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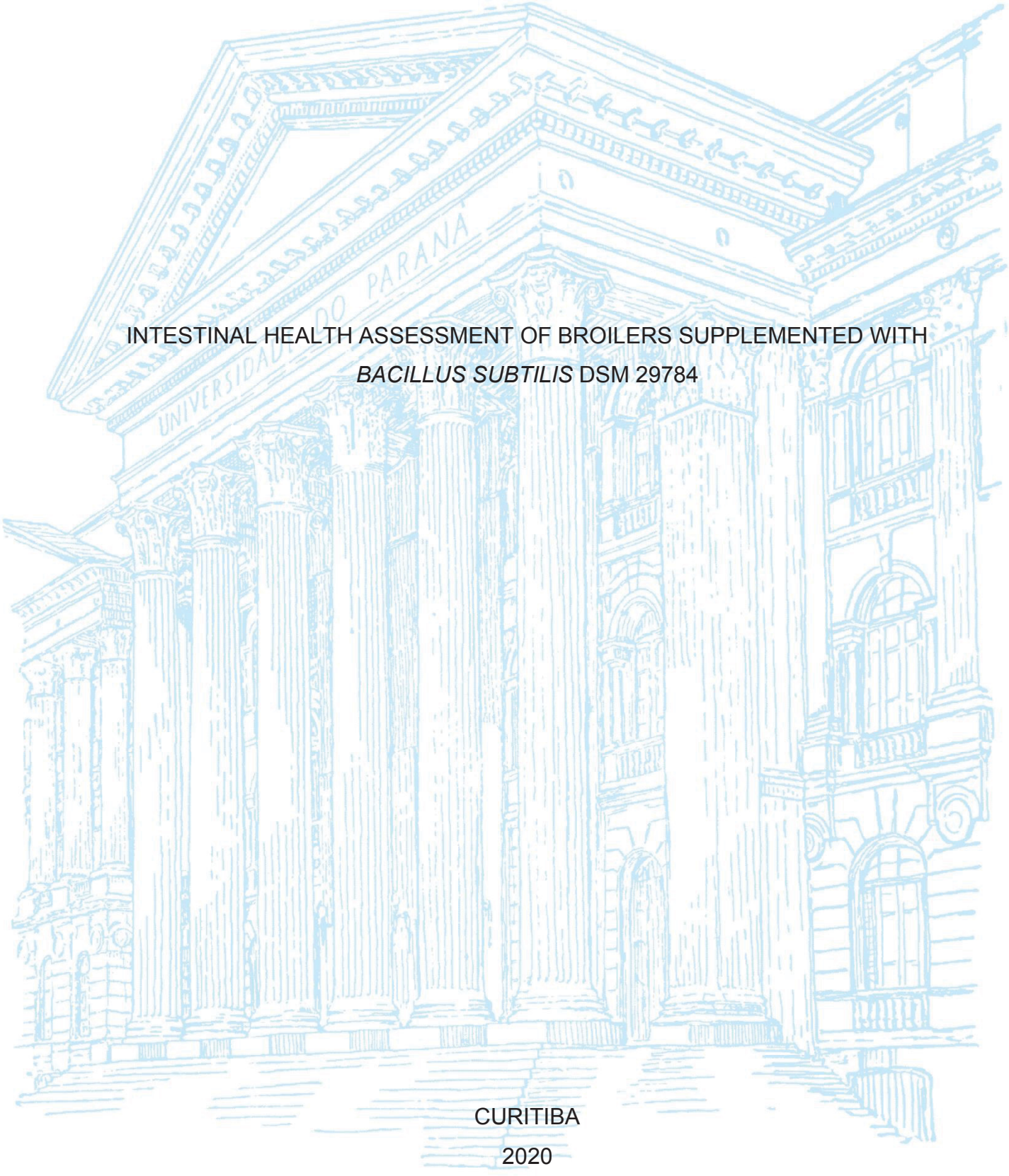


# INTESTINAL HEALTH ASSESSMENT OF BROILERS SUPPLEMENTED WITH *BACILLUS SUBTILIS* DSM 29784

CURITIBA

2020

AMANDA GABRIELA COBUCCI TIRADO

# INTESTINAL HEALTH ASSESSMENT OF BROILERS SUPPLEMENTED WITH BACILLUS *SUBTILIS* DSM 29784

Dissertação 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 Mestre em Ciências Veterinárias.

Orientador: Prof. Dr. Rafael Felipe da Costa Vieira

Co orientadora: Profa. Dra. Julia Arantes Galvão

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PROGRAMA DE PÓS-GRADUAÇÃO CIÊNCIAS VETERINÁRIAS - 40001016023P3

# TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS VETERINÁRIAS da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **AMANDA GABRIELA COBUCCI TIRADO** intitulada: **INTESTINAL HEALTH ASSESSMENT OF BROILERS SUPPLEMENTED WITH *BACILLUS SUBTILIS* 29784**, sob orientação do Prof. Dr. RAFAEL FELIPE DA COSTA VIEIRA, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

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

CURITIBA, 14 de Fevereiro de 2020.

RAFAEL FELIPE DA COSTA VIEIRA
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**ATA Nº 64**

## ATA DE SESSÃO PÚBLICA DE DEFESA DE MESTRADO PARA A OBTENÇÃO DO GRAU DE MESTRE EM CIÊNCIAS VETERINÁRIAS

No dia quatorze de fevereiro de dois mil e vinte às 14:00 horas, na sala Anfiteatro, do Hospital Veterinário do Setor de Ciências Agrárias da Universidade Federal do Paraná, foram instaladas as atividades pertinentes ao rito de defesa de dissertação da mestranda **AMANDA GABRIELA COBUCCI TIRADO**, intitulada: **INTESTINAL HEALTH ASSESSMENT OF BROILERS SUPPLEMENTED WITH BACILLUS SUBTILIS 29784**, sob orientação do Prof. Dr. RAFAEL FELIPE DA COSTA VIEIRA. A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação da Universidade Federal do Paraná em CIÊNCIASVETERINÁRIAS, foi constituída pelos seguintes Membros: RAFAEL FELIPE DA COSTA VIEIRA (UNIVERSIDADE FEDERAL DOPARANÁ), ALEX MAIORKA (UNIVERSIDADE FEDERAL DO PARANÁ), WANDERLEY MORENO QUINTEIRO FILHO (ADISSEO).A presidência iniciou os ritos definidos pelo Colegiado do Programa e, após exarados os pareceres dos membros do comitê examinador e da respectiva contra argumentação, ocorreu a leitura do parecer final da banca examinadora, que decidiu pela APROVAÇÃO. Este resultado deverá ser homologado pelo Colegiado do programa, mediante o atendimento de todas as indicações e correções solicitadas pela banca dentro dos prazos regimentais definidos pelo programa. A outorga de título de mestre está condicionada ao atendimento de todos os requisitos e prazos determinados no regimento do Programa de Pós-Graduação. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, RAFAEL FELIPE DA COSTA VIEIRA, lavrei apresente ata, que vai assinada por mim e pelos demais membros da Comissão Examinadora.

Observações: alterar o título para "Intestinal Health assessment of Broilers supplemented with Bacillus subtilis DSM 29784"

CURITIBA, 14 de Fevereiro de 2020.

