lymphocytes, especially T helper 17 (TH17) cells and innate lymphoid cells (iLCs), and by certain myeloid subsets, but unlike most cytokines, it does not target other leukocytes. Instead, IL-22 acts on epithelial cells and fibroblasts to stimulate proliferation inhibit death and delay terminal differentiation (NIKOOPOUR et al, 2015

The concept of Regenerative Inflammation, a process based in the overlapping effects of the inflammatory cytokines and the actions of the growth factors acting on the ISCs proliferation and differentiation. It encompasses not only infiltrating of immune cells, but also ISC microenvironment signals able to regenerate the epithelium (KARIN and CLEVERS, 2016. PANAYIDOU and APIDIANAKIS., 2013).

The concept of regenerative inflammation joined with the concept of microscopic enteritis well describes and allows the observed avian intestinal lesions of minor importance to be grouped as “Avian Proliferative Microscopic Enteritis” allowing a more detailed histopathological protocol in avian research.

1.4) Morphology of the Lesions of “Minor importance” and the ISI methodology.

1.4.1) Changes in the epithelium

The major change in this layer is the proliferation of epithelial cells in different intensities. This can be due the proliferation of immature enterocytes (**EP**), proliferation of goblet cells proliferated (**GCP**) or both. It leads to increased epithelial thickness (**ET**). Sometimes it culminates with a mushroom shape of the villi tips, villi detachment, villi fission and also a kind of mound shape fused villi. The proliferated cells invaded the lamina propria coming from the outer surface layer or from proliferated crypts below.

These crypts are long and shredded epithelial cells into the lamina propria. A widespread invasion of the lamina propria was the consequence. Associated with this proliferation epithelial folds are formed. In addition, the number of inflammatory cells in the epithelium can be found increased (**EI**).

The proliferated immature cells were characterized by small amount of cytoplasm (high nuclear-*cytoplasmic ratio*), round or polyhedral cytoplasm shape and one distinct nucleolus. No brush border is seen, and the tall columnar shape is lost (Figure 4E).

1.4.2) Changes in the Lamina propria

Lamina propria can express minor amounts of red blood cells in the capillaries seen named as congestion (**CO**), inflammation (**LPI**) and invasion by proliferated immature enterocytes. The blood cells invade the structure and could be classified as lymphocytes, plasm cells, macrophages, heterophils and eosinophils. The storage of inflammatory cells inside the lamina propria leads to the enlargement of this structure Edema, occasional foci of hemorrhage and fibrin could also add dimension to this structure (**LPT**).

1.4.3) Changes in the Basement Membrane

Basement membrane can be found dissociated or “foggy” under the proliferated immature enterocytes. It is well stained with the Mallory’s Trichrome and its dissolution allows the epithelial proliferation inside the lamina propria.

1.4.4) The “I See Inside (ISI)” methodology: concepts and parameters

The literature in poultry Science mentions few protocols that evaluate the intestinal microscopic histopathological profile linking it to the animal health and the performance. Current models still use linear measures such as the length, the thickness and perhaps the area of the villus and crypts to evaluate the intestinal mucosa in broilers. The inflammatory and proliferative trend in the avian gut is not considered. A protocol concerning more microscopic lesions (parameters) of no importance accompanied by the absence of gross lesions in most situations, for healthy broilers was published just quite recently (KRAIESKI et al; 2016; BELOTE et al, 2018, 2019). In addition, this same method tries to link how some specific histological changes could potentially lead to some malabsorption.

In this methodology, an impact factor (**IF**) is defined for each alteration in macro- and microscopic analysis according to the reduction of organ functional capacity, based on previous knowledge of literature and background research. It evaluates intestine and liver.

Table 1. ISI histological alterations (parameters) evaluated in intestine and liver.

Organ	Alteration	Impact Factor (IF)	Score	Final Score	Maximum Score
Ileum	Lamina propria thicknes – **LPT**.	2	3	6	45*
	Epithelial thickness – **ET**.	1	3	3	
	Enterocytes proliferation – **EP**.	1	3	3	
	Epithelial plasma cell infiltration – **EI**.	1	3	3	
	Lamina propria Inflammatory infiltration – **LPI**.	3	3	9	
	Goblet cells proliferation – **GCP**.	2	3	6	
	Congestion – **CO**.	2	3	6	
	Presence of oocysts – **OP.**	3	3	9	
Liver	Congestion – **CO**.	1	3	3	42*
	Cell vacuolization - **VAC.**	2	3	6	
	Bile duct proliferation – **BDP**.	2	3	6	
	Immune cells infiltration – **ICI**.	1	3	3	
	Necrosis – **NE**.	3	3	9	
	Pericholangitis – **PCH.**	3	3	9	
	Lymphocytic aggregate – **LAG**.	2	3	6	

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

The IF ranges from 1 to 3, where 3 is the most impactful for the organ function, e.g., necrosis, has the highest IF because the functional capacity of affected cells is totally lost. The extent of each lesion (intensity) or observed frequency compared to non-affected organs is evaluated in each organ/tissue per animal and the score ranges from 0 to 3. The score 0 (absence of lesion or frequency), score 1 (alteration up to 25% of the area or observed frequency), score 2 (alteration ranges from 25 to 50% of the area or observed frequency), and score 3 (alteration extent more than 50% of the area or observed frequency).

To reach the final value of the ISI index, the IF of each alteration is multiplied by the respective score number, and the results of all alterations are summed.

Figure 4: ISI parameters for ileum describing microscopic enteritis. A - Normal tissue depicting epithelium (1) and lamina propria (2) at 200X and close up of enterocytes and goblet cells at 400X. B – Epithelial inflammatory cells (EI) at 200X and close up at 400X. C – Lamina propria inflammation (LPI) at 200X and close up of plasma cells and lymphocytes at 400X. D – Goblet cell proliferation (GCP) at 200X and close up at 400X. E – Enterocyte proliferation (EP) at 200X and close up at 400X. The thickness of the epithelium is increased. F – Congestion (CO) at 200X and red blood cells close up at 400X. All samples stained with Hematoxylin and Eosin plus Alcian blue. Source: The author (2019).

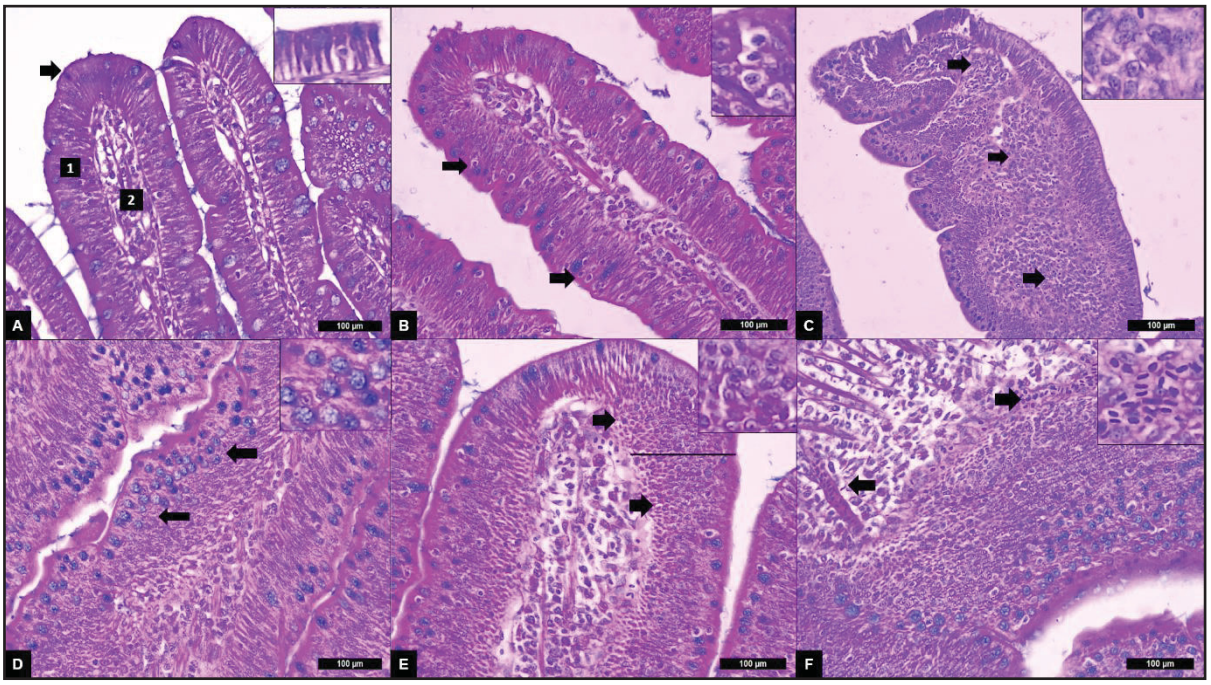
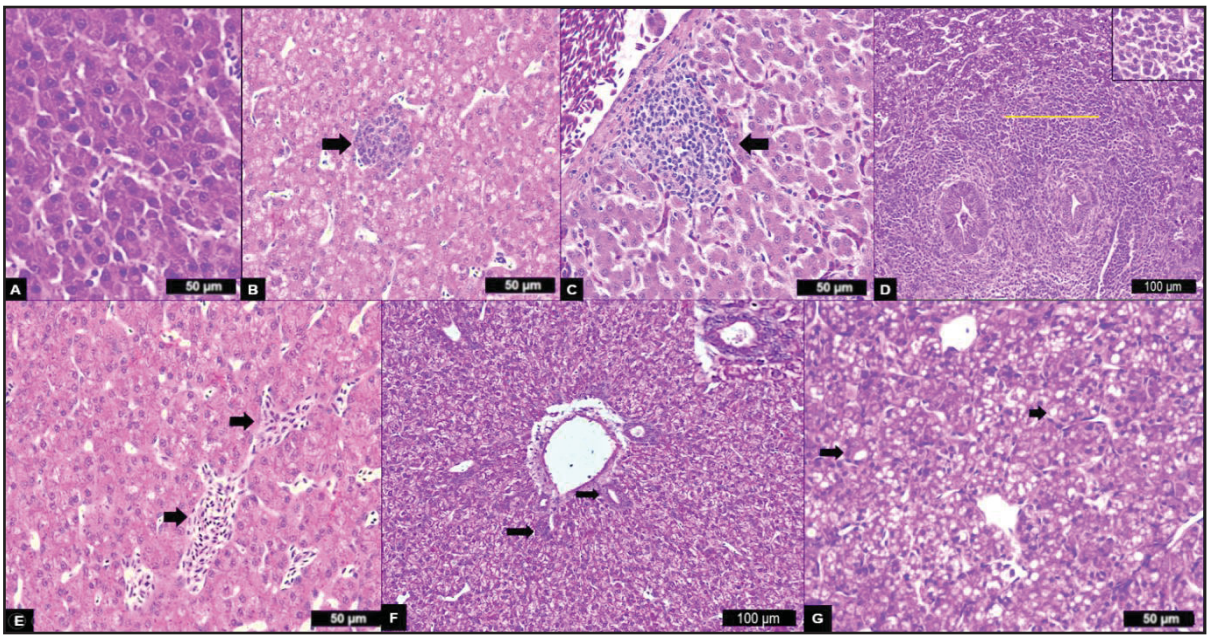


Figure 5: ISI parameters for liver. A - Normal tissue depicting hepatocytes. B - Lymphoid aggregate (LAG), 400X. C - Inflammatory cell infiltrate in the parenchyma (ICI), 200X. D - Per cholangitis (PCH), 200X. E – Congestion (CO), 400X. F – Bile duct proliferation (BDP), 200X. G – Vacuolization of hepatocytes (VAC), 400X. All samples stained with Hematoxylin and Eosin. Source: The author (2019).



The aim of this thesis was to describe the microscopic lesions presented in the broiler intestine. We compare the pattern of inflammation between different lineage of chickens and evaluate the “I See Inside” (ISI) protocol, as a tool to link the intestinal lesions to some deranged performance on industrial system flocks. In addition, it was tested the adaptability of the method to evaluate the digestive tract of other species such as the bovine.

In chapter 1 we present the microscopic lesions found in feral and domestic broilers and propose classifications, mechanisms and definitions to these histological changes.

In chapter 2 we describe the ISI parameters in a chronological way, linking and explaining their behavior using immunohistochemical, interleukin and performance data.

In the final chapter, we present the results of the adaptability of the method in other species such a as feedlot cattle.

CHAPTER 2: HISTOPATHOLOGY OF MINOR LESIONS IN THE INTESTINES OF BROILERS: A NEW PERSPECTIVE

Histopathology of microscopic changes in the intestinal tract of birds: A new perspective comparing domestic and feral chickens.

SANCHES, A.W.D. [1], HAYASHI, R.M. [2], HÜMMELGEN P.H.[1], PEREIRA A[1]. , B. L. BELOTE. [1], SANTIN E[1]. [*]

[1] Department of Veterinary Medicine, Federal University of Paraná, CEP 80035-050 Curitiba/PR Brazil. Microbiology and Avian Pathology Laboratory; [2]BRF SA.

***Corresponding author:**
Rua dos Funcionários, 1540 - Cabral, Curitiba - PR, 80035-050
E-mail: besantin@hotmail.com Tel +55(41) 3350 5859

Abstract

Losses of genetic diversity during G. *gallus* domestication, describe the individual commercial breeding lines as having lost 70% or more genetic diversity of which only 25% of this loss can be recovered by combining all stocks of commercial poultry. The theoretical genetic rescue could be achieved by the study and use of domestic animals that undergo feralization. Studies focusing on the microscopic inflammatory response of the intestinal tissue in feral chickens are absent. Nevertheless, even gastro enteric diseases in domestic poultry are devoid of proper morphological microscopic study protocols, classifications and conceptual definitions. The goal of this trial is to demonstrate, describe and compare the morphology of microscopic ileum responses between two distant strains of *Gallus gallus,* domestic and feral. In addition, comparative concepts are suggested as mechanisms for the tissue changes observed. Samples of ileum from Cobb®500 28Days of age and adult Feral animals from the Bahamas Islands were stained with Hematoxylin and Eosin plus Acian Blue and had their villi scored for parameters adapted from the “I See Inside” ISI protocol. In addition, the number of crypts and villi age/size was considered.

Both groups were characterized by the presence of epithelial inflammation (EI), inflammation in the lamina propria (LPI), proliferation of enterocytes (EP) proliferation of goblet cells (GCP) and angiogenesis (ANG).The presence of ANG was more

pronounced in the feral animals (p≤0.001) and was associated with higher epithelial inflammation (p≤0.002). The scores (intensity) for ANG (p≤ 0.001), LPI (p≤0.003) and EI (p≤0.002) were higher in the feral animals. Cobb®500 animals presented more (p≤0.023) nascent villi (NC), higher numbers (p≤0.046) of common crypts (CC) and higher numbers (p≤0.018) of accessory crypts (AC). It was considered that the cleaner industrial environment and the younger age (28 days) of the Cobb®500 determined these results. In the reactive villi, the enterocyte proliferation invaded the lamina propria and destroyed the smooth muscle tissue, the basement membrane and the collagen tissue. Fusion of the villi was seen and ended in the formation of new villi from the fused ones. Inflammatory cells in the lamina propria comprised lymphocytes, macrophages, heterophils and plasma cells, in different combinations being associated with proliferation of enterocytes and goblet cells. The samples of the feral and the Cobb®500 group demonstrated a strong similarity in the microscopic intestinal inflammatory proliferative response, despite the domestication and the feralization processes. The use of concepts from human pathology such as Regenerative Inflammation combined with Microscopic Enteritis would allow a better understanding of the avian intestinal lesions of minor importance. In addition, grouping of the findings in both groups as “Avian Proliferative Microscopic Enteritis” which would help to create more precise histopathological protocols in avian research.

Keywords – microscopic enteritis, regenerative inflammation, ISI, feral chicken.

2.1) INTRODUCTION

The chicken was domesticated around 6.000 to 10.000 years ago in Southeast Asia from the Red Junglefowl (Tixier-Boichard, Bed'hom et al. 2011), and has probably also seen some introgression from the Grey Junglefowl (Eriksson, et al. 2008). Domestication, understood as the evolutionary process when a wild population changes in response to a life with humans, clearly has multiple causes (Larson and Fuller 2014) and deleterious consequences.

There is evidence of losses of genetic diversity during G. *gallus* domestication, which may limit its resilience to future environmental challenges such as pathogens, extreme temperatures and drought. Decades of intense selection primarily for meat or egg production led to a decrease in the domestic poultry biodiversity. Genetic evaluations indicated that individual commercial breeding lines have lost 70% or more genetic diversity of which only 25% of this loss can be recovered by combining all stocks of commercial poultry (Muir and Cheng, 2008).

For this genetic rescue at least theoretically, options in domestic animals that undergo feralization, reversing the domestication process could be found. In this situation, environmental selective and neutral processes might promote genetic variation which could therefore assist evolutionary rescues of genetically depleted domestic populations (Price et al, 1984).Some recent studies on feral chickens include microbiota and genetic evolution (Chiara et al, 2017; Johnson et al, 2016) but studies focusing on the inflammatory response of the intestinal tissue are still absent. Nevertheless, even gastro enteric diseases in domestic poultry are still devoid of proper morphological microscopic study protocols, classifications and conceptual definitions. Gastrointestinal disorders (GI) in both, animals and humans, have already been demonstrated and similarities among them have been described. As in humans with enteric malabsorption diseases, poultry production is also affected by similar conditions that results in losses on the zoo technical performance parameters and well-being flock (KRAYESKY et al., 2016; BELOTE et al., 2018, 2019).