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## RESUMO

A saúde intestinal dos frangos de corte é um fator importante que influencia a produtividade na avicultura de forma que, uma mucosa intestinal prejudicada tem baixa digestão e absorção de nutrientes e isso afetará as funções fisiológicas do hospedeiro, resultando em um sistema imunológico enfraquecido, mais susceptível a doenças e, consequentemente, causará perda econômica e de bem-estar animal. Desde que os antibióticos foram banidos na produção animal de vários países, métodos alternativos foram desenvolvidos para melhorar a performance dos frangos, como por exemplo os probióticos, que estimulam o crescimento de bactérias produtoras de ácido-lático consideradas benéficas para o intestino, aumentam o ganho de peso, promovem o equilíbrio da microbiota, melhoram a morfologia intestinal e modulam a imunidade intestinal. Neste trabalho foi realizado um estudo sobre probióticos e um experimento específico sobre os efeitos do *Bacillus subtilis* DSM 29784. O experimento foi realizado com frangos de corte desafiados com *C. perfringens* e *Eimeria* spp. ou não desafiados, e suplementados com *B. subtilis* DSM 29784, enramicina ou não-suplementados. Aos 7, 14 e 21 dias de idade foi avaliada a performance das aves, a saúde intestinal com o método histológico *I See Inside* (ISI) e a reposta imunológica com quantificação celular de linfócitos T (CD4+, CD8+ e macrófagos). Não foi encontrado diferença estatística para resultado de performance em aves suplementadas com o probiótico ou com a Enramicina, apenas entre desafiados e não desafiados. O resultado do ISI demonstrou maior escore de índice histológico em aves desafias, quando comparadas com não desafiadas em todos os períodos. O grupo desafiado e suplementado com *B. subtilis* DSM 29784 apresentou menor escore total ISI aos 7 e 14 dias quando comparado com o desafiado e não-suplementado. Aos 21 dias, o grupo não-desafiado e suplementado com Enramicina teve o menor escore total ISI dentre todos os grupos. Aves suplementadas com o probiótico tiveram um menor escore de células CD4+ e CD8+ aos 7 dias, um pico aos 14 e posterior queda aos 21 dias. A contagem de macrófagos foi elevada em aves não desafiadas aos 7 dias de idade. Os resultados deste experimento demonstraram que o probiótico teve um impacto positivo na saúde intestinal de frangos de corte desafiados.



Palavras-chave: Probiótico. Antibiótico promotor de crescimento. Enterite necrótica. Clostridiose. Linfócitos T.

## ABSTRACT

Chicken intestinal health is an important factor that concerns aviculture productivity somehow that disruptive mucosa has poorer nutrient digestion and absorption that will physiologically affect the host resulting in weaker immunological system, more susceptible to diseases and, consequently will cause economical losses and failed animal well-being. Since antibiotic ban in animal production of many countries, alternative methods have been developed to enhance broiler chicken performance, for instance probiotics stimulate the grown of lactic-acid producer bacterias considered beneficial for intestinal health, improving body weight gain, promoting microbiota balance, enhancing intestinal morphology and modulating intestinal immunity. In this dissertation it was accomplished a study about probiotics and then it was performed a trial specifically on *Bacillus subtilis* DSM 29784. In the experiment it was evaluated broiler chickens challenged or not with *C. perfringens* e *Eimeria* spp. and supplemented with *B. subtilis* DSM 29784 or Enramycin or not supplemented at all. At 7, 14 and 21 days of age it was evaluated bird's performance, intestinal health with the histological method I See Inside (ISI) and immunological response through lymphocyte T cell (CD4+, CD8+ and macrophage) quantification. No statistical difference was found on performance in birds supplemented or not with probiotic or Enramycin, except among challenged or non-challenged birds. ISI results demonstrated higher total score in challenged birds when compared to non-challenge ones at all ages. The challenged group supplemented with *B. subtilis* DSM 29784 presented lower ISI total score at 7 and 14 days, when compared to challenged and non-supplemented group. At 21 days, the non-challenged and supplemented with Enramycin group had the lowest ISI total score among all groups. Birds supplemented with the probiotic had a lower CD4+ and CD8+ cell counts at 7 days, followed by a peak at 14 days and posterior decrease at 21 days. Macrophage cell counts was higher in non-challenged birds at 7 days of age. Results demonstrated that *B. subtilis* DSM 29784 had a positive impact over intestinal health of challenged birds.



Keywords: Probiotic. Antibiotic growth promoter. Necrotic enteritis. Clostridiosis. T Lymphocyte.

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**SUMMARY**

# CHAPTER 1:

# COCCI-CLOSTRIDIOSIS AND PROBIOTIC INFLUENCE ON INTESTINAL HEALTH AND IMMUNE RESPONSE OF BROILER CHICKENS

# CHAPTER 1: COCCI-CLOSTRIDIOSIS AND PROBIOTIC INFLUENCE ON INTESTINAL HEALTH AND IMMUNE RESPONSE OF BROILER CHICKENS

## 1 INTRODUCTION

For a long period, it was considered that the intestine role was nothing but nutrient absorption and waste excretion. This concept is now improved, since it is known that the intestinal has the largest interface with the external environment and it is important to protect the host from external threats (MACDONALD et al., 2011; WAN et al., 2016).

Enteric pathogens, such as *C. perfringens* and *Eimeria* spp., are a significant problem for chicken production for causing intestinal disorders. In this way, maintaining the intestinal health is a condition to enhance performance of the birds (JACQUIER et al., 2019). Necrotic enteritis and coccidiosis act synergically accentuating intestinal damage (TIMBERMONT et al., 2011) resulting in a worldwide economic impact of U$ 6 billion and U$ 3 billion annually, respectively (DALLOUL and LILLEHOJ, 2006; WADE and KEYBURN, 2015).

Antibiotic growth-promoter (AGP) supplemented in the feed of broilers have been used to control diseases such as necrotic enteritis over the last decades (BORTOLUZZI et al., 2019), despites improving feed efficiency and growth performance (GADDE et al., 2018). However, AGP for animal production was banned or controlled in many countries because it may develop bacteria resistance (AL-KHALAIFAH, 2018) and is considered a public health issue (WHO, 1997).

In the search to improve intestinal health without using AGP, new studies have demonstrated that probiotic supplementation in feed can impact positively the intestine of broilers balancing the microbiota population (PARK et al., 2016; JACQUIER et al., 2019), strengthening barrier functions (PRIETO et al., 2014) and improving immunity (RAJPUT et al., 2013). Specifically, a novel isolated probiotic, *Bacillus subtilis* strain 29784, demonstrated to have strain-specific properties that could be effective in improving intestinal health (RHAYAT et al., 2017). Recently selected, this strain has no antibiotic resistance genes, absent hemolytic and cytotoxic properties, can tolerate pelleting and digestive circumstances and demonstrated to have in vitro anti-inflammatory and anti-*Clostridium perfringens* activities (JACQUIER et al., 2019).

The aim of the first chapter is to deeper clarify how necrotic enteritis and coccidiosis act together and impact the host, review AGP and probiotics mechanisms of action and how they affect intestinal health including structural, physiological, immunological systems.

## 2 COCCIDIOSIS

Coccidiosis is caused by *Eimeria* spp., a host and infection-site specific parasite, affecting the digestive tract of poultry (DALLOUL and LILLEHOJ, 2005). The coccidia is an obligate intracellular parasite and there are seven species widely recognized to cause coccidiosis in broilers: *E. acervulina, E. maxima, E. tenella, E. praecox, E. brunetti, E. mitis* and *E. necatrix*, (SHIRLEY, 1986; GYÖRKE et al., 2016), being the last two unusual (GYÖRKE et al., 2016; HAUG et al., 2008A; SUN et al., 2009; HAMIDINEJAT et al., 2010; OGEDENGBE; HUNTER; BARTA, 2011).

### *2.1 EIMERIA* LIFE CYCLE

*Eimeria* spp. have several life cycle phases, which is already well described in literature (DALLOUL and LILLEHOJ, 2005; SHIRLEY; SMITH; TOMLEY, 2005). Brief, oocysts present in the litter sporulate under moisture and right temperature condition (between 15 to 30°C) and are ready to infect birds upon ingestion of sporocysts. Through enzymatic and mechanic action of the gizzard there is a release of sporozoites followed by epithelial cell invasion on the intestine (the infection site in the gastrointestinal tract will depend on each strain). *E. brunetti* and *E. praecox* take place within the enterocytes of the villi while other species are in the cells of crypts more superficially. Inside the cell, the parasite will suffer asexual replication (2 to 4 times depending on the strain) and posterior fecundation. The zygote will then transform to oocysts and when released from the intestinal cell will destroy it and be excreted with the feces (DALLOUL and LILLEHOJ, 2005).

Each *Eimeria* specie infect specific spots in the intestinal, for example, the small intestine can be infected by *Eimeria* acervulina (along duodenum loop and sometimes jejunum), praecox (duodenum), maxima (around Meckel diverticulum and mid-intestine section), necatrix (mid-intestine section), mitis (second half) and brunetti (distal section). Yet, it can be found in the cecum *E. necatrix* and *tenella*. And finally,

in the rectum, *E. brunetti* invade for their development (SHIRLEY; SMITH; TOMLEY, 2005).

## *2.2* CLINICAL, MICROSCOPICAL AND MACROSCOPICAL FINDINGS

Five *Eimeria* species can be easily identified in the necropsy, such as *E. acervulina, E. brunetti, E. maxima, E. necatrix,* and *E. tenella*, since they have typical gross lesions. Their pathogenicity ranges from moderate to severe. While *E. praecox* and E. mitis do not result in mortality or have specific clinical signs and are often considered benign (ALLEN and FETTERER; 2002). Clinical findings include different lesions in different segments of intestine while subclinical coccidiosis can be hard to be diagnosed during macroscopical analysis and therefore can be neglected. In addition, most of the economic losses are due to the subclinical form (BERA et al., 2010).

Microscopically, *Eimeria* spp. modifies the structure of the villi, specifically, the species *E. acervulina, E. maxima* and *E. tenella* reach the crypt epithelium of the host (SHIRLEY and LILLEHOJ, 2012) causing a local inflammatory response (HONG et al., 2006) and consequently epithelial cell destruction (SHIRLEY and LILLEHOJ, 2012). Histological analysis showed that broilers at early stages of necrotic enteritis had strong epithelial inflammation, hyperemic lamina propria infiltrated with numerous inflammatory cells, mainly heterophilic granulocytes (OLKOWSKI et al., 2006). Using the I See Inside methodology (ISI), a metric tool that converts a microscopic alteration to a numeric score and allows correlation with animal performance (KRAIESKI et al., 2017; BELOTE et al., 2018), it was observed that the presence of *Eimeria* spp. in the ileum increases the inflammatory cells in the lamina propria and epithelium, the lamina propria thickness and the presence of oocysts when compared to non-challenged group. In addition, they suggested that ISI is an effective tool to evaluate immune reaction to *Eimeria* spp. since it is possible to observe their presence at different stages (BELOTE et al., 2018).

## *2.3* IMMUNITY AGAINST COCCIDIOSIS

Many aspects of immune response are involved in the host immunity against coccidia, including innate and adaptive immunity (DALLOUL and LILLEHOJ, 2005).

Components of innate immunity avoid pathogen invasion with the assistance of physical barrier, microbiota members, phagocytes and complement components (RITZI, 2015). Galt-associated lymphoid tissues (GALT) act as a platform to trigger the adaptive immunity, since it is responsible for detecting and presenting the antigen, producing antibodies (IG) by humoral immunity stimulation and, finally, cell-mediated immunity activation (DALLOUL and LILLEHOJ, 2005; RITZI, 2015). Adaptive immune response is activated upon *Eimeria* spp. infection via increased levels of antibodies (IgM, IgG and IgA), but they play a minor role to fight against coccidiosis. Some studies suggest that antibodies only reduce the pathogen and do not eliminate them (DALLOUL and LILLEHOJ, 2006; RITZI, 2015). Finally, cell-mediated immunity is the most relevant mechanism of action against the coccidia. It is possible to observe increased proliferation and infiltration of T lymphocytes at the infection spot, specially by CD8+ T lymphocytes (RITZI, 2015) and consequent reduced oocyst shedding (BESSAY, 1996).

## *3* NECROTIC ENTERITIS

Globally found in the environment of most commercial poultry-producer regions of the world, necrotic enteritis deteriorates poultry performance and increases veterinary and disinfection costs in U$ 6 billion annually worldwide (TIMBERMONT et al., 2011; WADE and KEYBURN, 2015; DELPHINE et al., 2015). Since 65-75% of chicken production cost is related to feed expenses, this intestinal illness has a significant financial impact because necrotic enteritis can decrease nutrient digestion due to the presence of gross lesion in the mucosa (HOFACRE; SMITH; MATHIS, 2018).

*Clostridium perfringens*, a gram-positive spore forming bacterium, is the agent associated with necrotic enteritis in broilers, breeders, commercial layers and turkeys (SONGER, 1996; VAN IMMERSEEL et al., 2004). The disease is caused mainly by type A strains, which produce the α toxin and the pore-forming toxin NetB (necrotic enteritis B-like) (KEYBURN et al., 2008; DELPHINE et al., 2015). For a long time, α toxin was considered to cause the illness but it was already recognized that the NetB toxin can cause the disease itself (KEYBURN et al., 2008, 2010; VAN IMMERSEEL et al., 2009; DELPHINE et al., 2015) since it forms pores in enterocytes leading to cell death (KEYBURN et al., 2008, 2010).

Some strains produce bacteriocins and are more virulent because it inhibits the growth of other *C. perfringens* strains in order to take advantage during competition for nutrients (TIMBERMONT et al., 2009; DELPHINE et al., 2015). Recent trials identified perforin, a novel bacteriocin produced by a NetB- positive strain, isolated from a chicken with necrotic enteritis that has no sequence homology to other bacteriocin proteins, suggesting a new class of bacteriocin (TIMBERMONT et al., 2014).

*C. perfringens* colonizes the intestine as early as after hatch or day one at the farm and becomes a commensal bacterium of the microbiota, being found in the concentration lower than $10^5$ cfu/g in ileal digesta not prejudicial for the host (TIMBERMONT et al., 2009; MILLER et al., 2010) while birds who suffer from necrotic enteritis carrying $10^6$ to $10^8$ cfu/g in ileal digesta (LONG; PETTIT; BARNUM, 1974; BABA et al., 1997; TIMBERMONT et al., 2011). However, cell count is not an exclusive parameter to determine the disease (PEDERSEN et al., 2003; TIMBERMONT et al., 2011). Since it can be normally found in the intestine of healthy birds, it is necessary a predisposing factor to trigger necrotic enteritis, for example diets with high levels of indigestible fiber or protein, like wheat, rye, oats, barely and fish-meal can change the digesta viscosity, prologue intestinal transit time and decrease digestion (JIA et al., 2009) creating a surplus of nutrients in the intestinal environment that will be used for *C. perfringens* proliferation (GHOLAMIANDEHKORDI et al., 2007; TIMBERMONT et al., 2011). In addition, other infectious diseases, like coccidiosis and infectious bursal disease, threatens immunity and makes the animal more susceptible to necrotic enteritis. The most reported and acknowledged factor in the field associated with necrotic enteritis is coccidiosis and its reasons will be discussed later in this review (MCDEVITT et al., 2006; COLLIER et al., 2008; WU et al., 2014).