The current literature in broiler pathology focus in the standard routine of stablish the disease and/or the causative agent. This background is very important for diagnostic but minor lesions that could affect the health and performance of the flocks are neglected and labeled as “lesions without importance”.

However, these minor lesions can be scaled and linked with some amount of negative performance and health status along the life spam of the flock (KRAYESKY et al., 2016; BELOTE et al., 2018, 2019). Samples from experimental and even backyard chicken units in southern Brazil, examined by our group LABMOR-UFPR-BRAZIL demonstrated inflammatory and proliferative innate response of the avian intestine, mainly of the epithelial lineage, leading to areas in the villi that do not contain the traditional well-differentiated enterocyte attached to a healthy basal membrane as presented in morphological textbook villus. In addition, in these samples, inflammation in the lamina propria is overexpressed in many of the villi. These changes when presented in the same villus lead to a very different morphology not considered in the experiments and evaluations found in the literature. In or view, these changes represent the general responses of the avian intestinal mucosa facing an environmental challenge and are not diagnostic for any specific etiology. The use of “pathogen free animals” or very clean environmental conditions in many researches are very helpful in describing the growing and development of the normal avian intestine but do not properly link the experiments to real field immune challenges in the avian industrial houses. The experiments in the area of avian enteropathology still uses the traditional linear morphometry that consider just the high and thickness of the villi and the crypts depth, without evaluating the changes in the different layers and structures even when very similar or the same measures are found. Evaluation methods should consider the quality of the enteric tissue by the search of a histopathological profile, which express the real status of the organ. Under this background, the goal of this trial is to demonstrate, describe and compare the morphology of microscopic ileum responses between two distant strains of *Gallus gallus,* domestic and feral, raised under different environments and managements. In addition, comparative concepts are suggested as mechanisms for the tissue changes observed.

2.2 MATERIALS AND METHODS

This study used ileum samples from two strains of birds, six adult feral chickens from the Bahamas Islands and six 28 days old, domestic Cobb 500® lineage males from an experimental facility of the Avian Diseases and Microbiology Laboratory – LABMOR/UFPR in southern Brazil. For the study, only archive slides, paraffinized blocks and special stains were used.

2.2.1 The Feral chickens

2.2.1.1 Animals location and basic characteristics

The feral chickens were from the St. George's Island, Bermuda are a common feature in the islands of Bermuda and in the Caribbean region. These birds appear to be an admixed flock originating from several breeds that are popular sources of meat and eggs in the Western hemisphere. The Cobb evaluation of the gut content suggests a highly variable diet including locally occurring invertebrates (e.g., snails and insects), local ornamental and/or natural vegetation (e.g., seeds and shoots) and garbage from Bermuda households and businesses.

2.2.1.2 Sacrifice and tissue gathering

The adult animals were sacrificed by cervical dislodgement and had their intestines gathered right after the death and examined outside and inside. The Bermuda Department of Environment and Natural Resources in support of the Bermuda Biodiversity Project approved the gathering and export of the samples. The Institutional Animal Care and Use Committee of the Michigan State University, under permit number 06/17-093-00, approved the protocol.

2.2.1.3 Tissue process and staining

For the fixation, segments of the ileum were stippled on a cartoon piece to avoid bending and immersed in fixed in 10% formalin solution. All the samples were transversally trimmed, dehydrated, infiltrated, and embedded in paraffin following common histological routine. Blocks were cut in 5 μm sections. All the tissues were stained with Hematoxylin, Eosin, and Alcian Blue in the both slides.

2.2.2 The Commercial Cobb® Lineage chickens.

Six paraffinized tissue blocks from Cobb Lineage® animals, negative control, were obtained from the Avian Diseases and Microbiology Laboratory (Center of Avian Immune Responses) – LABMOR/UFPR in Brazil. These blocks come from one trial that were carried out in the conditions described below

2.2.2.1 Housing and management

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

2.2.2.2 Sacrifice and tissue gathering

The animals were sacrificed by cervical dislodgement and had samples of their ileum gathered right after the death and examined outside and inside. The Institutional Animal Use Ethics Committee of Agricultural Sciences of the Federal University of Parana (Protocol 041/2016) approved this trial.

2.2.2.3 TISSUE PROCESS AND STAINING

For the fixation, segments of the ileum were stippled on a cartoon piece to avoid bending and immersed in fixed in Davidson's solution (100 mL glacial acetic acid, 300 mL 95% ethyl alcohol, 200 mL 10% neutral buffered formalin, and 300 mL distilled water) for at least 24 hours.

All the samples were transversally trimmed, dehydrated, infiltrated, and embedded in paraffin following common histological routine. Blocks were cut in 5 μm sections. The tissues were stained with Hematoxylin and Eosin plus Alcian Blue in the same slide, Hematoxylin and Eosin and the Mallory's trichrome only. The Mallory's trichrome stain was tested to evaluate collagen, smooth muscle and general cytoplasmic enzyme presence. The same paraffin block was trimmed three times to obtain similar cuts of the same sample.

2.3 HISTOPATHOLOGICAL PARAMETERS AND DATA OBTAINMENT

Slides were examined in a LEICA ICC50 - E light microscope and photographed with the camera included. The LAZ V4.8 software processed the images. The separated villi images were obtained in 200X magnification and after were “stitched” by the same software. To the examination, the “stitched” images files were opened and all types of villi in the length of the shortest sample, 8,000 micrometers measured in the base of the mucosa, were numbered with the AperioImage Scope also, from Leyca Microsystems. The villi were also checked on microscope at 400X augmentation while the images were scored. The parameters evaluated in ileum mucosa described changes of epithelial cell lineage and inflammation. The lamina propria inflammation **(LPI**), epithelial inflammation (**EI**), goblet cells proliferation (**GCP**) and “enterocyte proliferation” (**EP**) were adapted from the “I See Inside” (ISI) methodology (Kraiesky *et al*., 2016; Belote *et al*., 2018) and the angiogenesis (**ANG**) was added. **Intensity** of these parameters was obtained by their scores. The **presence** of the parameters per villi was obtained by separating the scores in two groups: the villi with scores 0 and the villi containing any scores from 1 to 3.

The analysis also included the **presence** of the total number of crypts (**NC**), the number of common crypts (**CC**), the number of accessory crypts (**AC**), the number of “nascent” villi (**NV**), the number of “juvenile one” villi (**Juvenile-1**), the number of “juvenile two” villi (**Juvenile-2**), the number of adult villi (**AV**), the number of fused villi (**FV**) and the number of shed villi (**shed**).

2.4 SCORING AND CLASSIFYING THE VILLI

The score for each parameter evaluated ranged from zero (0) to three (3). Zero represented no changes or a minimum, one (1) represented up to 25 % of the field presenting the alteration or change in intensity compared to normal tissue, two (2) up to 50 % of the field or intensity difference compared to normal tissue and three (3) more than 75% of the field area or change in intensity compared to normal tissue. The villi “age and size” classification as **NV**, **juvenile-1**, **juvenile-2**, **adult** and **shed**, regarded the general size and height of the villi compared with the tallest ones divided in four quarters.

The smaller ones, pointing just above the lower surface of the mucosa were the “nascent” and had a quarter of the total high of the tallest villi. The juvenile one had two quarters in high of the tallest villi, the juvenile two had three quarters of the tallest villi and the adults had the full size. The size of the quarters was optically estimated. Shed villi were considered when the total fission occurred in the lower part of the villus detaching it from the mucosa. The nascent villi that were located right below the detached villus, was named as shed, not nascent. For the crypt evaluation, the **lateral** ones were named as **common** crypts (**CC**). For this, in the lateral site of crypt, all crypts below the common ones in a vertical line and very close to each other were counted as one. Just the accessories crypts (**AC**) located between the lateral ones, were counted individually. The villi were classified as fused when they had more than two accessories crypts between the **CC** since their maximum number was considered as two (Figures 1 and 2).

2.5 STATISTICAL ANALYSIS

The data were evaluated according to the *H*0 hypothesis, where the parameters of both groups were not different. The *H*1 hypothesis stated the presence of difference among these means. *P* value less than 0.05 was considered significant. The software Statistix 9.0 ® from Analytical Software processed the data. The Shapiro–Wilk test for normality preceded the Student's t-test for the parametric data and Kruskal Wallis, for the non-parametric values. Within the groups, the Pearson’s correlation was applied to the parametric data and the Spearman’s correlation was used in the non-parametric data.

2.6 RESULTS

2.6.1 Gross findings

The description of the samples did not reveal gross diagnostic lesions or strong distinct lesions. Generally, few petechiae and mucous were seen in the feral group. As for the Cobb group, just the normal amount of mucus was seen.

2.6.2 Histopathology

The evaluation at 40X and 100X of the villi, revealed many villi with inflammation in the lamina propria and proliferative epithelial cells increasing the thickness of the epithelium (Figures 1 and 2) in both groups. In both groups, the normal villi (Figure 3) had an epithelial outer monolayer made of epithelial cells. Most were enterocytes disposed in a single layer of elongated and closely attached to each other cells**.** The central frame of the villi was represented by the Lamina propria that kept the shape of the villi holding the blood vessels, the lymphatic vessels, collagen tissue and smooth muscle tissue had no changes. The mucous layer over it was also seen (Figures 3A and B). Both groups were characterized by the presence of inflammatory responses in the epithelium (EI) and in the lamina propria (LPI), proliferation of enterocytes and goblet cells and angiogenesis. The presence of angiogenesis was more pronounced in the feral animals and was associated with higher epithelial inflammation (Table 2).It was observed that the scores (intensity) for angiogenesis, lamina propria inflammation and epithelial inflammation were higher in the feral animals (Table 4). The types and distribution of villi and crypts were also different. The Cobb animals presented more nascent villi, number common crypts and accessory crypts than Feral chickens (Table 3).

Table 1: Means (normal distribution), median (for non-normal distribution) and standard deviations for the total number of different types of villi between Feral and Cobb chickens.

Group	NV*	Nascent*	Juvenile1*	Juvenile-2'	Adult*	Shed(IQR)**
Feral	37.83±4.00	2.83±2.63b	5.00±4.80	7.16±6.40	20.83±0.61	4.00±3.22a
Cobb	49.00±6.00	20.83±8.08a	5.33±2.25	8.00±6.22	13.83±0.73	0.00±0.00b
P value	0.051	≤ 0.001	0.871	0.823	0.113	≤0.032

Different letters in the same column indicate a significant difference ($P < 0.05$).* normally distributed, **non-normally distributed. NV – total number of villi.

Table 2: Means (normal distribution), median (for non-normal distribution) and standard deviations for the number of villi (presence) containing any degree (scores1 to 3) of the ileum parameters between Feral and Cobb chickens.

Group	ANG(IQR)**	LPI *	EI *	GCP *	EP *
Feral	9.5±6.17a	31.66±8.31	29.33±10.85a	23.66±15.00	23.66±13.17
Cobb	3.5±0.00b	32.00±5.96	8.16±6.24b	20.00±8.24	29.66±4.17
p value	≤ 0.001	0.938	≤ 0.002	0.611	0.312

Different letters in the same column indicate a significant difference ($p < 0.05$). * Normally distributed, **non-normally distributed. **ANG –** angiogenesis, **LPI** – lamina propria inflammation, **EI-** epithelial inflammation, **GCP –** goblet cell proliferation and **EP –** epithelial proliferation

Table 1: Means (Normal distribution), median (for non-normal distribution) and standard deviations for the total numbers (presence) of ileum parameters between Feral and Cobb chickens

Group	NC*	CC*	AC*	FV*
Feral	155.00±31.62b	75.33±75.33b	79.50±12.22b	12.16±3.31
Cobb	195.17±19.06a	98.00±98.00a	97.66±10.17a	12.83±2.92
p value	≤0.023	≤0.046	≤0.018	0.719

Different letters in the same column indicate a significant difference ($P < 0.05$). * Normally distributed, **non-normally distributed. **NC** – number of crypts, **CC** – number of common crypts, **AC** – number of accessory crypts, **FV –** number of fused crypts.

Table 4: Means (normal distribution), median (for non-normal distribution) and standard deviations for the scores of the parameters (intensity) of ileum between Feral and Cobb chickens.

Group	ANG(IQR)**	LPI *	EI *	GCP *	EP *
Feral	0.36±0.28a	1.34±0.25a	1.67±0.89a	0.78±0.48	1.27±0.72
Cobb	0.0±0.0b	0.56±0.11b	0.19±0.18b	0.56±0.23	1.33±0.21
p value	≤ 0.001	≤ 0.003	≤ 0.002	0.339	0.837

Different letters in the same column indicate a significant difference ($p < 0.05$). * Normally distributed, **non-normally distributed. **ANG –** angiogenesis, **LPI** – lamina propria inflammation, **EI-** epithelial inflammation, **GCP –** goblet cell proliferation and **EP –** epithelial proliferation

Figure 1: Ileum, Feral group. Inflammatory proliferative profile. The lamina propria thickness is increased due inflammatory cells (arrowheads). The epithelium has some proliferation (double head arrows). A nascent villus (black arrow), one juvenile -1 villus (red arrow) and a fused villus with four crypts (FV) are pointed, HE, 40X. Source: The author (2019).

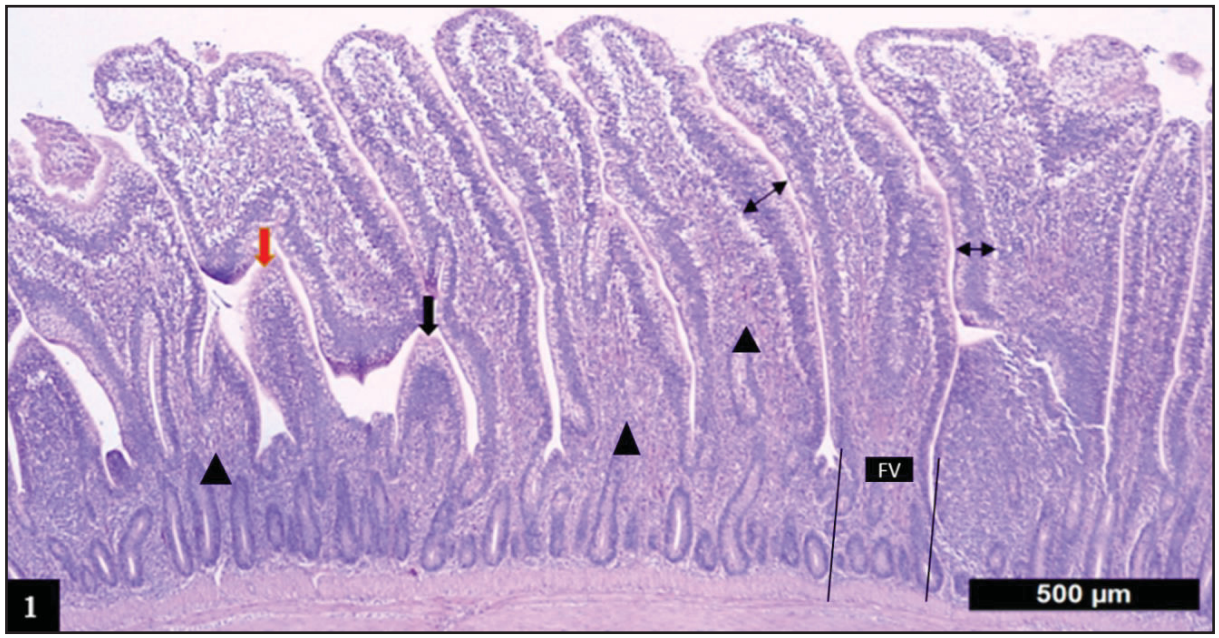


Figure 2: Ileum, Cobb group. Inflammatory proliferative profile. Nascent villi (black arrows) and one juvenile-1 villus (red arrow) are among a majority of proliferated villi and fused (FV) villi. The thickness of the epithelium is increased (double head arrows), HE, 40X. Source: The author (2019).