## 3.1 HOW *C. PERFRINGENS* AND *EIMERIA* SPP. SYNERGICALLY WORK TOGETHER?

*Eimeria* spp. colonizes the intestine of the host and, as a process to complete its intraepithelial life cycle, it damages the intestinal integrity (DALLOUL and LILLEHOJ, 2005). The destruction of mucosa structure creates gaps and it is known as “leaky gut”, a syndrome that result in outflow of plasma protein in the intestinal lumen (VAN IMMERSEEL et al., 2004; PRESCOTT et al., 2016). Because of the

damage caused there is increased inflammation by the adaptive immune response, mediated by T cells, that results in enhanced intestinal mucogenesis (COLLIER et al., 2008; RITZI, 2015). The surplus of mucus, plasma protein released from the leaky gut and, if any, indigestible nutrients in the diets are used by *C. perfringens* as a substrate for further proliferation (VAN IMMERSEEL et al., 2004; COLLIER et al., 2008).

Coccidia impact the microbiota composition decreasing the microbial diversity of the intestine (WU et al., 2014; ZHOU et al., 2017), while a higher variability of bacteria in the gut is considered healthier. For instance, members of the family *Ruminococcaceae* are decreased in this situation (WU et al., 2014), consequently allowing intestinal colonization by other pathogenic agents such as *C. perfringens* (COLLIER et al., 2008). Likewise, it was reported alterations in the intestinal microbiota by *C. perfringens* infection (ANTONISSEN et al., 2016) such as decrease of SCFA-producing bacteria (AL-KHALAIFAH, 2018). The interaction of *C. perfringens* and other microorganisms has a major objective to compete against each other to favors their proliferation, production of toxins and the severity of the disease (ANTONISSEN et al., 2016).

## 3.2 FORMS OF NECROTIC ENTERITIS AND CLINICAL SIGNS

Necrotic enteritis can be subclinical or acute, but most cases are followed by reduced feed intake. In the subclinical form the birds do not present any evident clinical signs nor peak of mortality. For this reasons it is often neglected and leads to chronical intestinal mucosa damage, resulting in mucoid enteritis at necropsy (TIMBERMONT et al., 2011). This enlarged mucus production may impair the digestion and nutrient absorption in the small intestine, consequently affecting the feed intake and body weight gain. The subclinical form has the biggest economic impact in the industry because it is harder to diagnose and it affects feed efficiency (HOFACRE; SMITH; MATHIS, 2018; VAN DER SLUIS, 2000).

The acute form of the illness does not have specific clinical signs, other than depression and lower feed intake, but there are indicators like wet litter and sudden increase of flock mortality that can lead to diagnose (TIMBERMONT et al., 2011). In necropsy examination it can be found ballooned small intestine with brownish diphtheritic membranes and a brown blood fluid, followed by putrid odor. The mucosal surface of the intestine has a “fluffy towel” appearance and the intestine walls becomes

friable or fragile. Normally, lesions are found in the descending loop of the duodenum into the jejunum and sporadically in the ileum (HOFACRE; SMITH; MATHIS, 2018).

### 3.3 NECROTIC ENTERITIS PREVENTION AND TREATMENT

Biosecurity practices and good hygiene measures, essential oils, prebiotics, probiotics, enzymes are strategies that can be used as a preventive measure to prevent necrotic enteritis (ALBORNOZ; NAKANO; AVILA-CAMPOS, 2014). The enzymes, for instance, can minimize the levels of indigestible protein and fibers on the diets (JIA et al., 2009) that could generate surplus of nutrients and be used by *C. perfringens*. Probiotics can also be a good option to enhance the microbiota balance and prevent *C. perfringens* by some mechanisms of action that will be later discussed (PARK et al., 2016; JACQUIER et al., 2019).

In the last decades, many countries used antibiotics like penicillin, cephalosporin, quinolone, bacitracin and ionophore for treatment or prevention of necrotic enteritis (ALBORNOZ; NAKANO; AVILA-CAMPOS, 2014). However, since European and North American countries initially prohibited the use of antibiotic as growth promoters for animal production, it started a search for solution to deal with this disease more effectively, focusing on avoiding the predisposing factors (TIMBERMONT et al., 2011). In addition, the ban of AGPs increased the need to use coccidiosis vaccines which got the birds in contact with *Eimeria* spp. earlier in life and it was associated with more necrotic enteritis cases (DALLOUL and LILLEHOJ, 2005).

Although AGP's use is becoming an old-fashion practice in animal production industry, it is still interesting to deepen our knowledge on their mechanisms of action to find better alternative methods that could have a similar impact in the zootechnical results of production animals.

## *4* ANTIBIOTICS GROWTH PROMOTER

Antibiotics have been used in poultry feed as growth-promoters in low-doses to improve growth and feed efficiency, as preventive in intermediate doses during critical transition periods or as therapeutic in high-doses for infectious diseases (GUBAN et al., 2006). Its use as growth-promoters was considered by some scientists

the main cause of antimicrobial resistance to some antibiotic drugs (COSBY et al., 2015; GADDE et al., 2017).

The beneficial effect of AGP was first associated with a reduction in intestinal microbiota diversity because of the antibacterial action (FRANCOIS, 1961; VISEK, 1978), that consequently could decrease competition for nutrients and reduce microbial metabolites production that affect growth (FEIGHNER and DASHKEVICZ, 1987; GASKINS, 2002; KNARREBORG, 2004). Later, it was suggested that AGP impact the host positively because of the interaction with host immune cells lowering the inflammatory response and the production of pro-inflammatory cytokines (NIEWOLD, 2007). This could reflect in reduced energy cost of the host's immune system that could be directed to metabolizable energy and used for growing, for instance. In addition, it can minimize microscopical lesion in the tight junctions when the cytokines are released (RHAYAT et al., 2019) not harming intestinal integrity. Moreover, intestinal cells shed naturally, or when there is pathogen invasion, in order to promote tissue turnover. Deeper crypts indicate a fast turnover, and, in this case, the maintenance cost is higher. The use of AGP also saves this type of energy cost that can be used for other important physiological functions such as tissue maintenance, nutrient absorption, immune functions and production (MILES, 2006). Briefly, AGP favors the host improving growth rate, reducing the mortality and increasing resistance to pathogens (ESCELI and DEMIR, 2010).

However, many trials were performed under perfectly sanitized conditions, which do not represent the commercial farms, therefore it is known that AGP is not always effective (BROOM, 2018) and other alternatives should exist to improve intestinal health. Furthermore, the use of AGP in feed have been associated to development of antimicrobial resistance (COSBY et al., 2015; GADDE et al., 2017) but there are still divergent opinions among scientists if AGPs are the main reason to cause antibiotic resistance and transfer it from animal to human.

Since 1999, the European Union decided to ban the use of AGP for poultry production just like the Food and Drug Administration (FDA) did in the USA at the same time (DIBNER and RICHARDS, 2005; GADDE et al., 2017), increasing the incidence of certain infectious diseases, necrotic enteritis for instance, and economic losses for the industry (VAN IMMERSEEL et al., 2004; DIBNER and RICHARDS, 2005; RHAYAT et al., 2017). In Brazil, MAPA (Ministério da Agricultura, Pecuária e Abastecimento) put for public consultation a list of AGPs forbidden for animal production since 2003,

which was recently updated with the addition of tylosin, lincomycin and tiamulin (MAPA, 2020).

Facing the antibiotic resistance concern of some authorities world-wide, industry and scientists have been studying other methods that could be efficient to enhance animal intestinal health and have a positive impact on performance, such as probiotics.

## *5* PROBIOTICS AND *BACILLUS SUBTILIS* DSM 29784

Probiotic inclusion in the feed of farm animal is considered an “alternative” to AGP, but its unique mode of action demonstrated that they are, in fact, a strategy to improve intestinal health and not only act as a bacteriostat. These live microorganisms are non-pathogenic bacteria with positive health impact on the host (FAO/WHO 2002), including body weight gain (RHAYAT et al., 2017), balanced microbiota (PARK et al., 2016; JACQUIER et al., 2019), improved intestinal morphology (PRIETO et al., 2014; JACQUIER et al., 2019) and intestinal immunity (RAJPUT et al., 2013).

*Bacillus* (Gram-positive spore forming bacteria), lactic acid producing bacteria (*Lactobacillus, Bifidobacterium, Enterococcus*), and yeast are the most commercially known microorganisms used as probiotics in animal production (MOHAMMADIGHEISAR et al., 2018; JACQUIER et al., 2019). Probiotics can be classified as “feed additive” (FAO/WHO, 2002), but not all microbes can be defined as probiotic since it is necessary to isolate, characterize and prove its beneficial action (WAN et al., 2019). In fact, a potential strain can proliferate and colonize the intestine and stands bile salts, heat and osmotic or oxidative stress throughout feed processing or inside the host (ROSS et al., 2005; JACQUIER et al., 2019). *Bacillus subtilis* strain 29784 is a recent discovered probiotic strain, freshly approved by European Union (RYCHEN et al., 2018), that was rigorously selected due to its lack of antibiotic resistance, hemolytic and cytotoxic properties, capacity to tolerate pelleting and digestive circumstances, and in vitro anti-inflammatory and anti-*Clostridium perfringens* activities (JACQUIER et al., 2019).

Recent studies demonstrated that *B. subtilis* strain 29784 improves performance of broilers reared under different conditions associated to improved intestinal health (RHAYAT et al., 2017). A healthy gastrointestinal tract has conserved physical integrity and a balanced and diverse microbiota which are both important for

the host regarding not only digestive function (SHANG et al., 2018). Recent studies established that probiotics influences the whole immune system, defense against infections and, in addition, it has immunomodulatory effects (PENNISI, 2013; WAN et al., 2019).

Probiotics favors the host through several mechanisms of action, such as competitive exclusion of colonization spots in the intestinal mucosa is a manner to control and keep balanced the microbial populations (AL-KHALAIFAH, 2018). This action is mediated through acid polysaccharide cell wall compounds present in the bacteria (SOERJADI et al., 1982) that is responsible for attaching to the intestinal epithelium, this way physically obstructing other bacteria adhesion to the mucosa and further proliferation (FULLER, 1975).

Luminal pH affects this competition (AL-KHALAIFAH, 2018) since these live microorganisms intensifies the growth of bacterial population responsible for releasing volatile fatty acids in the ceca (acetic, butyric, propionic, and lactic acids) (JACQUIER et al., 2019) and, therefore, foment their binding to the intestinal wall, excluding pathogens (FULLER 1977, 1978). For instance, a trial demonstrated that *B. subtilis* strain 29784 stimulated a greater proliferation of *Ruminococcus, Anaerostipes* and *Lachnospiraceae* (JACQUIER et al., 2019), which are microorganisms known to produce butyrate (EECKHAUT et al., 2011; RIDLON et al., 2015) an energy source for enterocytes differentiation and proliferation in the intestinal mucosa (BEDFORD and GONG, 2018; SIKANDAR et al., 2017). This can result in increased tissue intestinal weight (LE BLAY et al., 2000; FUKUNAGA et al., 2003) and improved barrier functions by the microbiota (BORDIN et al., 2004; PENG et al., 2007). The production of such fermentation products was related to better growth performance (BROOM, 2018; LEY et al., 2005; BORTOLUZZI et al., 2019) and improved intestinal morphology (JACQUIER et al., 2019). These products, particularly the butyrate, has been shown to reduce bacterial colonization, modulate immunity, suppress inflammation (ZHOU et al., 2014; 2017).

Other studies indicated that probiotic improves intestinal morphology by increasing villus height and width (RAJPUT et al., 2013) and decreasing the depth of crypts in poultry (SAMANYA and YAMAUCHI, 2002; MARKOVIĆ et al., 2009; RITZI, 2015). Villus enlargement indicates bigger intestinal surface area with improved nutrient absorptive capacity. As mentioned before, a deeper crypt, due to natural shedding or external insults, means a faster turnover and wastes more metabolic

energy. Shallower crypts and higher villi are associated to decreased cell replacement, longer enterocyte lifespan and better performance (MARKOVIĆ et al., 2009; RITZI, 2015). Even though the villus height and width resulted in better performance in chickens, only these two histological criterions are not enough to suggest a healthier intestine. Other accurate morphology parameters, such as lamina propria thickness, presence of inflammatory immune cells, proliferation of goblet cells and others, as it is evaluated with the I See Inside (ISI) methodology, are important alterations to be considered (KRAIESKI et al., 2017; BELOTE et al., 2018).

Probiotic can also support intestinal barrier integrity exerting influence in the tight junctions (WAN et al., 2016), that is a type of cell that unites epithelial cells in order to delimit inter and extracellular space, adjust permeability and paracellular diffusion (NIESSEN, 2007). An even intestinal barrier regulates properly nutrient absorption and host homeostasis (RAJPUT et al., 2013) and a good indicator to measure its integrity is the transepithelial electrical resistance (TEER) of cell monolayer (OSWALD, 2006; WAN et al., 2016). Epithelial mucosa is vulnerable to pro-inflammatory cytokines stimulation by microbial compounds (RHAYAT et al., 2019) that, consequently, impairs tight junction integrity and starts an inflammatory response (RHAYAT et al., 2019). Damaged or loose tight junctions means lower TEER and are related to increased intestinal permeability (NIESSEN, 2007). Recent trials demonstrated that, when comparing *B. subtilis* 29784 to two other commercial *B. subtilis* strains, it was found higher expression of proteins involved in tight junctions like ZO-1, occluding and claudin-1 and this resulted in higher TEER, or strengthened barrier function (RHAYAT et al., 2019). Furthermore, the *B. subtilis* 29784 controlled the release of pro-inflammatory cytokines by intraepithelial cells in response to a stimulus (MCALINDON; HAWKEY; MAHIDA, 1998; RHAYAT et al., 2019) suggesting an anti-inflammatory effect.

Some probiotic strains modulate innate immune molecules like mucins, trefoil factors, antimicrobial peptides, toll-like receptors (TLRs) and macrophage (WAN et al., 2016; AL-KHALAIFAH, 2018). Mucins are produced by goblet cells and form the intestinal mucus, which is responsible to detain and eliminate pathogens from the gastrointestinal tract, lubricate and support colonization of commensal bacteria (ZHANG; EICHER; APPLEGATE, 2015; BROOM, 2018), through modification of pH environment (OSWALD, 2006; WAN et al., 2016). Studies suggest that there is an optimal mucus layers thickness since the lack of it is related to enteric infections due

to impaired intestinal barrier (WLODARSKA et al., 2011) and its abundance to poorer performance, perhaps because of poorer distribution of digestive enzymes and nutrients (BONTEMPO et al., 2006). A trial demonstrated that probiotic supplementation was able to regulate mucin production by increasing MUC2 gene expression in HT29 cells and reducing the possibility of Escherichia coli adhesion (MACK et al., 1999; WAN et al., 2016).

Still not very known, trefoil factors are protease resistant peptides produced by goblet cells (WONG and POULSOM, 1999; LILBURN and LOEFFLER, 2015) that protect the mucosa from insults, stabilize the mucus layer, and restores epithelial cells (KJELLEV, 2009; LILBURN and LOEFFLER, 2015). This protein is secreted in the mucus gel layer (WAN et al., 2016) along with mucins to work synergically (KJELLEV, 2009; LILBURN and LOEFFLER, 2015). Although there are few evidences over this topic and no study demonstrate probiotic supplementation benefit on trefoils production in chickens (PENDER et al., 2017), it was suggested that both over and sub production of trefoil factors disturb intestinal integrity and probiotic could equilibrate their level back to normal standard (WAN et al., 2016) but it still necessary more studies on this protein.