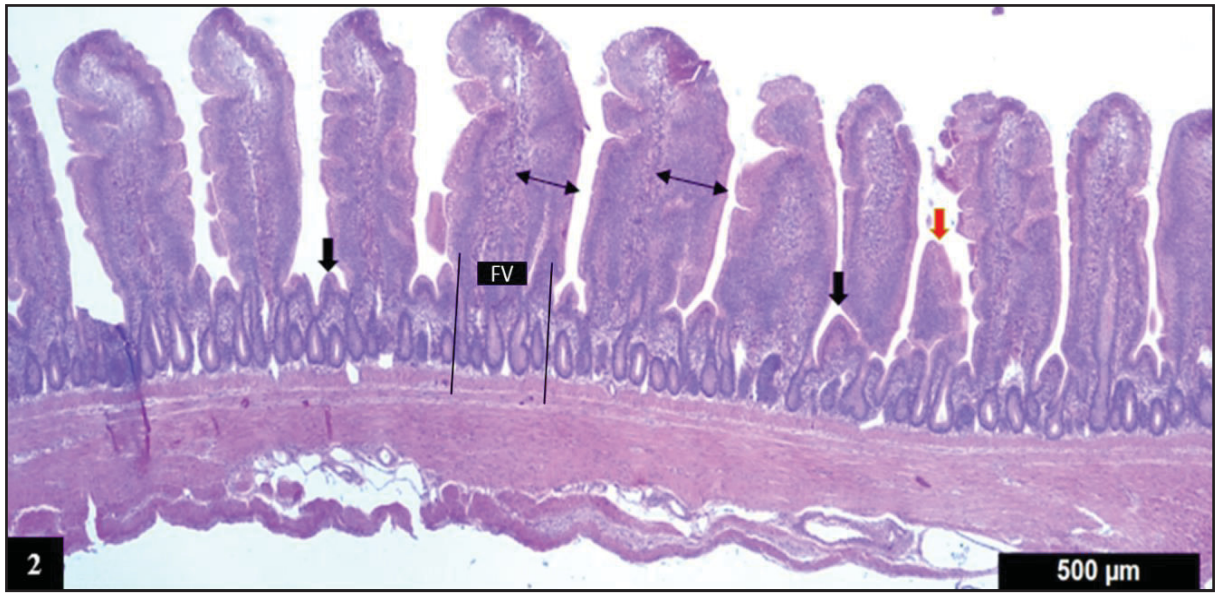
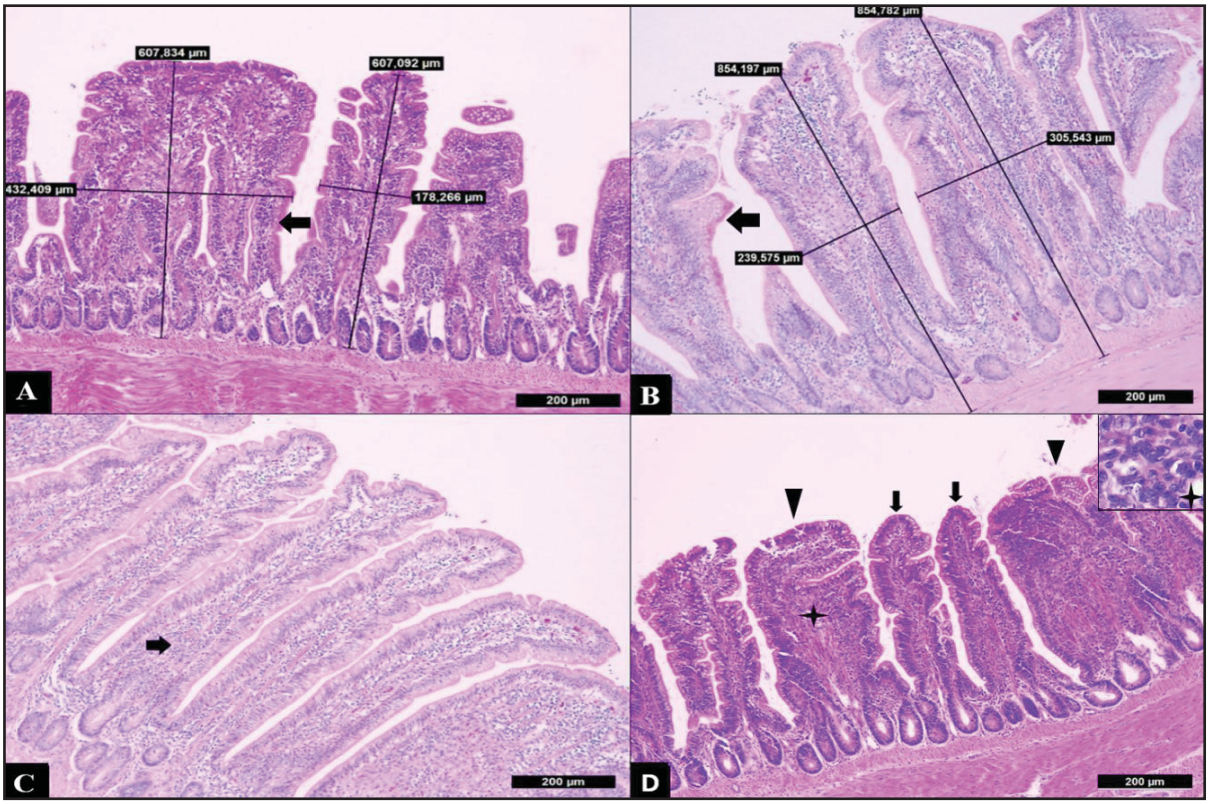


Figure 3: Ileum**. A –** A fused villus (left) with the same high of a normal (right) villus. The mucous layer can be seen (arrows), Cobb group, HE, 100X. **B** - Villi with the same high having different histopathological profiles. The mucous layer is pointed (arrows) Feral group, HE, 100X. **C**- Four non-proliferated villi having inflammation in the lamina propria (arrow) beside an enlarged villus (right), Feral group, HE.100X. **D** - Two normal villi (arrows) among proliferated ones (arrowheads), Cobb group. The small window magnifies in 400X the lamina propria with lymphocytes (star), HE. 100X.Source:Theauthor(2019).



Lesions not statistically quantified observed in both groups are described in their nature as inflammatory and proliferative (Figure 1, 2 and 3). In the inflammatory process, the major lesion in the lamina propria was the invasion of incoming inflammatory cells (Figure 3C and D) as lymphocytes, plasm cells, macrophages, heterophils and eosinophils. Congestion seen as red blood cells infiltration was noticed in the tissue (Figure 4D). In the feral group, the angiogenesis is more evident alongside the inflammation process in lamina propria (Figure 4B). The proliferation of epithelial cells was also a common observation in ileum mucosa of both groups of birds (Figures 1 and 2).

Mallory's Trichrome staining was used to describe the maturity of the epithelial proliferate cells and highlighted the presence of general enzymes in the brush border and cytoplasm of the well-matured cells, mainly in the tip of the villi, were well stained

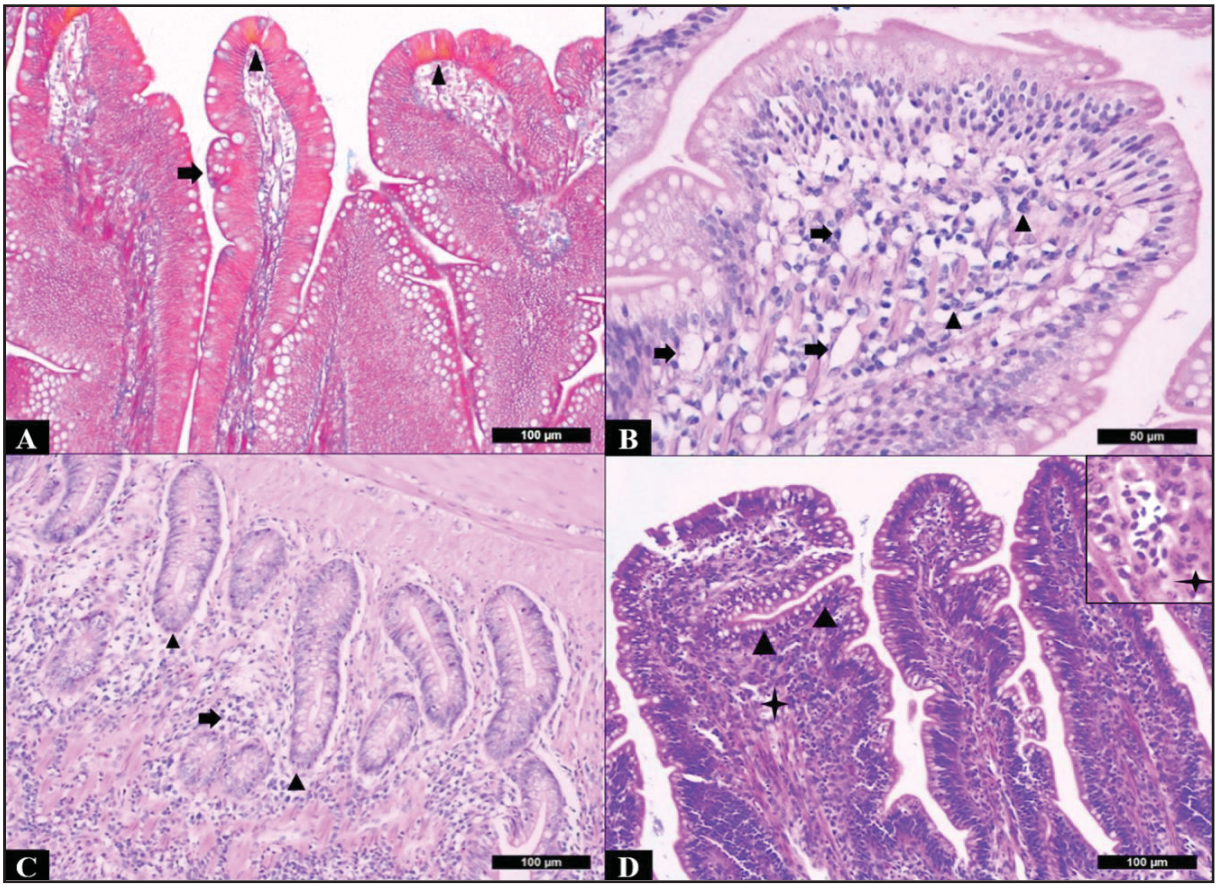
in dark red/orange was observed (Figure 4A). During the epithelial proliferation, the villi with partial alteration had an asymmetrical surface portrait (Figure 5A).

On this situation, the epithelium of one “face” of the villus remained with normal morphology having tall well-differentiated matured columnar enterocytes attached to a non-changed basement membrane. In this “face”, the cells stain well red/orange by the Mallory’s Trichrome and the basement membrane in light blue (Figure 5B). The “opposite side” in the same villus (Figure 5A) had epithelial cells proliferated in an intense and diffuse degree causing an invagination that we called fold. In this, a “tail” like face composed by the proliferated cells is observe (Figure 5A and B).

The proliferated immature cells were characterized by small amount of cytoplasm (high nuclear-*cytoplasmic ratio*), low polymorphism, round or poliedric cytoplasm shape and one distinct nucleolus. No brush border was seen and the tall columnar shape was lost (Figure 5D). The Mallory’s Trichrome highlighted the progressive reduction in the red dye of the cytoplasm and its differentiation as the epithelial proliferation increased (Figure 5A and B).

Enteroendocrine cell (Figure 5D) proliferated as well in the immature epithelial tissue. The immature cells invaded the lamina propria and destroyed the smooth muscle, their basement membrane and the connective tissue (Figures 5A and B).

Figure 4: Ileum. **A**- Normal well differentiated epithelial cells in a healthy isolated villus. The most matured cells stain in orange or dark red. Mallory's trichrome, Cobb group, 200X. **B -** Angiogenesis (arrows) and inflammatory cells (arrowheads) in the lamina propria, Feral group, HE, 400X. **C –** Immune cell infiltration in the lamina propria (arrow) and hyperplastic crypts (arrowhead), Feral group, HE, 200X. **D -** Invagination (arrowheads) of the epithelium (epithelial fold) lined by goblet cells creating a horizontal line of fission, HE, 200X. The small window magnifies the lamina propria with congestion, HE, 400X. Source: The author (2019).



The proliferate epithelial cells presented pyknosis (Figure 6A) and apoptotic bodies (Figure 6B). It is not clear if the proliferation of epithelial cells induce the inflammation of the lamina propria or vice-versa or if they are independent process, but we observe that as the lamina propria is progressively destroyed by the epithelial invasion the immune cell infiltration in lamina propria increases. The invasion by the epithelial cells in many cases acted as a detaching horizontal mechanism that we denominated fold (Figure 4D) or as a vertical fission line in the fused villi (Figure 8C). Once the progressive destruction of the lamina propria was finished, the whole villi was detached from the area previously characterized by a “bottle neck” constriction (Figures 7 A and B).

At the site of the fissure, before the detach, one or both opposite surfaces were invaded by immature epithelial cells. Further, the fissured surface was lined by goblet. These proliferated cells destroyed the lamina propria components on the site and

finished the detach process. Frequently, growing new villi were observed under the detached villi (Figure 7B).

Figure 5: Ileum. A - Epithelial fold in a proliferated surface (arrows). Poorer staining in the proliferated cells and undifferentiated portion is noticed, Cobb group**,** Mallory's trichrome, 200X. The opposite surface is yet monolayer (arrowhead), with adequate enzymatic staining. Cobb group, Mallory's trichrome, 200X. B - Discontinuity of the basement membrane under the proliferated cells (arrowheads), Cobb group, Masson's trichrome, 400X. C **-** Crypts fusing of their basement membranes, Mallory's Trichrome. Cobb group, 40X. D **-** Enteroendocrine cells (arrows) proliferated amidst immature epithelial cells, Cobb group, HE, 1000X. Source: The author (2019).

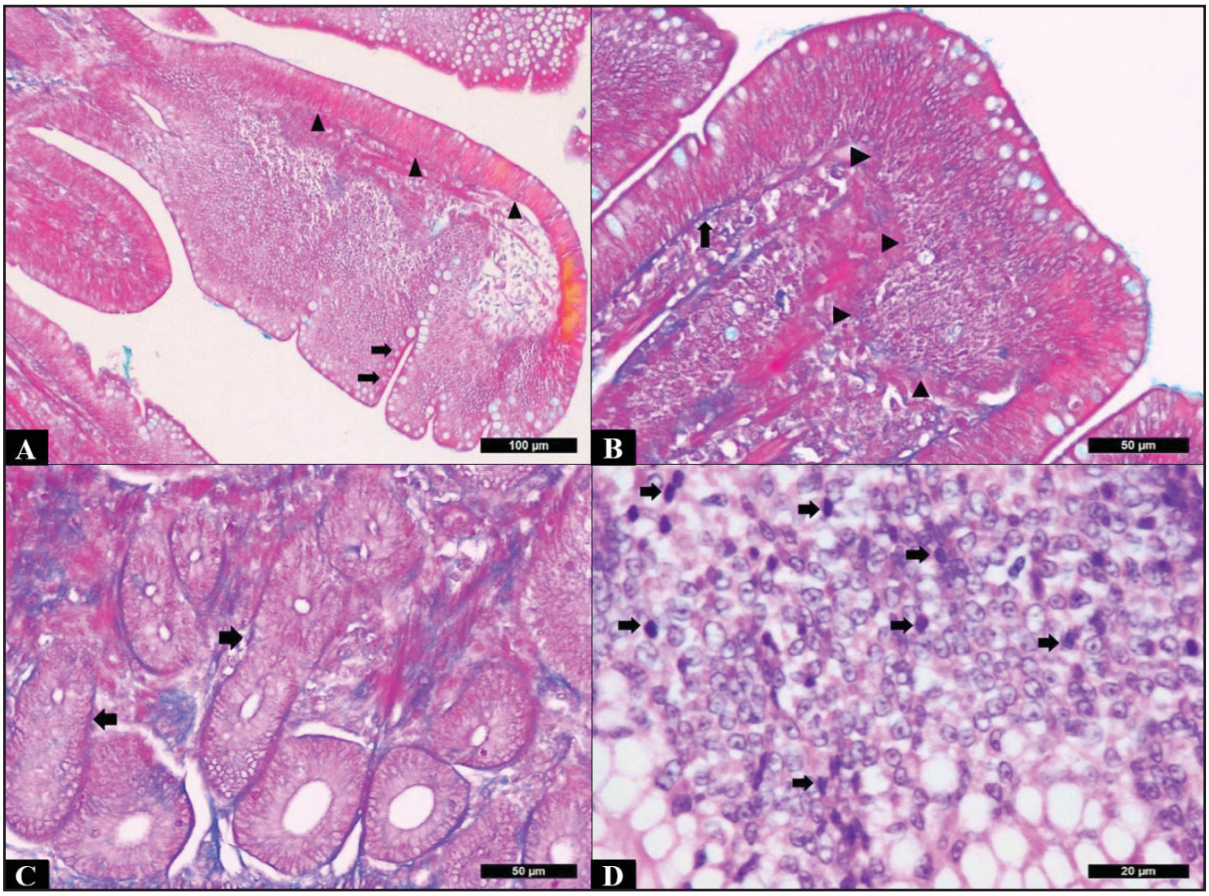


Figure 6: Ileum. **A -** Proliferated immature epithelial cells (arrowheads), many undergoing pyknosis (arrows) **-** Cobb group HE, 1000X. **B -** Apoptotic bodies (arrows) amidst immature enterocytes in the top of the villus, Masson's trichrome, Cobb group, 1000X. Source: The author (2019).

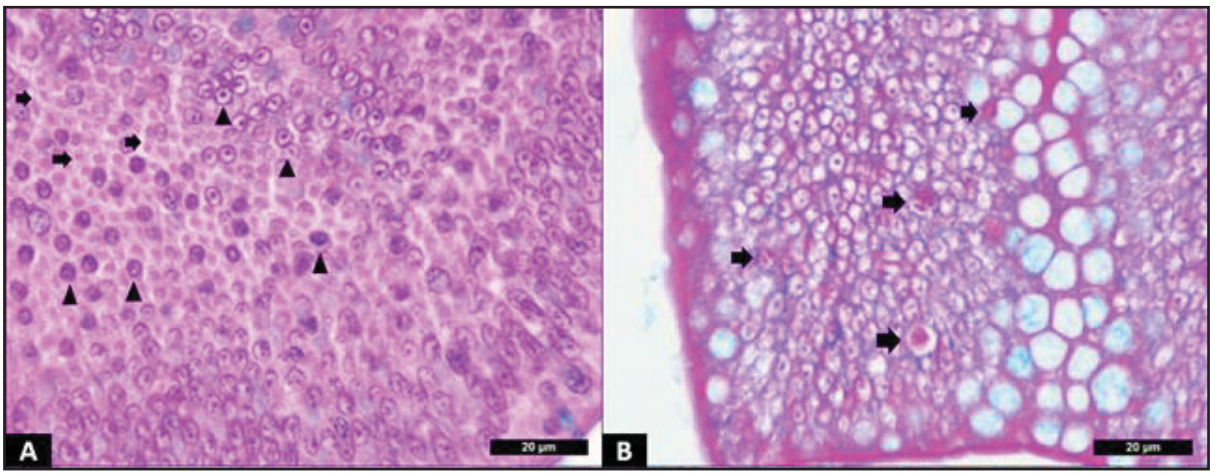


Figure 7: Ileum. **A** - Horizontal fission in a villus, Mallory's trichrome **B** – Detached villus (left) and "bottle neck" villus. New villi can be seen under the detached villi. Cobb group, HE, 100X. Source: The author (2019).

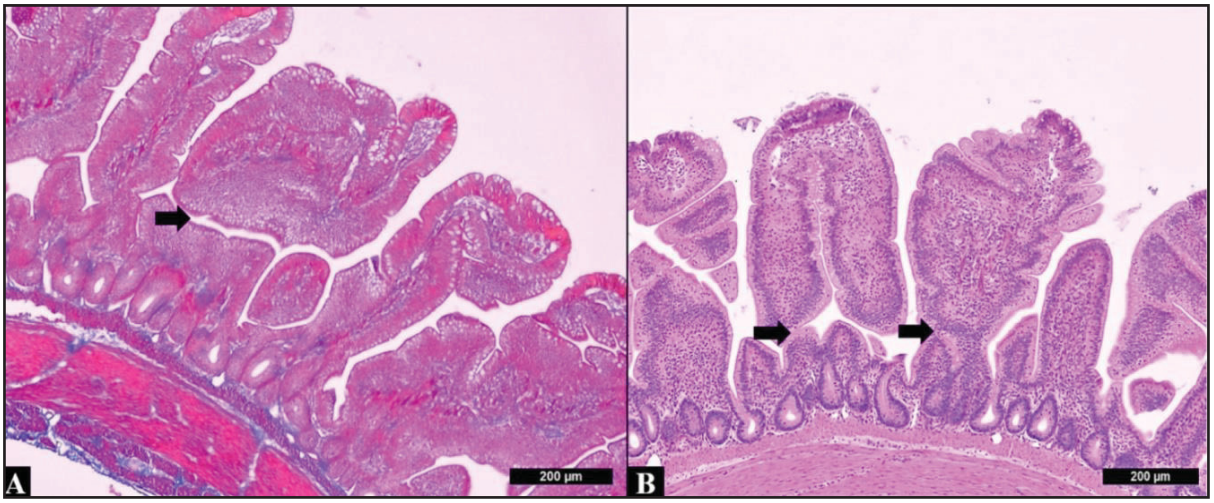


Figure 8: Formation of new villi from the proliferated fused ones in Ileum. **A** and **B** - Hyperplastic crypts (arrow) shedding cells inside the lamina propria. **C** - Vertical lines of epithelial cells proliferate and invade the villus creating new ones. All samples from Cobb group and stained with Hematoxylin and Eosin plus Alcian Blue, 200X. Source: The author (2019).

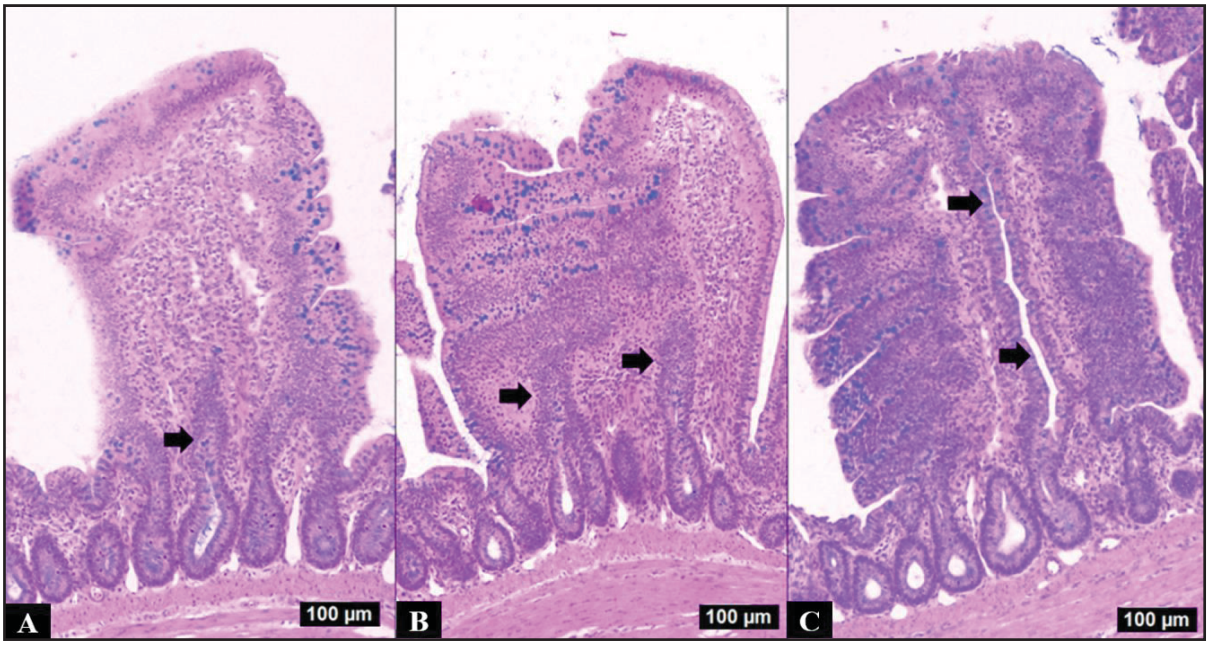
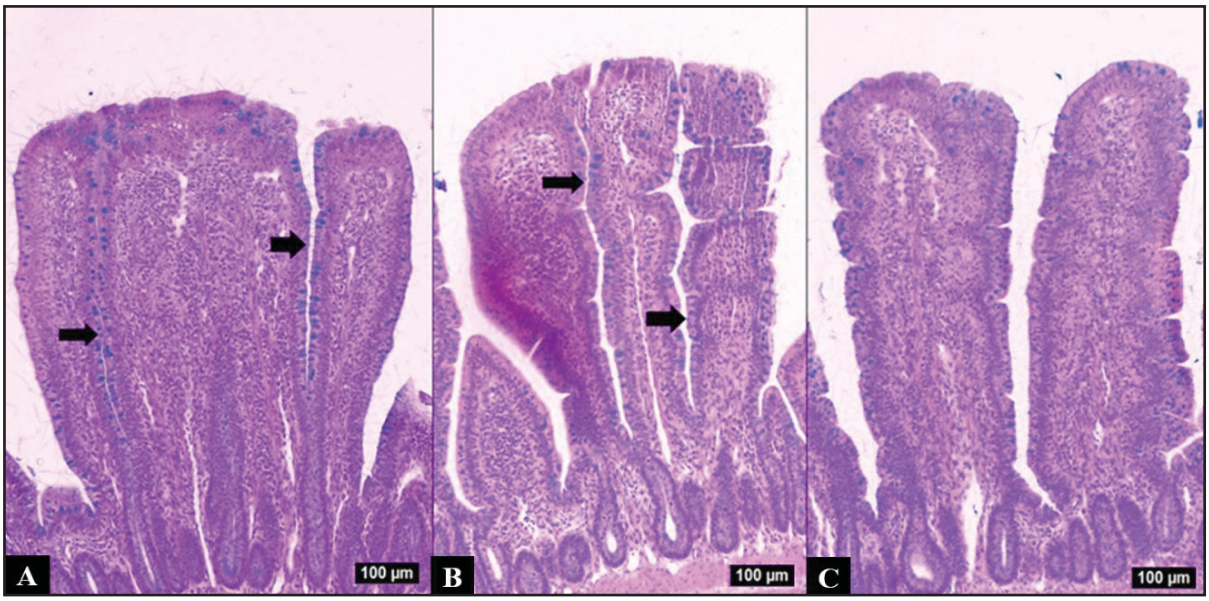


Figure 9: Formation of new villi from the proliferated fused ones in Ileum (cont). **A** and **B** -Vertical lines of epithelial cells kept to proliferate and invade the villus lamina propria creating new ones villi. **C** - New villi already formed. All samples from Cobb group and stained with Hematoxylin and Eosin plus Alcian Blue, 200X. Source: The author (2019).



Formation of the crypts were observed as small multifocal aggregations of epithelial cells inside the lamina propria, below the villi (Figure 8A). As their cells multiplied, a lumen could be observed and these niches fused their basement membranes (Figure 5C) aggregating the epithelial cell pools and by this creating a new bigger crypt that shed cells upward inside the lamina propria and also joined the epithelial layer in the common crypts (Figure 8A). From the crypt, the immature cells invaded the lamina propria and destroyed the smooth muscle, their basement membrane and the connective tissue (Figure 9A). In the sequence, a line of fission comes from the upper, the lower or even both surfaces creating new villi in the same area (Figure 9B). The final step was the formation of new villi (Figure 9C).

2.7 DISCUSSION

As observed in our study (Figure 1 and 2) the monolayer healthy villus was not the predominant type and most of samples. The proliferative epithelial status (hyperplasia of goblet cells and immature enterocytes) associated with inflammation was the hallmark of the sample evaluated. These histological reactions demonstrate how the simple measurements as villi high and crypt depth does not could be always related to better absorption function as described in previous study (SANTIN et al , 2002). The Feral group showed more severe intensities of **ANG**, **LPI** and **EI** than domestic chickens. **EI** and **ANG** were present also. The controlled and cleaner environment of the experimental Cobb groups and the diet could be linked to these differences, suggesting that environmental affect the mucosal response. The early age, 28 days in the Cobb 500® group could explain the results for the presence of **Nascent**, **NC**, **CC** and **AC** but could only be a speculation once we do not have all information about the age in the feral group

Epithelial proliferation of goblet cells and/or immature enterocytes and enteroendocrine cell were the standard response in both groups and were associated to inflammation. The direct consequence of this epithelial hyperplasia was the observation of shed villi and it pointed for a life cycle (**villi turnover**) on them in both evaluated groups.

Once the adult villi were affected by inflammation and proliferation, they became detached through the action of immature enterocytes invading the lamina propria.

Despite the domestication and the feralization processes, the nature of the innate intestinal tissue response (epithelial proliferation) was found similar between the groups. This proliferative trend in the avian gut can also be found, associated with inflammation, at least in one type of reptile, the leopard gecko (*Eublepharis macularius*) representing the birds' ancestor great group (TERREL et al., 2003). It could suggested the inheritance and preservation of these responses.

The structural immaturity of the invading hyperplastic cells could lead to a halt in the production of the epithelial basement membrane (**BM**) components causing its collapse allowing the passage of the immature cell. In the mammals the immature enterocytes are nominate Transition Amplifying cells (PANAYIDOU and APIDIANAKIS, 2013). We suggest that this proliferation of immature cells could be an innate response process of shedding resulting in the elimination of inflammation causative agents in the mucosa.

Similar conditions had been described in other animal species, under the general heading of proliferative enteropathy or enteritis**.** As for the mammals, the proliferative response in the intestine mucosa varies from a simple mucosal proliferation through adenoma to a more carcinomatous form (ERIKSEN and LANDVERK, 1985). Occasional reports of the condition in the fox (ERIKSEN and LANDVERK, 1985), the horse (DUHAMEL and WHEELDON, 1982) and guinea pig (ELWEL, 1980) describe a hyperplastic condition of the mucosa. In pigs, these kind of epithelial proliferations were named Proliferative Enteropathy and were proven to be induced by *Lawsonia intracellularis*.

In our study, the tissues evaluated showed very mild and microscopic lesions. This situation matches the one found in humans with Celiac Disease (**CD**) were the concept of Microscopic Enteritis (**ME)** has arisen from the mucosal changing our from score 0 to 3 (MARSH, 1992). This model leaded to the first description of the ME as a standard descriptive name or classification to be used in the histological descriptions of human chronic enteropathies (ROSTAMI, 2009).

The **ME** is characterized by mucosal abnormalities without prominent lesion of inflammation, villous effacement, erosions or ulcerations on conventional light microscopy.

The pathologists (SHAHRAKI, 2012) and veterinary pathologists generally overlook the weak intensities and distributions of the inflammatory infiltrate in the mucosa and the predominant absence of lesions. In the description of **ME** in humans, villi structure is largely preserved, but the epithelium is variably infiltrated by small lymphocytes, and there may be increases in crypt depth caused by crypt hyperplasia. An increase in plasma cells, eosinophils and other inflammatory cells is likely to be present in the lamina propria (ROSTAMI *et al*., 2015). In our avian samples, despites of absence of macroscopic lesion, the inflammation was also observed in the lamina propria and in the epithelium. The grading of the parameters observed in our evaluation from 0 to 3 also correspond with the Marsh classification. In the birds of our study, crypt hyperplasia was observed associated with the inflammation. In the chicken production, inflammation is associated with worse in weight gain, feed intake, feed efficiency and survivability (KOGUT, 2013; ISERI and KLASING, 2014).

In clinically and healthy broilers, some impairment in the performance was recorded and linked the microscopic proliferative enteritis as well (KRAIESKY *et al*., 2016 ; BELOTE *et al*., 2018).

Similar condition was described in humans were the presence of pro-inflammatory cytokines such as TNF seem to act at the level of the enterocyte inhibiting the uptake of the micronutrients such as iron (SHARMA *et al*., 2005) and phosphate (CHEN *et al*., 2009). The observed associated of inflammation and proliferation can be described under the Regenerative Inflammation (**RI**) concept.

Given the above, It encompasses the overlapping effects of the inflammatory cytokines and the actions of the growth factors acting on the Intestinal Stem Cells (ISCs) proliferation and differentiation. It also considers the infiltration of immune cells and the ISC microenvironment signals able to regenerate the epithelium (KARIN and CLEVERS, 2016; PANAYIDOU and APIDIANAKIS, 2013). The concomitant actions of the **ME** and **RI,** as observed in our study, would restore the microscopic health of the mucosa.

2.8 CONCLUSIONS

The logistics and difficultness in obtaining more intestinal feral samples limited more detailed results but the few samples of the feral group demonstrated a strong similarity in the intestinal inflammatory proliferative microscopic response between the two groups, despite the domestication and the feralization processes.

Moreover, the use of concepts from human pathology such as Regenerative Inflammation combined with Microscopic Enteritis allows a better understanding of the avian intestinal lesions of minor importance.

In addition, they allow the grouping of the findings in both groups as “Avian Proliferative Microscopic Enteritis” which would help to create more precise histopathological protocols in avian research.

CHAPTER 3: BASAL AND COCCI-CLOSTRIDIAL ENTERITIS IN BROILERS UNDER THE "I SEE INSIDE" METHODOLOGY: A CHRONOLOGICAL EVALUATION

Basal and cocci-clostridial enteritis in broilers under the "I See Inside" methodology: A chronological evaluation.

SANCHES, A.W.D [1], BELOTE, B. [1], HÜMMELGEN, P. [1], HEEMANN, A.C.W. [1], TUJIMOTO-SILVA, A.[1], SOARES, I. [1], TIRADO, A.G.C. [1], A. F. Cunha.[2], SANTIN, E. [1] *

[1]Department of Veterinary Medicine, Federal University of Paraná, CEP 80035-050 Curitiba/PR Brazil. Microbiology and Avian Pathology Laboratory. [2]Laboratório de Bioquímica e Genética Aplicada - Departamento de Genética e Evolução – Centro de Ciências Biológicas e da Saúde - UFSCar - São Carlos, SP, Brazil

***Corresponding author:**
Rua dos Funcionários, 1540 - Cabral, Curitiba - PR, 80035-050
E-mail: besantin@hotmail.com Tel +55(41) 3350 5859

Abstract

Recently, the inflammation of the intestinal mucosa have been related to many disease in human and animals. The concept of the Microscopic Enteritis **(ME**) used in human pathology through the Marsh classification system has no counterpart in veterinary medicine. In poultry science, the I See Inside (ISI) methodology, unlike the current linear measures of villi and crypts, displays possibilities to describe and understand the avian ME. Through specific parameters, graded from zero to three, the model links the proliferative and/or inflammatory reactions in the intestinal layers to some losses in performance. Herein, two trials were conducted in order to describe the development of ME through the ISI methodology in chickens challenged or not with *Eimeria* spp and *Clostridium perfringens*. In each trial, a total of 64 birds were divided in 2 treatments of 4 replicates with 8 birds each: non-challenged (**NCH**) and challenged (**CH**) through gavage with an *Eimeria* spp vaccine at 1 day of age and 10^8 CFU/mL of *Clostridium perfringens* administered at 10, 11, and 12 days of age. At 7, 14, 21 and 28 days of age birds were euthanized and samples of ileum and liver were collected for ISI evaluation, cytokines and presence of macrophages, CD4+ and CD8+ cell. The

results allowed the description of the avian Microscopic Enteritis and of its two basic components: the infectious enteritis and the Basal Enteritis (**BE**). In addition, the chronology of the ISI methodology parameters were associated to losses in zootechnical performance.