Antimicrobial peptides (AMPs), such as defensins and cathelicidins, are released by the host as a defense mechanism (OSWALD, 2006; WAN et al., 2016), through leukocyte cells (monocyte, macrophage, mast cells and natural killers) and epithelial cells. AMPs have bactericidal effect and are secreted to the intestinal lumen (AUVYNET and ROSENSTEIN, 2009) when exposed to damaging bacteria, disrupting microbial membrane (AUVYNET and ROSENSTEIN, 2009; OSWALD, 2006; WAN et al., 2016). These small peptides are released by the host and they participate in the innate immune response indorsing neutrophil recruitment, in mammals for example, improving phagocytosis and dendritic cell maturation, stimulating pro-inflammatory cytokines production and modulating anti-inflammatory mediators to avoid an exacerbated inflammatory response or to dismiss immune response (AUVYNET and ROSENSTEIN, 2009; WAN et al., 2016). Several Lactobacillus and Bacillus produce bacteriocins, a type of AMP, with anti-*C. perfringens* properties (DELPHINE et al., 2015) and modulates intestinal epithelium-derived antimicrobial synthesis by the host (WAN et al., 2016). Indeed, an important criterion to frame an efficient probiotic for animal production is if it produces such compounds (AL-KHALAIFAH, 2018) which are responsible to spot harmful bacteria, impede the adhesion or even the production of

pathogenic toxins (JOERGER, 2003; PAN and YU, 2014). Several *Bacillus* strains (*licheniformis, pumilus, subtilis*) isolated from broiler feces presented activity against *C. perfringens* in vitro (BARBOSA et al., 2005).

A hypothesis of another mechanism of action is that, to protect the intestinal lumen, intestinal epithelium express pattern-recognition receptors (PRR) such as toll-like receptors (TLR) to identify pathogen-associated molecular patterns (PAMP) of damaging bacteria (WAN et al., 2016). This mechanism of defense regulates several antimicrobial immune responses like pro-inflammatory cytokines, chemokines and antimicrobial peptides production (ARTIS, 2008; UEHARA et al., 2007), recruitment of B cells and production of secretory IgA in the lamina propria (SHANG et al., 2018), through initiation of TLR signalling (ARTIS, 2008; UEHARA et al., 2007). Pathogen and commensal bacteria can also be distinguished by PRR, as proposed by some authors. Even harmless commensal bacteria can initiate the pro-inflammatory signal pathway (WAN et al., 2016), this way probiotics could maintain 'a state of awareness' in the host since it is considered to possibly modulate TLR in the intestinal epithelial cells (VIZOSO PINTO et al., 2009; WAN et al., 2016). Studies have proposed that modulation in TLR by probiotics can influence the activation and monitoring of innate defense responses such as production of antimicrobial peptides and cytokines by the host (WAN et al., 2016).

Moreover, probiotics employ a part in the modulation of the adaptive defense response influencing the humoral and cell mediated immunity. In the humoral immunity, probiotic addition showed to stimulate the production of Immunoglobulin A (IgA) and thereby enhance barrier function by those agents (OHASHI and USHIDA, 2009; WAN et al., 2016). Probiotics can be incorporated into the Peyer's patch (GALDEANO et al., 2007; WAN et al., 2016) to modulate the intestinal associated lymphoid tissues defense responses. This interaction promotes the IgA cycle, thus increasing the amount of B lymphocytes that will produce IgA in the mucosal sites distant to the intestine (GALDEANO et al., 2007). Immunoglobulin A (IgA) is an abundant antibody that plays a crucial role in the immune functions of mucous membranes (MALDONADO-CONTRERAS and MCCORMICK, 2011). It becomes secretory IgA (SIgA) when released to intestinal lumen to attach to the mucus covering epithelial cells. To avoid pathogen invasion, it builds a hydrophilic and epithelial-glycocalyx excluding opportunistic bacterium and preserve beneficial microbe

communities (OHLAND and MACNAUGHTON, 2010; SHERMAN; OSSA; JOHNSON-HENRY, 2009; WAN et al., 2016).

Probiotics can stimulate the cell mediated immunity through an early release of intraepithelial lymphocytes expressing the cell surface markers CD4+ and CD8+ (MUNIZ et al., 2013; HAYASHI et al., 2018). In fact, when pathogens reach the epithelial barrier, it stimulates the release of pro-inflammatory chemokines of innate immunity which could trigger, if necessary, the adaptive immunity such as the emergence of T helper lymphocytes CD4+ cells. *Lactobacillus* and *Bacillus*-based probiotics can modulate the levels of several cytokines including pro-inflammatory cytokines (IL-1ß, IL-6, IL-17a, IL-18), Th1 cytokines (IFN-γ, IL-2, IL-12), and Th2 cytokines (IL-4, IL- 10, IL-13) (DALLOUL and LILLEHOJ, 2005; LEE, 2010a; RITZI, 2015).

A trial compared the immunomodulatory property of *Bacillus subtilis* 29784 with two commercial *Bacillus subtilis* through the release of IL-8 on Caco-2 cells (RHAYAT et al., 2019). These cells were stimulated with several pro-inflammatory compounds to increase the release of IL-8, which initially was also potentiated by the probiotic. However, the strain 29784 showed a posterior lower IL-8 release in all inflammatory circumstances compared to the other probiotics (RHAYAT et al., 2019). At first, all *Bacillus subtilis* naturally stimulates a slight secretion of IL-8 by intraepithelial cells (HOSOI et al., 2003; RHAYAT et al., 2019), that consequently will program these cells to cause a milder inflammatory response the next time they are in touch with pro-inflammatory molecules. Moreover, it creates an attenuating effect that will limit a subsequent inflammation caused by intestinal damage or when intestinal macrophages fail to downregulate IL-1β production in a chronical intestinal inflammation (MCALINDON; HAWKEY; MAHIDA, 1998; RHAYAT et al., 2019).

# CHAPTER 2:

# INTESTINAL HEALTH AND IMMUNE RESPONSE OF BROILERS SUPPLEMENTED WITH *BACILLUS SUBTILIS* STRAIN 29784 AND CHALLENGED WITH *CLOSTRIDIUM PERFRINGENS* AND *EIMERIA* SPP.

# CHAPTER 2: INTESTINAL HEALTH AND IMMUNE RESPONSE OF BROILERS SUPPLEMENTED WITH BACILLUS SUBTILIS DSM STRAIN 29784 AND ENRAMYCIN IN NECROTIC ENTERITIS EXPERIMENTAL MODEL

## 1 INTRODUCTION

Even with all recent advances in poultry industry, enteric diseases still represent a major challenge causing intestinal health disorders, disbalancing intestinal microbiota, disrupting physical integrity and being energetically expensive to the host due to the immune response (JAYARAMAN et al., 2017; JACQUIER et al., 2019). *Clostridium perfringens* is the etiological agent of necrotic enteritis in broilers (VAN IMMERSEEL et al., 2004), a multifactorial disease, with huge impact on intestinal microbiota diversity that affects performance and flock mortality (TIMBERMONT et al., 2011; WADE AND KEYBURN, 2015; DELPHINE et al., 2015). It has been controlled over the past few years with the use of antibiotic growth-promoters (AGP) (BORTOLUZZI et al., 2019), but since its restriction in many countries (AL-KHALAIFAH, 2018) it became a reemergent disease.