Key words: microscopic enteritis, basal enteritis, ISI, regenerative inflammation.

3.1 INTRODUCTION

The concept of the microscopic enteritis (ME) has been in use in human pathology through the Marsh classification system (ROSTAMI, 2009) to describe an inflammatory process in the intestinal mucosa histology in the absence of macroscopic alteration. In this system, scores from 0 to 3 describe the intensities of established morphological parameters such as inflammation in lamina propria (ILP) and epithelial inflammation (EI) (ROSTAMI et al., 2015). In veterinary medicine, similar concepts are still not currently applied except by the I See Inside (ISI) methodology that is a similar model which apply specific parameters to evaluate the intestinal mucosa and correlating the histologic changes to some losses in performance (KRAIESKI *et al*., 2016; BELOTE *et al*., 2018). In addition, as a specific property of the avian mucosa and unlike mammals, the strong proliferative reaction of the epithelial is describe in specific parameters for the enterocyte (EP) and goblet cell proliferation (GCP). The method concurs with the score intervals as in the Marsh classification, describing the histopathology of the ME in broilers in a more dynamic way that the traditional linear measurements of villi and crypts still used in the current poultry science. Since it encompasses inflammation in minor scale, it also accepts the concepts of ME up to the score 3, when after this level, the gross lesions star to show up. Under experimental conditions, the ME is better described through comparisons among challenged and not challenged groups. The final scores of challenged groups can be separated from the scores of the negative controls, resulting in a more realistic and specific impact of the challenging element on the tissues. The goal of this trial was to demonstrate and describe the existence of a constant basal intestinal inflammatory proliferative response (Basal Enteritis-BE) observed even in negative control birds raised in an experimental clean environment and how this response changes after the challenge with Eimeria *spp* plus *Clostridium Perfringens*.

3.2 MATERIAL AND METHODS

The two trials performed for this study were approved by the Institutional Animal Use Ethics Committee of Agricultural Sciences of the Federal University of Parana (Protocols 041/2016 and Protocol 081/2016).

3.2.1 *In vivo* experiment: birds, experimental design, diet and housing

The experiments were conducted in previously disinfected isolated rooms with negative pressure, containing vertically stacked cages (replications) with sterilized wood shaving litter (to avoid external contamination), nipple drinkers, and automatic control of temperature and lighting. These facilities are integrated to the Laboratory of Microbiology and Avian Diseases – LABMOR/UFPR, Curitiba, Brazil. At both trials, 64 male broilers Cobb® 500 were housed from 1 to 28 days of age, totalizing in two trials 128 birds. Both experiments followed a randomized design with 2 treatments of 4 replicates with 8 birds each. The treatments were: non-challenged (**NCH)** and challenged **(CH)** – birds inoculated with *Eimeria* spp and *Clostridium perfringens*. Animals were maintained in comfortable temperature according to their age, with feed and water *ad libitum*. The diet was based on corn and soymeal, following Brazilian nutritional recommendations for poultry (ROSTAGNO, 2011).

3.2.2 Performance

At 1 day of age, birds were distributed into treatments in a way to obtain an equal initial body weight in each cage (replicate) of a treatment. Birds and feed were weekly weighed (zero, 7, 14, 21, and 28 d) to evaluate feed intake (**FI**), body weight gain (**BWG**), and feed conversion ratio (**FCR**).

3.2.3 Challenge and sampling.

On the first day of age, all birds from CH treatment received the *Eimeria* vaccine (Bio-Coccivet – Biovet) by gavage. The doses were 15 times higher than the manufacturer recommendation (7.1 x 10^4 oocysts per bird) and the vaccine was stated to contain oocysts of *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. praecox, E. tenella* and *E. mitis*. At 10, 11, and 12 days of age, an inoculum of10^8 CFU/mL/bird of *Clostridium perfringens* was administered by gavage.

At 7, 14, 21 and 28 days of age, 6 birds per treatment (one bird per replicate) were euthanized by cervical dislocation and samples of ileum were collected for fixation and histological slides manufacture. At 7, 14 and 21 days samples of ileum and liver are also collected and placed in eppendorfs with 1mL of RNAlater for cytokines gene expression evaluation.

3.2.4 Cytokine expression

For cytokines mRNA expression, total RNA from ileum and liver was isolated using Trizol reagent (15596-018, Invitrogen, Carlsbad, CA, USA) following the manufacturer's procedures instructions. Turbo-DNAse kit (AM1907, Applied Biosystems, Foster City, CA, USA) was used for the collected samples. RNA concentrations were quantified by NanoDrop Spectrophotometer (ND1000, Thermo Scientific, Bonn, Germany) A total of 1 μg of RNA was treated with DNAse (Invitrogen-Thermo Fisher Scientific, Waltham, Massachusetts, USA) and was reverse transcribed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems-Thermo Fisher Scientific, Waltham, Massachusetts, USA). RT-qPCR was conducted using Power Sybr® Green PCR Master Mix (Applied Biosystems-Thermo Fisher Scientific, Waltham, Massachusetts, USA) on StepOnePlus Real Time PCR System (Applied Biosystems-Thermo Fisher Scientific, Waltham, Massachusetts, USA). All the primers were designed using OligoAnalyzer 3.1 (Integrated DNA Technologies, Coralville, Iowa, USA) and are listed in Table 1. The concentration of primers was optimized prior to the efficiency curve reaction and their efficiency ranging from 95%-105%. Relative fold change in mRNA quantity was calculated according to $2^{(-\Delta\Delta Ct)}$ method and all values were normalized to the expression of the human β-actin (BAC) gene.

Table 1: Primers used for cytokines gene expression

CYTOKINE	F 5' – 3'	R 5' – 3'
IL-10	AAGCAGATCAAGGAGACGTTC	GATGAAGATGTCGAACTCCCC
IL-8	CAGTTTCCTAGTCAGAGTCAGC	ACCAAACCCACAGTCTTACAG
GAPDH	TCTCTGGCAAAGTCCAAGTG	TCACAAGTTTCCCGTTCTCAG

3.2.5 Histopathology

For the fixation, segments of the ileum were stippled on a cartoon piece to avoid bending and immersed in Davidson's solution (100 mL glacial acetic acid, 300 mL 95% ethyl alcohol, 200 mL 10% neutral buffered formalin, and 300 mL distilled water) for at least 24 hours. All the samples were transversally trimmed, dehydrated, infiltrated and embedded in paraffin, following common histological routine. Blocks were cut in 5 μm sections. All the tissues were stained with Hematoxylin and Eosin plus Alcian Blue in the same slide for goblet cells staining (RAPP and WURSTER, 1978).

3.2.6 ISI methodology

The ISI® methodology was applied on the ileum samples from the two experiments and the evaluated parameters were listed in Table 2. In this methodology, an impact factor (**IF**) is defined for microscopic alteration according to the reduction of organ functional capacity, based on previous knowledge of literature and background research. The IF ranges from 1 to 3, where IF = 3 is provided to the most impactful alterations for the organ function (e.g., necrosis has the highest IF because the functional capacity of affected cells is totally lost). The extent of each lesion (intensity) or observed frequency compared to non-affected organs is evaluated though a score (**S**) that ranges from 0 to 3, where: score 0 – absence of lesion or frequency; score 1 – alteration up to 25% of the area or observed frequency; score 2 – alteration ranges from 25 to 50% of the area or observed frequency and score 3 – alteration extent more than 50% of the area or observed frequency.

Table 2. ISI histological alterations evaluated in ileum

Organ	Alteration	Impact Factor (IF)	Maximum[1] Score
Intestine	Lamina propria thickness - (**LPT**).	2	45
	Epithelial thickness - (**ET**).	1	
	Enterocytes proliferation - (**EP**).	1	
	Inflammatory cell infiltration in the epithelium - (**EI**).	1	
	Inflammatory cell infiltration in the lamina propria - (**LPI**).	3	
	Goblet cells proliferation – (**GCP**).	2	
	Congestion (**CO**).	2	
	Presence of oocysts (**OP**).	3	

*Maximum score represents the sum of all alterations according to the formula ISI = Σ (IF ∗S), where IF = impact factor (previous fixed) and S = Score (observed) considering the maximum observed S. For example, the lamina propria thickness has IF = 2; this number will be multiplied by observed score (range from 1 to 3), if in a villus, a score S = 3 (maximum score) was observed for lamina propria thickness, so the ISI for this parameter in the villi will be ISI = (2∗3) = 6. The average of 20 villi in the ileum for each bird will be the final ISI value for each bird. (BELOTE *et al*., 2018)

To reach the final value of the ISI index, the IF of each alteration is multiplied by the respective given score and the results of all alterations are summed, according to the formula ISI = Σ (IF x S). Twenty intestinal villi per bird were evaluated proportionally to the morphological distribution (merged and normal), in 10X objective (also used 40X objective to confirm alterations) of an optical microscope (Nikon Eclipse E200, Sao Paulo-SP- Brazil).

3.2.7 Immunohistochemical analysis

All samples were dehydrated, infiltrated, and embedded in paraffin following common histological routine. Immunohistochemistry slides were horizontally placed in a humid incubation chamber and incubated with 100 to 500 *µ*L of primary specific antibody for macrophages, CD4+, or CD8+ T-lymphocyte. Each antibody was placed on a different slide and washed 3 times with PBS. The slides were incubated for 30 to 60 min with HRGP conjugated antibody specific for the primary antibody, then peroxidase activity was blocked using DAB kit for immunocytochemistry (HRP-conjugated rabbit anti-mouse Ig, Dako North America, Carpinteria, CA, USA). The slides were counterstained with hematoxylin solution.

The labeled cells were counted in an optical microscope (400X magnification objective). Five fields per bird totalizing 30 fields per treatment of intestine were measured.

3.2.8 Statistical Analysis

The data were processed by the software “Statistix 9.0 ®” (Analytical Software) submitted to Shapiro–Wilk normality test, followed by one-way analysis of variance (ANOVA) and Tukey tests. Within the groups, the Pearson’s correlation was applied. The analysis compared the scores between NCH and CH groups at the ages of 7, 14, 21 and 28 days, the scores of the different ages within NCH and within CH. In addition, the performance data had the same treatment. Results were graphically and chronologically disposed.

3.3 RESULTS

The scores of the ISI© parameters were arranged in chronological graphics comparing the two groups for congestion (Figure 1A), goblet cell proliferation (Figure 1B), epithelium inflammation (Figure 1C), oocyst inflammation (Figure 1D), lamina propria inflammation (Figure 2A), Enterocyte Proliferation (Figure 2B), Epithelium Thickness (Figure 2C), Lamina Propria Thickness (Figure 2D) and I ISI Total Score (Figure 2E). In general, the scores for the CH group were higher than non-challenged groups for all parameters in all the period evaluated

In Figure 3 are presented the temporal difference between treatments in feed conversion ratio (3A) and body weight gain (3B). It was also observed the worst parameters in challenged groups compared to non-challenged ones. Figures 3C, 3D, and 4A to 4D presented immunohistochemical results of liver and ileum. Figures 5A to 5D.Tthe results of cytokines in the two groups at the different periods evaluated.

Figure 1: Ileum ISI parameters from 7D to 28D between challenged (CH) and non-challenged groups (NCH). A- Congestion (CO). B- Goblet cell proliferation (GCP). C-Epithelium inflammation (EI). D- Oocyst proliferation (OP). Lower case letters represent significant differences between groups.

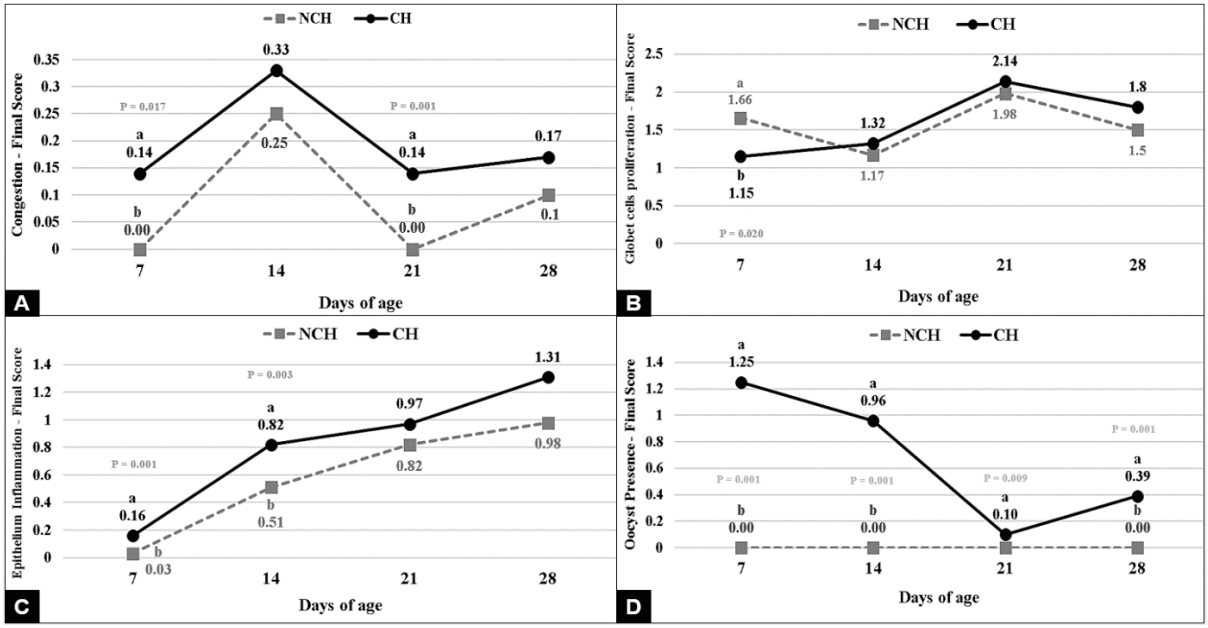
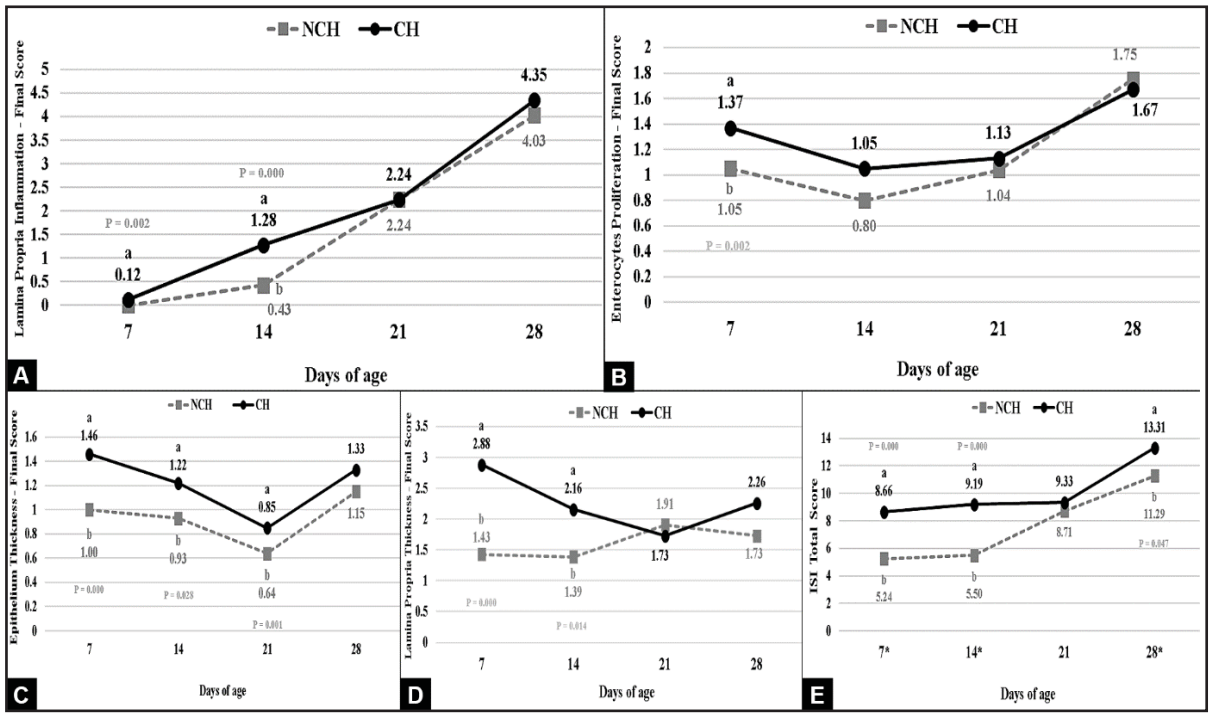


Figure 2: Ileum ISI parameters from 7D to 28D between challenged (CH) and non-challenged groups (NCH). A-Lamina propria inflammation (LPI). B- Enterocyte (immature) proliferation (EP). C- Epithelium thickness (ET). D- Lamina Propria thickness (LPT). Lower case letters represent significant differences between groups.



At 7 D days, it was possible to compare the groups according to the challenge with “15X” the *Eimeria* vaccine. The challenged group presented higher score of ileum congestion, epithelium inflammation, presence of oocyst, lamina propria inflammation, enterocytes proliferation, epithelium thickness, lamina propria thickness and total ISI score but lower goblet cell score compared to non-challenged ones.

These alterations are associated to inflammation of the mucosa where the higher presence of macrophage and CD8+ cell in ileum and liver and initial lower CD4+ cell in ileum and higher CD4+ cell in liver. There was also higher expression of RNAm IL-8 and IL-10 in liver and ileum in challenged group. Ileum IL-10 had a peak of expression at 14d and the same occurred in liver. At 21 D for the liver, there was no expression of IL-10 in ileum (Figure 5).

There was no difference in FI but the CH groups presented worst FCR at 7 and 21 days. It was interesting to observe that whatever the challenge the inflammation parameters increase, the chronological evaluation of ISI parameters show the same tendency in challenge and non-challenge birds, except for presence the oocysts. Macrophages, CD4+ cells show also a linear positive response in the ileum mucosa but CD8+ cell show a reduction from 7 to 28 days in the two groups.

In the liver the same tendency in the two groups for presence of macrophage, CD4+ and CD8+ cell were observed (Figures 14 to 16) and show also that this organ respond to the challenge with increase of this cell in the parenchyma compared to liver of non-challenged birds

Figure 3: Performance and immunohistochemical (ileum and liver) data from 7 to 28D between challenged (CH) and non-challenged groups (NCH). A-Feed Conversion ratio (FCR). B- Body weight gain (BWG). C- Ileum macrophage counting. D-Liver macrophage counting.

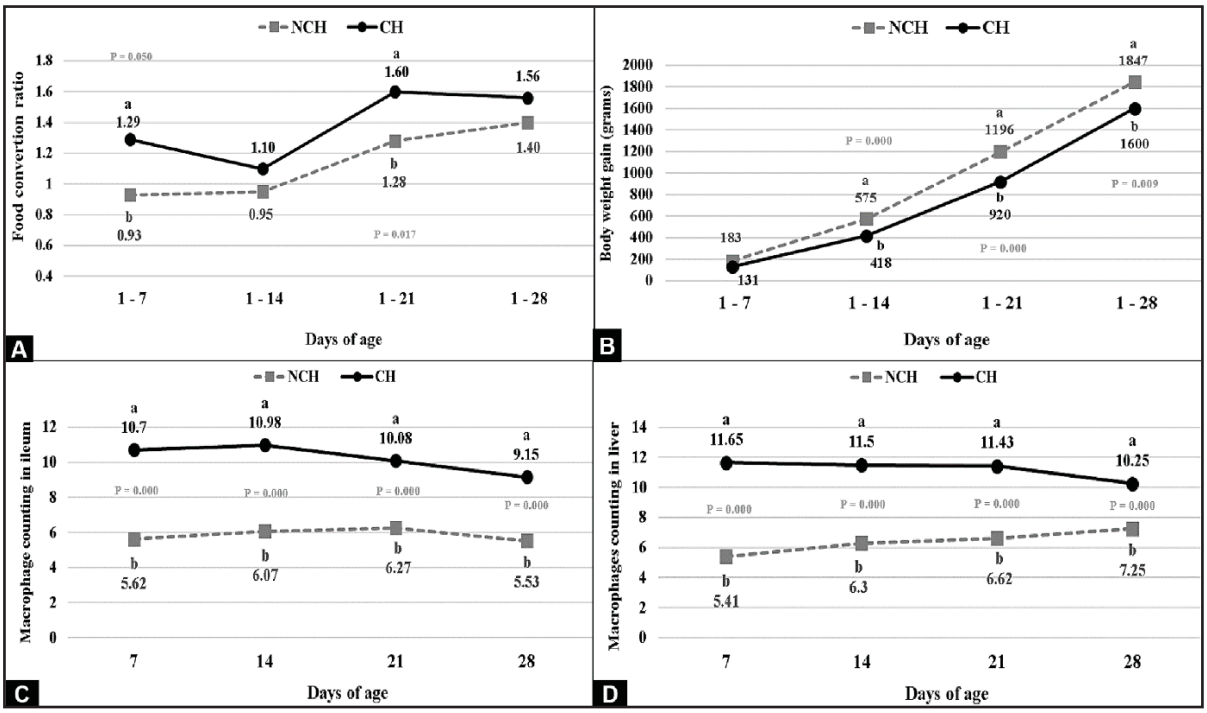


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Figure 4: Immunohistochemical (ileum and liver) data from 7 to 28D between challenged (CH) and non-challenged groups (NCH). A- Ileum TCD8+ lymphocytes. B- Liver TCD8+ lymphocytes. C- Ileum TCD4+ lymphocytes macrophage counting. D-Liver TCD4+ lymphocytes.

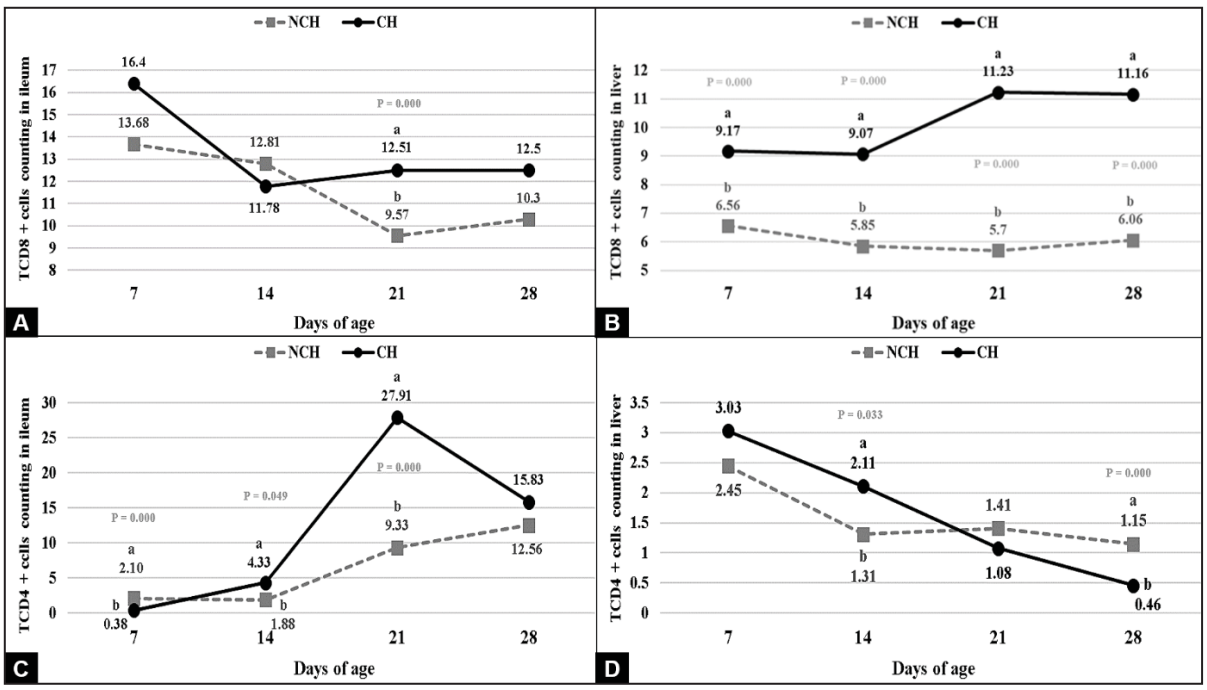

Figure 5. Interleukin data from 7 to 28D between challenged (CH) and non-challenged groups (NCH). A- Ileum interleukin 8 (IL-8). B- Ileum interleukin 10 (IL-10). C-Liver interleukin 8 (IL-8).D- Liver interleukin 10 (IL-10). Lower case letters represent significant differences between groups

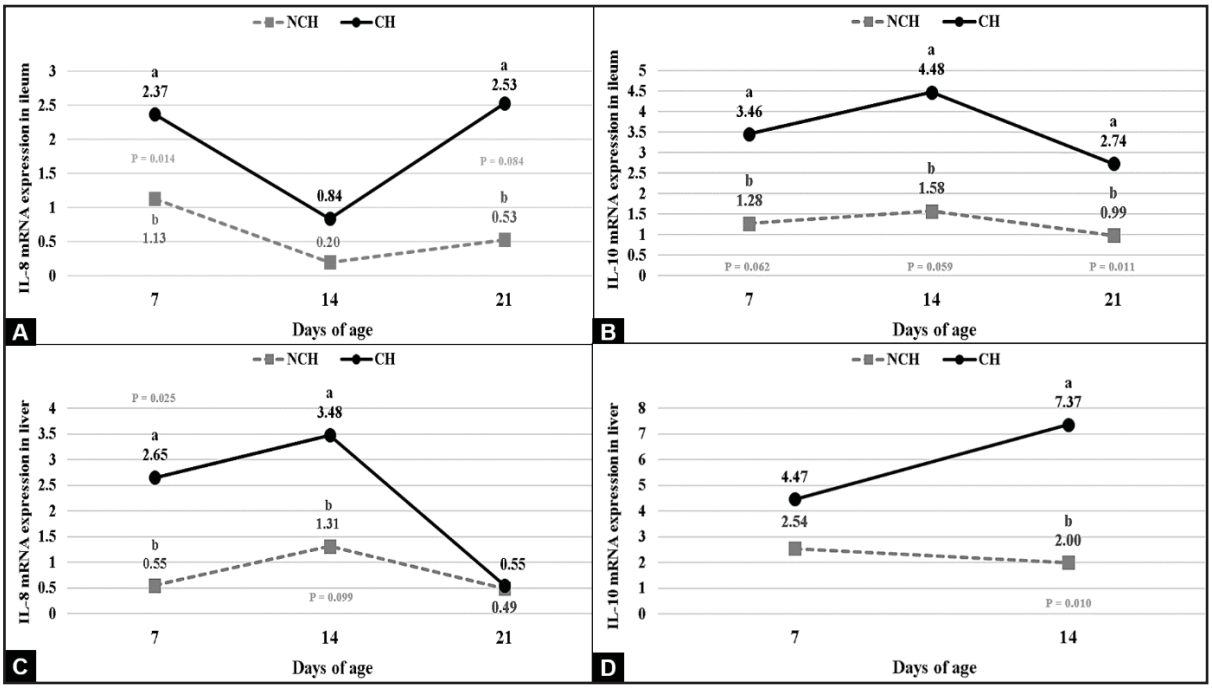

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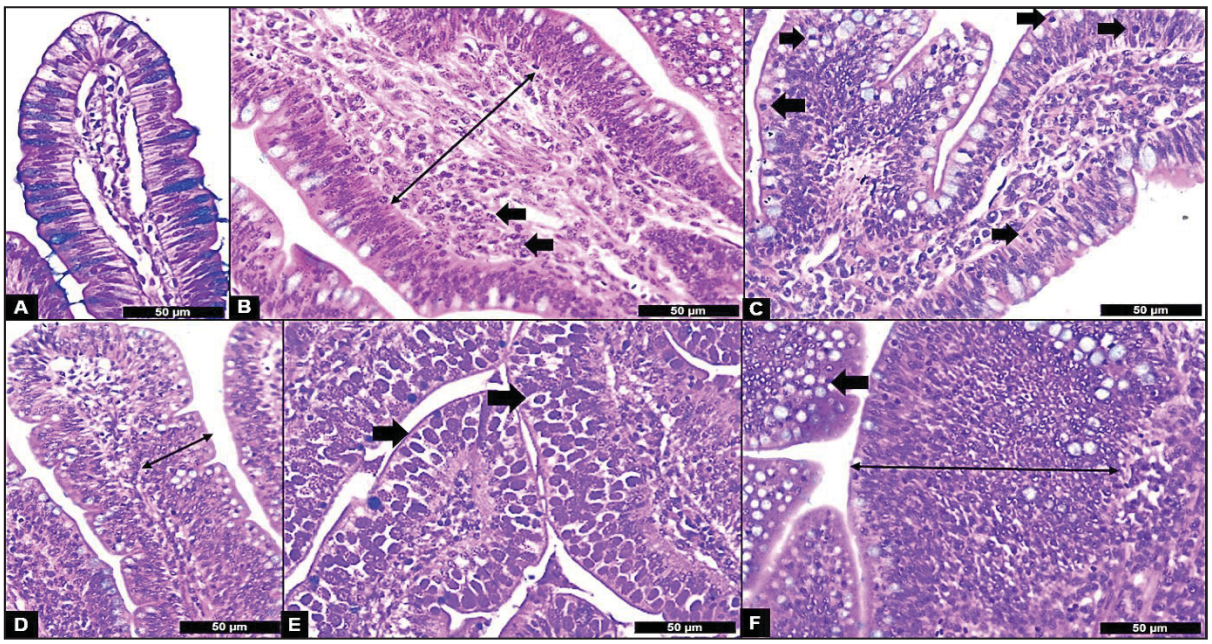
Figure 6: Microscopic Enteritis lesions and ISI parameters. A-Normal villus. B-Lamina propria thickness (LPT) grade 2 (long arrow) and lamina propria infiltrate (LPI), in arrows. C- Epithelial inflammation by lymphocytes (EI). D- Epithelial thickness (ET) grade 2. E- Oocysts proliferation (OP). F – Goblet cells proliferation (GCP) grade 2 (arrow) and Enterocyte proliferation (EP) grade 3 (long arrow). All samples stained with Hematoxylin and Eosin plus Alcian Blue and 400X. Source: The author (2019).



3.4 DISCUSSION

In animal production, enteritis, here described as congestion, ILP and/or EI, is associated with a decrease in weight gain, feed intake, feed efficiency and survivability, (KOGUT, 2013; ISERI and KLASING., 2014). The paradox of the ongoing poultry science and industry is the acceptance of inflammation, as an entity that requires study, associated with the lack of appropriate histopathological descriptions of the inflammation on mucosa. The situation is worsened by the solely use of current linear measures of villi and crypts dimensions and areas that do not consider the changed histology of the mucosa.

Such status is also reinforced by the fact that many pathologists do not regard lesions of minor importance (SHAHRAKI, 2012) and the daily fact these mild inflammatory response do not have evident or distinct gross changes. Herein we demonstrate that in *Eimeria* challenged and in healthy broilers it is possible to describe a microscopic proliferative enteritis and that increase in the intensity of this parameters are associated to lower zootechnical performance as presented in our previous studies (KRAIESKY et al., 2016 ; BELOTE et al., 2018, 2019). In human pathology, the idea of a microscopic inflammation in the absence of gross lesions has been accepted and described for patients with Celiac Disease (**CD**) were the concept of Microscopic Enteritis (ME**)** has arisen from the mucosal changes and described by the Marsh 0 to 3 categories (MARSH, 1992).

This model leaded to the first description of the human ME as a standard descriptive name or classification to be used in the histological descriptions of human chronic enteropathies. Mucosal abnormalities without prominent inflammation (no gross lesions), villous effacement, erosions or ulcerations on conventional light microscopy (ROSTAMI, 2009) characterize it. In avian medicine, such concept is not in use and the only closest protocol is the ISI methodology (KRAIESKY et al., 2016; BELOTE et al., 2018, 2019). This method allows use the scores for inflammatory parameters and do mathematical interpretation of the changes in the microscopic helm.

In the preset study we observe that, however the challenge present an increase in the inflammation parameters, the chronological ISI evaluation show the same tendency in challenge and non-challenge birds, except for presence the oocysts.

Through this, the chronological behavior of parameters can be evaluated, in experimental conditions, using graphics that compare the scores of the non-challenged (NCH) groups versus the ones of the challenged groups (CH). The NCH data would represent the Basal Enteritis (BE) and the CH data would represent the real pathogenic enteritis, in this case, the *eimeria*-clostridial enteritis (ECE) that could be explained by the results of mRNA expression of cytokines and the presence of macrophages and CD8+ cell on intestinal mucosa and liver. In the graphics, from day 7, all the scores already started with some intensity ($p \leq 0.01$). The values at 7D can express the first contact of the mucosa with the feed, litter, water, feces and other factors.

The scores of CO, EI ($p \leq 0.003$), LPI ($p \leq 0.001$) and TO ($p \leq 0.001$), raised from 7 D to 14 D. All the three represented the general inflammatory process. The LPI (both 7d and 14d $p \leq 0.001$) and EI (all 7d, 14d and 28d $p \leq 0.001$) of CH, had a continuous rising up to 28 D, despite the falling in the OP scores from 7 D to 21 D and a minimum rise from 21 D to 28 D (all $p \leq 0.001$). The LPI and EI in the NCH raised constantly up to 21 D, going along with the inflammations in the CH group, except from 21 to 28 D when EI reduced ($p \leq 0.050$). CO did not accompany the inflammatory process from 14 D to 28 D (21d $p \leq 0.013$). It could describe the congestion as being reduced as the inflammation become more chronic and associated with OP which reduced from 14 d to 21 D.