Since *C. perfringens* is a commensal bacteria of chicken microbiota, it requires a predisposing factor to develop the disease, for instance diets rich in fiber or animal protein, nonstandard climatic or management conditions and coccidiosis (MCDEVITT et al., 2006; WU et al., 2014), being the latter, the most important triggering factor (COLLIER et al., 2008). Coccidiosis is caused by the protozoan Eimeria spp., a host and infection-site specific parasite, affecting the digestive tract of poultry (DALLOUL AND LILLEHOJ., 2005). The parasite infects the intestinal epithelial cells of the host and as a result of its replicative cycle, it causes cell damage for the release of new oocysts (DALLOUL AND LILLEHOJ., 2005). The cell impairment caused by the coccidia increases the inflammatory response of adaptive immunity, mediated by T cells, that results in enhanced intestinal mucogenesis (COLLIER et al., 2008; RITZI, 2015) and plasma outflow to the intestinal lumen (VAN IMMERSEEL et al., 2004; PRESCOTT et al., 2016). The mucus and plasma surplus are used by *C. perfringens* as a substrate, increasing its population and possible toxin liberation (VAN IMMERSEEL et al., 2004; COLLIER et al., 2008). Intestinal microbiota is also influenced by the coccidia, decreasing the diversity of microbes, specially the member

of the family *Ruminococcaceae* (WU et al., 2014, ZHOU et al., 2017), and therefore allowing intestinal colonization by pathogenic agents such as *Clostridium perfringens* (COLLIER et al., 2008).

AGP mechanism of action are related to reduced microorganism's diversity composing the microbiota due to the antibacterial action (FRANCOIS, 1961; VISEK, 1978) and anti-inflammatory action (NIEWOLD et al., 2007). However, probiotics are often referred by scientists as an "alternative" to AGP, in fact they should be considered a strategy to improve intestinal health. They are non-pathogenic bacteria that might improve body weight gain (RHAYAT et al., 2017), microbiota balance (PARK et al., 2016; JACQUIER et al., 2019), intestinal morphology (PRIETO et al., 2014; JACQUIER et al., 2019) and modulate intestinal immunity (RAJPUT et al., 2013).

*Bacillus subtilis* DSM 29784 is a recent discovered probiotic strain that was rigorously selected due to its lack of antibiotic resistance, hemolytic and cytotoxic properties, capacity to tolerate pelleting and digestive circumstances, and in vitro anti-inflammatory and anti-*Clostridium perfringens* activities (RHAYAT et al., 2017, JACQUIER et al., 2019). Among many species of *B. subtilis,* it was observed its capacity to balance microbiota population, (AL-KHALAIFAH, 2018), intensify the growth of lactic-acid producing bacteria (JACQUIER et al., 2019), improve intestinal morphology by the increase of villus height and width (RAJPUT et al., 2013), decrease of depth of crypts (SAMANYA and YAMAUCHI, 2002; MARKOVIĆ et al., 2009, RITZI, 2015), and modulate innate and adaptive immunity (WAN et al., 2016; AL-KHALAIFAH, 2018; HAYASHI et al., 2018).

The aim of this trial was to observe *B. subtilis* DSM 29784 and enramycin effect in the intestinal mucosa histology and immune response of broilers challenged or not with *C. perfringens* and *Eimeria* spp. at different ages.

## 2 MATERIALS AND METHODS

### *2.1 ETHICAL COMMITTEE APPROVAL*

This trial was approved by the Institutional Animal Use Ethics Committee of Agricultural Sciences of the Federal University of Parana (Protocol 020/2018).

## *2.2 EXPERIMENTAL DESIGN, ANIMALS, DIET AND HOUSING*

The experimental design consisted of factorial of 2x3, resulting in six treatments with four replicates each and 10 birds/replicate. A total of 240 male Cobb 500® broilers (01 to 21 days of age) were distributed in a completely randomized design, into the treatments (table 1): negative control (NC) - non-challenged birds and no additive in the feed; non-challenged birds receiving *B. subtilis* in the feed (NCBS); non-challenged birds receiving enramycin in the feed (NCAGP); birds challenged with *Eimeria* spp. and *C. perfringens* and no feed additive in feed (CH); birds challenged with *Eimeria* spp. and *C. perfringens* , receiving *B. subtilis* in the feed (CHBS); birds challenged with *Eimeria* spp. and *C. perfringens*, receiving enramycin in the feed (CHAGP). Whenever it was added in the diet, the dose of enramycin was 0.12 g/kg (10 ppm) and *B. subtilis* 29784 0.5 g/kg and both the feed additives were provided in the feed at all stages.

**TABLE 1.** DESCRIPTION OF THE TRIAL TREATMENTS

| TREATMENT | CHALLENGE | ADDITIVE PROTOCOL |
|---|---|---|
| NC | No | No additive |
| NCBS | No | *B. subtilis* DSM 29784 |
| NCAGP | No | Enramycin |
| CH | *Eimeria* spp. and *C. perfringens* | No additive |
| CHBS | *Eimeria* spp. and *C. perfringens* | *B. subtilis* DSM 29784 |
| CHAGP | *Eimeria* spp. and *C. perfringens* | Enramycin |

*B. subtilis* DSM 29784 dose: 1 x $10^8$ CFU/kg of feed; Enramycin dose: 0.12 g/kg (10 ppm).

Birds were housed in isolation rooms with negative pressure. Each room had four stacked cages with litter, nipple drinkers, feeders and automatic temperature control. Birds were raised with water and feed *ad libitum*. All groups received a corn and soybean-based feed. For sampling it was necropsied 6 birds per treatment, per period.

## *2.3 CHALLENGE*

Challenged birds (CH, CHBS and CHAGP) have received 15 times the manufactured recommended dose of a commercial *Eimeria* vaccine (Bio-Coccivet R® live vaccine strains: *E. acervulina, E. brunetti, E. maxima, E. necatrix, E. praecox, E.*

*tenella* and *E. mitis*) at the first day of the trial, in order to induce experimental sub-clinical coccidiosis disease. Each bird from the challenge groups received 0.5 mL solution containing 330.000 oocysts of *Eimeria* spp. while the birds of the non-challenged birds NC, NCBS and NCAGP, received 0.5 mL of saline water instead. In the 10th, 11th and 12th day of the experiment, the animals received, by gavage, $10^8$ CFU of *C. perfringens/*mL, that was isolated from a necrotic enteritis field case, however this strain was not sequenced for the NetB toxin confirmation. All non-challenged groups received water via gavage at same amount and day.

*2.4 PERFORMANCE*

Animals and feed were weekly weighted (7, 14 and 21 days of age) for evaluation of feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR).

*2.5 I SEE INSIDE (ISI) METHODOLOGY*

At 7, 14 and 21 days of age, six birds per treatment were euthanized by cervical dislocation and necropsied to collect ileum samples for histopathological and immunochemistry analysis (Kraieski et al., 2017; Belote et al., 2018). Samples were fixed in Davidson's solution (100 mL glacial acetic acid, 300 mL 95% ethyl alcohol, 200 mL 10% neutral buffered formalin, and 300 mL distilled water) for at least 24 hours. All samples were dehydrated, infiltrated, and embedded in paraffin following common histological routine. Blocks were cut in 5 µM sections and stained with hematoxylin and eosin associated with Alcian Blue for goblet cells staining (RAPP and WURSTER, 1978) used for the histological analysis. In the intestinal morphology, a bird was represented by one slide containing 30 intestinal villi each, which was observed in 10X magnification (using 20X and 40X magnification to confirm alterations) under optical microscope (Nikon Eclipse E200, Sao Paulo, Brazil). The I See Inside (ISI) methodology was first described by Kraieski et al. (2017) and modified by Belote et al. (2018), and evaluation parameters are presented in table 2.