Despite no significant value between NCH and CH, GCP concurred with EI and raised from 7 D to 21 D, fell from 21 to 28 D and considering the common presence of both parameters in the epithelium and their close contact with the luminal content, the link was demonstrated. ET, was reduced (all $p \leq 0.001$) continuously from 7 to 21 D and concurred with the fall of OP. LPT (CH) had a constant fall from the beginning leveling the scores with the NCH group. From 21 D to 28 D, for both groups, an increase of TO, ET, EP, LPI, OP and EI was observed and a decrease of CO, GCP, and LPT. The concurrence of these parameters between the two groups, would point a modulatory immune event in the tissue at 21 D.

The common raise of ET (21D $p \leq 0.001$) and EP with the fall of GCP from 21D to 28D would point the proliferated enterocyte as the major cell present in the thickened epithelium reacting again due a small rise in OP ($p \leq 0.017$). Yet, the general fall in EP from 7D to 21D concurred with the decreased ET in these intervals.

The fact the challenge contained *Eimeria spp*, affecting the enterocytes, concurs with the data pointing the enterocyte as a major cause for changes in the ET.

The general behavior of the NCH scores as being smaller than those of the CH group concurs the effectiveness of the infection. In addition, it demonstrated the amount of the BE as a component of the ME observed and its modulation of the CCE responses. The presence of EI and LPI in both groups and in a constant rise can be explained by the constant recontamination of the litter by feces, urine and food rests, even in the advent of previous sterilization of the litter and food.

The final scores (**BE** plus **ECE**) would express the real scores found in field samples and the synergic effect between **BE** and **ECE** would lead to less efficiency in performance**.** In some situations, as the inflammation induces a strong proliferative portrait of the avian mucosa, the epithelial multiplication could act in favor of the bacterium, *Clostridium perfringens*, entry and colonization as seen in clostridial induced diseases interacting with oocysts. In this study, the Body Weight Gain (BWG) (all $p \leq 0.01$) had a decrease from 7 D to 14 D, so had the Feed Conversion Ratio (FCR) in the same interval but with no significant value.

The histological parameters that concurs (raised) on these same days were the constant raising LPI and EI, the GCP and CO as increase in presence of macrophage and CD4+ cell as mRNA expression of IL-8 and IL-10 in the mucosa and liver. CO associated with LPI and EI could explain the BWG and FCR results since the increased hydrostatic pressure in the capillaries of the lamina propria would decrease the assimilation of nutrients passing through diffusion or lead to a stasis in the blood flow resulting in a delayed transport into the blood stream.

As observed in humans, the presence of pro-inflammatory cytokines would seem to act at the level of the enterocyte inhibiting the uptake of the micronutrients such as iron (SHARMA et al, 2005) and phosphate (CHEN et al, 2009). The LPI would increase the congestion and the exudate in the interstitial portions of lamina propria, worsening the intake of the nutrients by the capillaries. At 7 D, OP, EP, LPT and ET were higher and this was associated with a significant higher level of IL-8 and CD4+ lymphocytes At this age, the oocysts proliferation (Figure 4) produced an inflammatory response that induced the enterocyte proliferation with immature cells and consequently the increasing in the thickness of the epithelium.

Considering the role of IL-8 as a heterophil attractant, the results well describe a still acute phase of inflammation. In addition, its production by mesenchymal cells such as fibroblasts and endothelium (ACKERMAN, 2007), describes injury in the lamina propria expressed significantly by CO (Figure 1), LPI (Figure 5) and LPT (Figure

8) at 7D in the CH group. EI was also significantly higher in the CH group at 7D. At 21 D, GCP was peaked and this was concomitant with a high expression of mRNA IL-10, CD8+ and CD4+ lymphocytes. No other parameter was peaked in this age. This could point the joining action of the IL-10 and the T lymphocytes over the GCP. The higher amount of CD4+ in the CH group in this age concurs with the high amount of IL-10 since this interleukin is linked to this cell population in the adaptive phase by stimulating the B-lymphocytes to make antibodies and inhibiting the innate response (ACKERMAN, 2007).

Reduced Ileum IL-8 at 14D and Ileum IL-10 peak concurs with each other since IL-10 is also described as having anti-inflammatory immune suppressive effect over the acute phase controlling the nature and extent of inflammatory responses during infection with viruses, bacteria, fungi, protozoa and helminths causing the observed reduction in IL-8 (COUPER et al, 2008; MOORE et al, 2001). At 21 D for the liver, there was no expression of IL-10 while there was significance at 21D in ileum. It demonstrated the relative independence of the liver when ileum IL-10 level were not high enough to affect liver.

In addition, it point the barrier function of the intestine and the liver codependency of the systemic blood flow from hepatic arteries.

The presence of a CD8+ population at 21D could be explained by some initial 21d to 28D, persistence of an acute phase response induced by the constant recontamination from the litter by oocysts (Figure 4). IL-8 raise in this age concurs with OP re-raising and with a reduced IL-10 levels allowing the acute phase to raise start again despite the reduced severity (COUPER et al, 2008; MOORE et al, 2001).

Macrophage response behaved as expected for the CH group as for any kind of challenge. The observed inflammations (**EI** and **LPI)** and proliferations (**EP** and **GCP**) can be described under the concept of the **Regenerative Inflammation (RI)**. It encompasses the overlapping effects of the inflammatory cytokines and the actions of the growth factors acting on the Intestinal Stem Cells (**ISCs**) proliferation and differentiation.

It also considers the infiltration of immune cells and the ISC microenvironment signals able to regenerate the epithelium (KARIN and CLEVERS, 2016; PANAYIDOU and APIDIANAKIS., 2013; MACDONALD, 1992). The concomitant actions of the **ME** and **RI** would work together restoring the health of the mucosa.

3.5 CONCLUSIONS

The data described the morphological persistence of a **constant basal intestinal inflammatory proliferative response,** the Basal Enteritis, as a component of the total level of the Aviary Microscopic Enteritis worsened by an experimental *eimeria*-clostridial challenge, producing the real observed enteritis.

CHAPTER 4 - ISOQUINOLONE ALKALOIS MITIGATES MICROSCOPIC DIGESTIVE TRACT LESIONS INDUCED BY SUB-ACUTE RUMINAL ACIDOSIS (SARA) IN FEEDLOT CATTLE

Isoquinolone alkaloids mitigates microscopic digestive tract lesions induced by sub-acute ruminal acidosis (SARA) in feedlot cattle.

Sanches, A.W.D[1].; Montiani-Ferreira, F*[1].; Santin, E[1]; Neumann, M[2]., Reck, Â.M[2].; Bertagnon,H.G[2].; Pachaly, J.R[2].

[1]Department of Veterinary Medicine, Federal University of Paraná, CEP 80035-050 Curitiba/PR Brazil. Microbiology and Avian Pathology Laboratory;[2] Programa de Pós Graduação em Ciência Animal da Universidade Estadual do Centro Oeste (UNICENTRO), Guarapuava, Paraná, Brasil.[7] Programa de Pós-Graduação em Ciência Animal da Universidade Paranaense (UNIPAR), Umuarama, Paraná, Brasil.

***Corresponding author:**
Rua dos Funcionários, 1540 - Cabral, Curitiba - PR, 80035-050
E-mail: besantin@hotmail.com Tel +55(41) 3350 5859

ABSTRACT

The current trend of removing growth-promoting antibiotics such as ionophores, used to control the sub-acute ruminal acidosis (SARA), has created an entire new focus on alternatives to control infectious diseases and enhance performance in livestock. Many natural alternatives have being investigated and the compounds found in *Macleaya cordata* (Papaveraceae), sanguinarine and chelerythrine, have demonstrated antimicrobial, anti-inflammatory and immune-modulatory effects in both humans and animals. The aim on this study, using histopathology and a score system, was to evaluate the differences between a non-treated and a treated group feed with these isoquinolone alkaloids, present in trade preparation Sangrovit-RS® as a source of sanguinarine (SG), chelerythrine (CH) and protropine (PA) standardized to 0.15% w/w SG, using feedlot cattle under a high-grain diet as an inflammatory model for gastrointestinal system. The samples of forestomachs were evaluated and graded using scores ranging from zero (0) to three (3) obtained at light-microscopic fields of 400X. Parameters such as inflammation, hydropic degeneration, hyperkeratosis, and

vesicle formation were accessed in the different layers of the tissues, considering the severity and dispersion of the microscopic lesions. The soft tissues such as the abomasum, small intestine, cecum and colon had their total amount of inflammatory cells counted at light-microscopic fields of 200X. The rumen of the SG-CH-PRO-treated group showed a significant reduction in the epithelial hydropic degeneration scores ($p \leq 0.001$) and lamina propria inflammation ($p \leq 0.001$). The reticulum had a similar reduction in all scores of epithelial ($p \leq 0.002$) and stratum corneum ($p \leq 0.001$) hydropic degeneration, All scores of hyperkeratosis ($p \leq 0.002$) and minor inflammation in lamina propria ($p \leq 0.001$) and epithelium ($p \leq 0.002$). The omasum had no significant differences. All non-keratinized tissues, except for ileum, had a significant decrease ($p \leq 0.001$) in the total counting of inflammatory cells. In this trial, the feedlot cattle feed with high grain diet and treated with isoquinolone alkaloids expressed lesions that indicate a weak anti-inflammatory effect associated with a reduction in weak epithelial cell edema alongside significant reducing in the amount of inflammatory cells of the small and large intestine.

Keywords*:* Isoquinolone alkaloids, Ruminitis, SARA.

4.1 INTRODUCTION

The demanding global meat market has induced a dietary change in feedlot cattle, replacing traditional diets based on 70% forage and 30% concentrate with more energy containing diets composed of higher percentage of grains (MILLEN and ARRIGONI, 2013). The direct side effects of these diets are the acute and the chronic form of ruminal acidosis (SARA). Ionophore antibiotics have been used to control SARA in ruminants feed high-carbohydrate diets because the absorption of very short chain fatty acids (VFAs) and H+ from the rumen are stimulated and thus reducing the ruminal pH (COSTA et al., 2008).

These diets also have a reduced proportion of fiber reducing ruminal motility and favor VFAs to pool in the rumen, helping to induce SARA, alongside lactic acid production (PLAIZIER et al., 2012). SARA results in negative effects on the animals' performance and health, as the microbiota and low pH changes even affects the ruminal wall integrity. Associated with Gram negative bacterial lysis and the releasing of endotoxins known as lipopolysaccharides (LPS), the start of an inflammatory cascade occurs and this may lead to rumenitis, laminitis, liver abscesses, damage digestibility and nutrients absorption. Therefore, lower performance feed and conversion ratio will take place consequently (SATO, 2015). However, the concerns raised about possible drug residues in meat-derived products and about the growing number of reports on multiple antibiotic resistant bacteria had led to alternative strategies to replace the additives (YANG et al., 2009). Natural products started to be recognized as important source of therapeutically effective substances in food animal production. These phytogenic feed additives are commonly defined as plant-derived compounds incorporated into feed to improve the productivity of livestock (WINDISCH et al., 2008).

A number of plant compound used in livestock are also used in traditional medicine. One of these plants, the *Macleaya cordata* (Willd.) R.Br. (Papaveraceae) is used in the commercial product Sangrovit-RS®. Its compounds are classified as benzo[c]phenanthridine alkaloids (QBAs), represented mainly by the sanguinarine (SG) and chelerythrine (CH). This group also includes protopine (PRO), allocryptopine (ALL), dihydrosanguinarine (DHSA), dihydrochelerythrine (DHCHE), oxysanguinarine (OSA) and berberine (BER) (ZHI-BI et al., 1979; XU-BIAO et al., 2005). SG and CH are known to have antimicrobial, anti-inflammatory and immune-modulatory effects in

humans (e.g. dental health) and animals (LENFELD et al., 1981; NEWTON et al., 2002; JANKOWSKI et al., 2009).

The best-known property of SG is an antibacterial effect, already described in the 1950s (JOHNSON et al., 1952) and confirmed *in vitro* and *in vivo* in later researches (WALKER, 1990; HERRERA-MATA et al., 2002; MAHODY et al., 2003). Sangrovit-RS® is used as a source of these alkaloids, obtained from intact aerial parts of the plant resulting in a fraction of quaternary benzo[c]phenanthridine alkaloids (QBAs), mainly SG, CH and protopine (PRO) being standardized to 0.15% w/w SG (ZDARILOVA et al., 2010; KOSINA et al., 2010).

The aim on this study was to evaluate the histopathological changes in tissue samples from the digestive tract of feedlot cattle receiving a high-grain diet as an inflammatory model, and compare results between the group treated with isoquinolone alkaloids (Sangrovit-RS®) and a control group.

4.2 MATERIALS AND METHODS

4.2.1 Location, facilities and nutrition

The research was conducted and executed at the Programa de Pós Graduação em Ciência Animal da Universidade Estadual do Centro Oeste – UNICENTRO (Animal Science Graduate Program of the UNICENTRO) in the state of Paraná, Southern Brazil. The pens and the animals were housed in the UNICENTRO's Animal Production Center, in the city of Guarapuava, State of Paraná, Brazil (lat-25º 23' 43" S, lon-51º 27' 29" W, and alt-1098 m). Sixteen Angus half-breed non-neutered bulls fed with a high grain diet were orally supplemented with trade preparation of Sangrovit-RS® made from *Macleaya cordata* by the Phytobiotics Feed Additives GmbH (Eltville, Germany). The bulls were eleven months in age, initially weighting 350 kg in average being allocated in two per pen. The study was double-blinded. One group of eight animals served as the control and received only a high grain diet and the other group of eight animals received the same diet plus 4 g / day of trade preparation Sangrovit-RS®, resulting in approximately in 6 mg of SG a day. The research lasted 116 days. On Day 0, the animals received a diet composed of concentrated mixture and corn silage.

The diet was balanced in 80 % of whole corn grain, 20 % of protein-vitamin-mineral. The pens were supplied with automatic waterers. Diets were provided *"ad-libitum twice"* a day, at 7:00 a.m. and 5:30 p.m. The treated group received corn kernel (10 g per animal) with Sangrovit-RS®, poured over the mixed feed (RTM), in the moment of the feeding. The RTM was prepared at the Agricultural Cooperative commercial feed plant located in the Entre Rios district (Guarapuava, PR, Brazil). The following food items were used to prepare the pelleted protein concentrate: soybean meal, wheat bran, malt radicle, calcitic limestone, dicalcium phosphate, livestock urea, vitamin and mineral premix, common salt, monensin sodium (75 mg/kg^{-1}) and virginiamicin (75 mg/kg^{-1}). This way, the diet protein nucleus presented the percentage mean contents of: 90.22% MS; 42.23% PB; 2.59% EE; 24.61% FDN; 12.28% FDA; 16.31% MM; 2.77% Ca; 1.11% P; 1.57% K and 0.48 mg based on the total dry material. The trade preparation Sangrovit-RS® was added over the ration in the feeding moment to assure the ingestion by the animals. The composition the ration included soybean meal, wheat meal, malt sprouts, lime, dicalcium phosphate, agricultural urea, vitamin-mineral premix, sodium chloride, sodic monensin (75 mg kg^{-1}) and virginiamicin (75 mg kg^{-1}).

4.2.2 Sampling, histopathological process and data acquisition

The slaughterhouse was located in the same city. During slaughtering, the removed gastrointestinal tissues were grossly evaluated and photographed when distinct lesions were seen. All sixteen forestomachs, abomasa, duodena, jejuna, ilea, ceca and colons had their mucosae exposed, evaluated and had their samples collected (5-10 cm in length) for fixation in 10 % buffered formalin.

Each larger tissue sample resulted in three trimmed samples per glass slide. Rumen was sampled in the cranial, middle and caudal portions of the ventral sac. The dorsal sac was sampled in the middle portion. Reticulum, omasum, abomasum, duodenum, jejunum, ileum, cecum and colon were sampled in their middle regions. All samples were routinely processed for confection of histological paraffin blocks with subsequent staining by Hematoxylin and Eosin. The scoring of keratinized tissues was accomplished by checking eight light-microscopy fields of 400X per sample.

The scoring model was based on the ISI® method (KRAISEKY et al., 2017; BELOTE et al., 2018), where determination of the scores considers extension, intensity and observed frequency of the histopathological change.

The following scores were used: Score 0 (absence of lesions or presence of minor ones), Score 1 (25% of the total area of the tissue showing lesions), Score 2 (25 to 50% of the area showing lesions), and Score 3 (more than 50% of the area examined showing lesions). The soft tissues such as abomasum, small intestine, cecum and colon had five fields per sample photographed under light-microscopy fields of 200X, with subsequent manual marking and counting of all types of their inflammatory cells, using the software Windows Photo Paint®.

4.2.3 Histopathological parameters accessed.

The ruminal, reticular and omasal parameters compared were: inflammation in lamina propria, stratum corneum (pustules), epithelium (micro abscesses), hydropic degeneration in the epithelial cells, hydropic degeneration in the stratum corneum, hyperkeratosis in the stratum corneum, vesicle formation in the epithelium and vesicle formation in the stratum corneum. For the omasum, the parameter edema in the lamina propria was added.

4.2.4 Statistical analysis

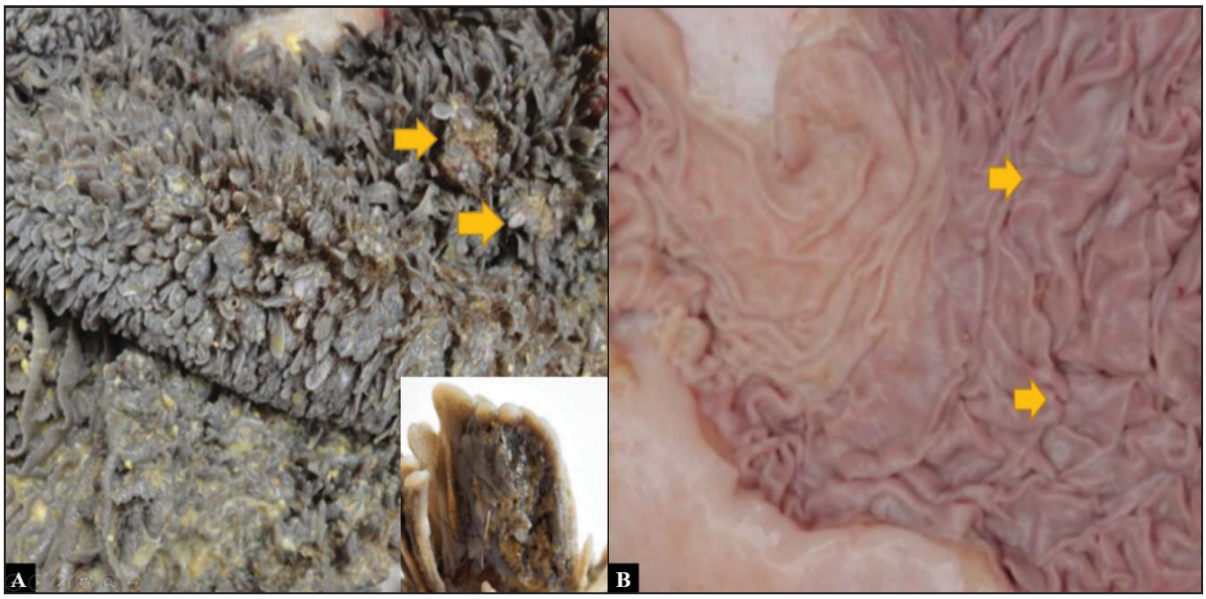
Shapiro-Wilk test demonstrated that the data was not normally distributed. A Mann–Whitney U test was used for data comparisons of the SG-CH-PRO-treated versus non-treated group regarding the 0-3 histopathology scores and an unpaired t-test was sued to compare the total number of inflammatory cells between the two groups, using StatView® 5.0.1 (SAS Institute, Cary NC).

4.3 RESULTS

4.3.1 Gross and Histopathology

In both groups, the gross evaluation of the rumen, reticulum and omasum revealed the presence of diffuse hyperkeratosis (parakeratotic) in the papillae with some multifocal areas of severe hyperkeratosis (fused and thicker papillae) in the ruminal mucosa (Figure 1A). Most mesenteric lymph nodes were enlarged. Occasionally, congested livers with a weak nutmeg portrait were seen. Hyperemia in the abomasum mucosa was seen in some animals (Figure 2B). Erosions/ulcers, sites of hemorrhage or scars were not observed. The microscopic evaluation of the rumen, reticulum and omasum revealed that most papillae had at least some amount of lesion and that a minor number of papillae were normal (Figure 2A).

Figure 1: Gross lesions of SARA. A - Rumen with multifocal areas of severe hyperkeratosis (arrows) containing fused and thicker papillae (window) in the mucosa. B - Abomasum with hyperemia (arrows).

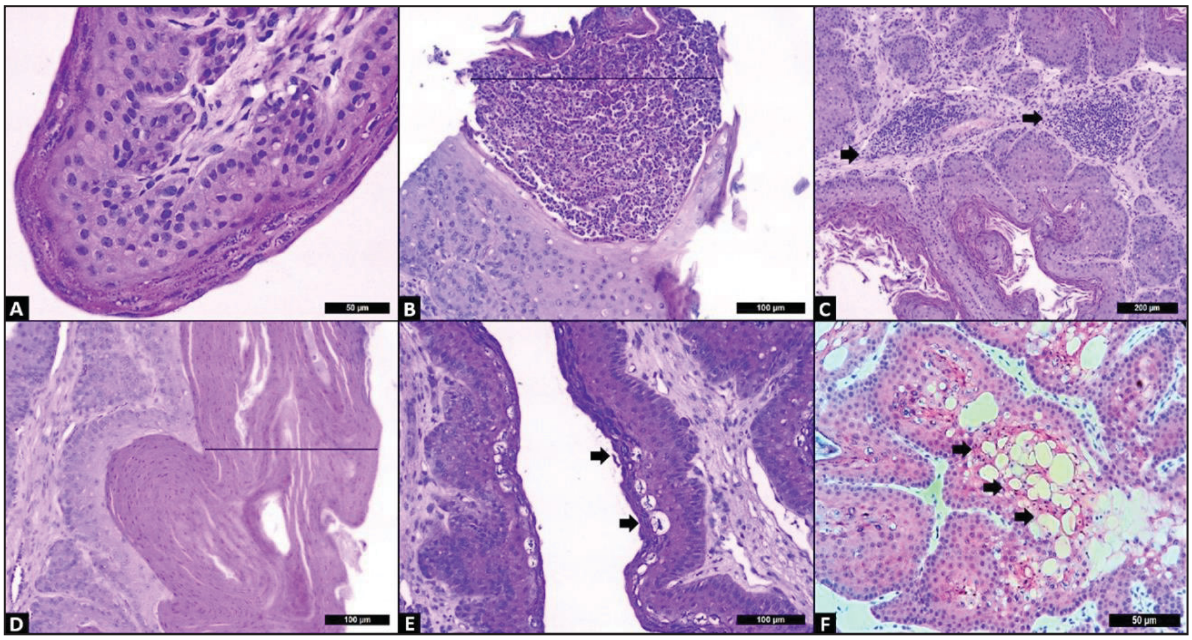


Occasionally, congested livers with a weak nutmeg portrait were seen. Hyperemia in the abomasum mucosa was seen in some animals (Figure 2B). Erosions/ulcers, sites of hemorrhage or scars were not observed. The microscopic evaluation of the rumen, reticulum and omasum revealed that most papillae had at least some amount of lesion and that a minor number of papillae were normal (Figure 2A). Microscopically, in the rumen, scattered neutrophils and multifocal micro abscesses were present in the stratum corneum **(**Figure 2B). In the lamina propria a diffuse chronic inflammatory infiltrate composed by lymphocytes and macrophages, with a small amounts of neutrophils was observed (Figure 2C). Additionally, hyperkeratosis (Figure 2D), epithelial inflammation, epithelial hydropic degeneration (Figure 2E), corneal pustules, corneal vesicles (Figure 2F) were also observed. Reticulum had normal papilla (Figure 3A) and similarly lesioned ones except for the absence of the corneal pustules/abscesses.

Microscopic lesions in the abomasum comprised inflammation (abomasitis) expressed by multifocal or extensive focal areas of inflammatory cells in the lamina propria of the gastric glands (Figure 3B). In some areas of the mucosa, a minor fibrinous exudate was be seen in the surface of the mucosa. The main microscopic change in the omasum was a chronic inflammatory infiltrate, located mainly in the lamina propria, composed by lymphocytes and less amount of macrophages with some amount of neutrophils. The intensity of the inflammation was smaller than the one

observed in the forestomachs. Edema in the lamina propria was evident (Figure 3C). The intestinal microscopic predominant lesion was a diffuse chronic inflammation (enteritis) located mainly in the lamina propria of the duodenum **(**Figure 3D), jejunum, ileum (Figure 3E), cecum and colon (Figure 3F). The thickness of the lamina was increased in these parts. The pools of inflammatory cells were composed by lymphocytes and macrophages with a minor amount of neutrophils. Foci of necrotic epithelial cells were not observed. In some fields, some amount the inflammation was located around the villi.

Figure 2: Forestomachs microscopic lesions in SARA. Normal ruminal papila, 400X (A); Ruminal corneal pustule, score III, 200X* (B); Ruminitis score II, 100X* (C); Ruminal corneal hyperkeratosis score III, 200X* (D); Ruminal epithelial hydropic degeneration, 200X* (E); Ruminal epithelium vesicles, 400X* (F); All samples stained with Hematoxylin and Eosin. *Different magnifications were chosen to better demonstrate the lesions. Source: The author (2019).



4.3.2 Statistical results

In the analysis of the rumen, treated animals had significantly higher numbers of score 0 (absence of lesions) per HPF for inflammation in the lamina propria ($p \leq 0.001$) and significantly less fields with higher scores of epithelial hydropic degeneration ($p \leq 0.001$). Conversely, in the non-treated group, significant less HPFs ($p \leq 0.017$) with higher scores for stratum corneum vesicles were found. Mean ruminal pH values, obtained the day before the inclusion of the diet, were 7.0 in both groups.

In the day after the inclusion of the diet, both groups had pH 6.0. In the day of the slaughtered, the control group had pH 5.5 and the SG group had pH 6.0.

For the reticulum, treated animals demonstrated significant reduced numbers of scores for lamina propria inflammation ($p \leq 0.001$), epithelial hydropic degeneration ($p = 0.025$), stratum corneum hyperkeratosis ($p = 0.002$), stratum corneum inflammation ($p = 0.001$), epithelial inflammation ($p = 0.002$), epithelial vesicles ($p \leq 0.001$), stratum corneum vesicle formation ($p \leq 0.001$), stratum corneum hydropic degeneration ($p = 0.013$). Soft tissues total number of inflammatory cells were significant reduced (all $p \leq 0.05$) in the treated group except for the ileum, that had in the treated group more cells ($p \leq 0.001$) than in non-treated animals (Table 1)

Figure 3. Reticulum and soft tissues microscopic lesions in SARA. Normal reticulum papilla, 100X* (A); Abomasum with inflammatory cells (abomasitis), 200X* (B); Omasum with edema in lamina propria (score III), 100X* (C); Duodenum with inflammatory cells (enteritis), in lamina propria, 100X* (D); Ileum with inflammatory cells in lamina propria, 100X* (E); Colon with inflammatory cells (enteritis) in lamina propria, 200X* (F). All samples stained with Hematoxylin and Eosin. *Different magnifications were chosen to better demonstrate the lesions. Source: The author (2019).

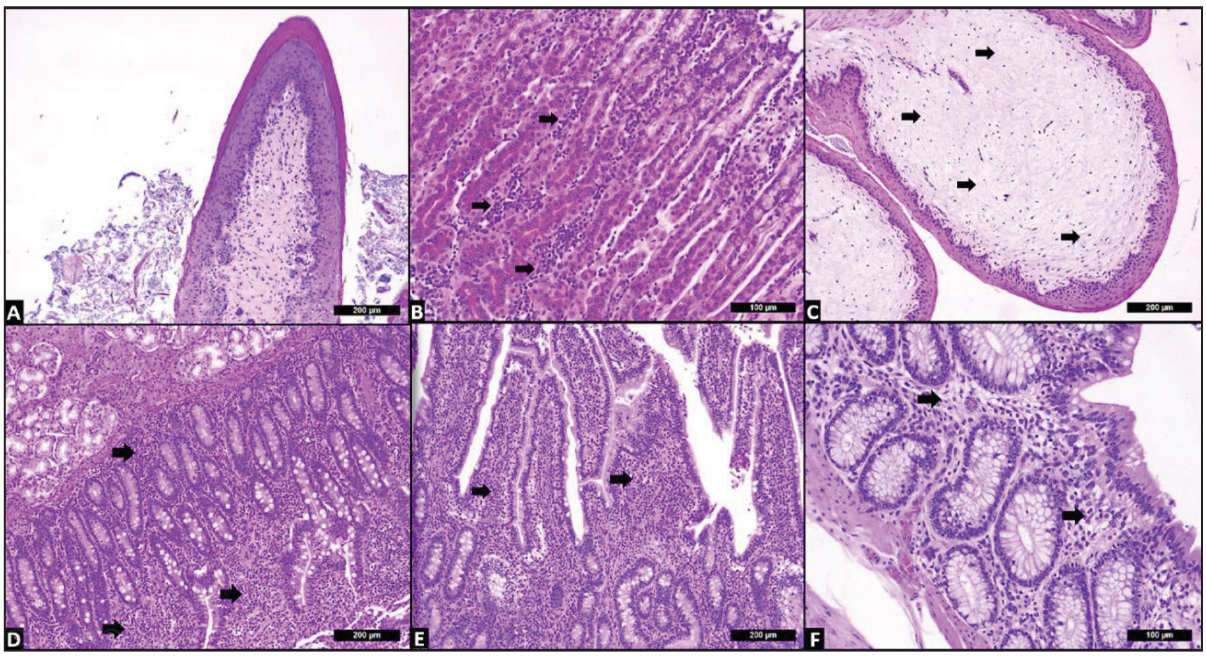


Table 1: Non-keratinized tissue amount (number) of inflammatory cells and p-values

	Non treated	Treated	p-value
Abomasum	18392	17595	p≤ 0.006
Duodenum	44975	28739	p≤ 0.001
Jejunum	45029	39088	p≤ 0.001
Ileum*	28175	34240	p≤ 0.001
Cecum	45675	43619	p≤ 0.001
Colon	43588	32926	p≤ 0.001

*The non-treated group demonstrated significantly less lesions for this parameter only.

4.4 DISCUSSION

This study obtained a pH of 5.5 at the slaughterhouse, against an average pH of 7.0 before the inclusion of the diet. This value meets those described for SARA that considers the fall of ruminal pH at or below 5.5 some hours after concentrate feeding (GARETT et al., 1999, OETZEL et al., 2000) and values between 5.6 and 5.7 as a transition towards SARA by some authors (GARRETT et al., 1999, MORGANTE et al., 2007). The chronic ruminitis in lamina propria with intra-corneal pustules or intra-epithelial neutrophils associated with the pH values and the length of diet well characterize a chronic ruminitis with recurrent acute episodes. In the rumen and reticulum significantly less inflammation in the lamina was found for the treated group. Thus, a weak morphological anti-inflammatory effect (MAIE) can be suggested.

Significant reduced epithelial hydropic degeneration scores demonstrated in the rumen and reticulum concurs with those found in the rumen of ewes (ESTRADA-ANGULO et al., 2016) being a consequence of the MAIE of the alkaloids.

The weak effect of the alkaloids was repeated but was not strong enough to create results in the very low ruminal pH associated with the bacterial lipopolysaccharides (LPS). The effect of the alkaloids under low pH resulting in additional damage to the epithelial cells could be considered.

Like pigs and rats fed these substances with about 98% of alkaloids excreted in feces (KOSINA et al., 2004), the topic effect of SG was demonstrated. The overall less effective results in rumen could also be attributed to the high amount of hyperkeratosis lessening the topic effect of the compound in the mucosa.

Reticulum stratum corneum hyperqueratosis scores were reduced in the treated group. Again, these results concur with those found in the rumen of ewes (ESTRADA-ANGULO et al., 2016) and can be a consequence of the MAIE of the alkaloids under better conditions. The ruminal pH results in the treated group, the small raise from 5.5 to 6.0 could be related to the reducing in the hydropic degeneration allowing more absorption of H+ and VFAs by the papilla and thus raising a little the pH.

Since the length of the papilla increases when cattle are feed high grain diets (DIRKSEN et al., 1985), this light improvement in the mucosa health could ameliorate the ruminal conditions. None of the omasum parameters had a statistical significance.

Considering the physiology of the organ, the dehydration of the luminal content would certainly raise the pH and the E *coli* endotoxins amount, erasing any possible effect of alkaloids in the histopathology. The strong edema in the lamina propria, not seen in the other tissues, seems to validate this idea. The statistical results in soft tissues (Table 1), except for the ileum, expressed a significant reduced amount of inflammatory cells in the mucosa (all p values ≤ 0.05). Ileum´s higher count could be explaining by its normal presence of lymphoid tissue and their modulated activity by the alkaloids used.

These results concurs with those found in rats with induced colitis having histological parameters less severe and statistically significant, including the inflammatory infiltrate in the treated group (VRUBLOVA et al., 2010) and with those in clinical induced enteritis by *Crytosporidium* spp. in calves resulting in amelioration of the clinical conditions in the treated group (MENDOÇA et al., 2016).

4.5 CONCLUSIONS

In this trial, SARA induced feedlot cattle treated with isoquinolone alkaloids, demonstrated morphological minor anti-inflammatory effects better expressed in reticulum and intestines than in rumen.

FINAL CONSIDERATIONS

The present research work allowed the description, classification and practical application of broiler intestinal histological changes considered as “lesions of minor importance”, offering a new tool to be used by poultry researchers. In its first chapter, through the comparisons between domestic broilers Cobb® 500 and feral chickens the microscopic lesions were described as parameters that can be scaled and classified using the concept of Microscopic Enteritis evaluated by the Marsh classification and the I See Inside” (ISI) methodology. Current concepts in human gastroenterology were proposed as mechanism for the lesions observed in broilers. In the second chapter, the experimental conditions allowed the separation of the Avian Microscopic Enteritis as having a Basal and Pathogenic components, In addition, the data of the parameters were displayed in a chronologic way that can be easily linked with interleukin, immunohistochemical and performance data to explain the behavior of the parameters. The final chapter demonstrated the flexibility of the adaptation of the ISI method to evaluate gastrointestinal tissues in feedlot cattle.

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