**TABLE 2.** ISI HISTOLOGICAL ALTERATIONS EVALUATED IN THE SMALL INTESTINE

| Sample | Microscopical Findings | Impact Factor (IF) | Score | Final score | [1]Maximum score |
|---|---|---|---|---|---|
| Ileum | Lamina propria thickness | 2 | 3 | 6 | 45 |
| | Epithelial thickness | 1 | 3 | 3 | |
| | Enterocytes proliferation | 1 | 3 | 3 | |
| | Epithelial plasma cell infiltration | 1 | 3 | 3 | |
| | Lamina propria inflammatory infiltration | 3 | 3 | 9 | |
| | Goblet cells proliferation | 2 | 3 | 6 | |
| | Congestion | 2 | 3 | 6 | |
| | Presence of oocysts | 3 | 3 | 9 | |

[1] Maximum score represents the sum of all alteration according to with the formula ISI =Σ(IF*S) where IF = impact factor (previous fixed) and S=Score (observed) considering the maximum observed S. For example, the lamina propria thickness has IF = 2, this number will be multiplied by observed score (range from 1 to 3), if in a villus it was observed a score S=3 (maximus score) to lamina propria thickness, so the ISI for this parameter in this villus will be ISI = (2*3) = 6. The average of 30 villi in ileum for each bird will be the final ISI value for each bird.

### *2.6 IMMUNOCHEMESTRY ANALYSIS*

For immunohistochemistry analysis, the paraffin block samples were sectioned with the microtome at 4µm and were placed in immunohistochemistry slides, dewaxed in xylene at 60º C for 20 min and rehydrated in water and alcohol. Slides were horizontally placed in a humid incubation chamber, covered with 100 to 500 µL of primary specific antibodies (SouthernBiotech, USA) for avian macrophages, CD4+ and CD8+ T lymphocytes (added in different slides for each cell type) and incubated overnight at 5◦C. Then, the slides were washed 3 times with phosphate buffered saline (PBS), covered with 100– 500 µL of antibody conjugated with horseradish peroxidase (HRP-conjugated rabbit anti-mouse Ig, Dako North America, Carpinteria, CA, USA) and incubated for 30 min. The peroxidase reaction was developed using a chromogen for 30 s. The slides were counterstained with hematoxylin, washed in water, dehydrated, and mounted. The labeled cells were counted in an optical microscope (400X magnification objective). Five fields per bird were measured, totalizing 30 fields per treatment of intestine.

*2.7 STATISTICAL ANALYSIS*

Data was evaluated using the statistical software Statistix 9 and analyzed by the Shapiro-Wilk normality test. Parametric data was submitted to analysis of variance (ANOVA) for the means with a significant difference. Nonparametric data was submitted to the Kruskal-Wallis test at 5% probability. Performance, histology and immunohistochemistry data were submitted to ANOVA using a 2 × 3 factorial design.

## 3 RESULTS

All data was analyzed in factorial arrangements. Firstly, the data was evaluated for the variable challenge (performed or not), then for the product supplementation (no product, *B. subtilis* 29784 or enramycin) and lastly, for the interactions (challenge*product) that were represented by the treatments already described in the methodology. The challenge statistically worsened ($p<0.05$) the feed intake (FI) by 14.5% at 1-14 d and by 2.80% at 1-21 days. Body weight gain (BWG) was worsened by the challenge by 17.26%, 22.06% and 15.53% at 1-7, 1-14 and 1-21 days, respectively. Feed conversion rate (FCR) was worsened 16.30%, 9.09% and 13.29% at 1-7, 1-14 and 1-21 days, respectively (figure 1). No statistical difference was found with *B. subtilis* or enramycin addition.

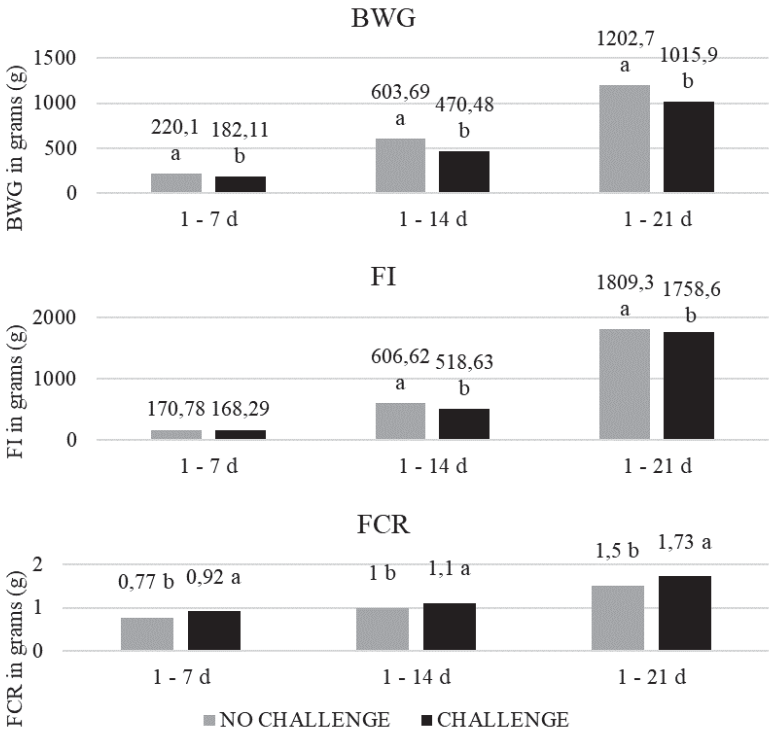



**FIGURE 1.** BWG (body weight gain), FI (feed intake) and FCR (feed conversion rate) in grams, among no challenge and challenge groups with *C. perfringens* and *Eimeria* spp. at different ages. Different superscript letters indicate significant difference ($p<0.005$).

The challenge efficacy was confirmed through the histologic data, once microscopical alterations were observed in the ileum of challenged birds at 7, 14 and 21 days of age, represented by statistically higher ($p<0.05$) ISI scores when comparing the challenged to the non-challenged groups (figure 2).

*Bacillus subtilis* 29784 controlled the challenge impact over the ileum integrity at 7 days, since the ISI total score in the CHBS group was statistically lower ($P<0.001$) in comparison to the CH animals at this age (figure 3), due to lower score of lamina propria thickness found at this age (figure 4). No statistical difference of total score was verified between the CH and CHAGP groups, although it was observed statistically lower score of lamina propria thickness of CHAGP.

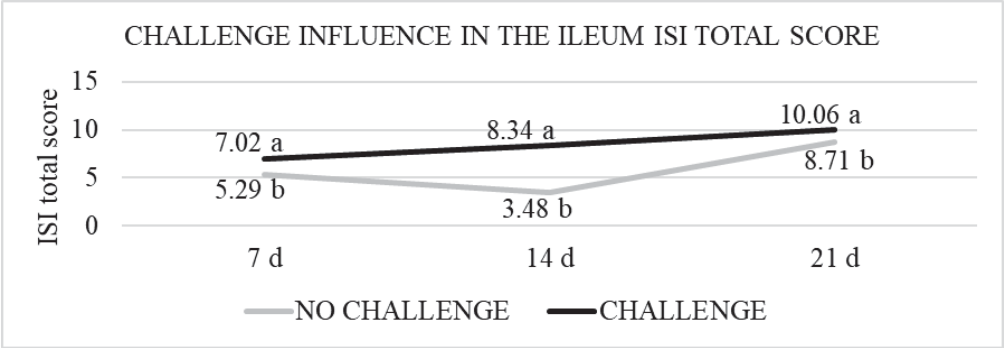



**FIGURE 2.** ISI total score in the ileum at each period considering the challenge factorial: NO CHALLENGE and CHALLENGE groups. Different superscript letters indicate significant difference ($p<0.05$).

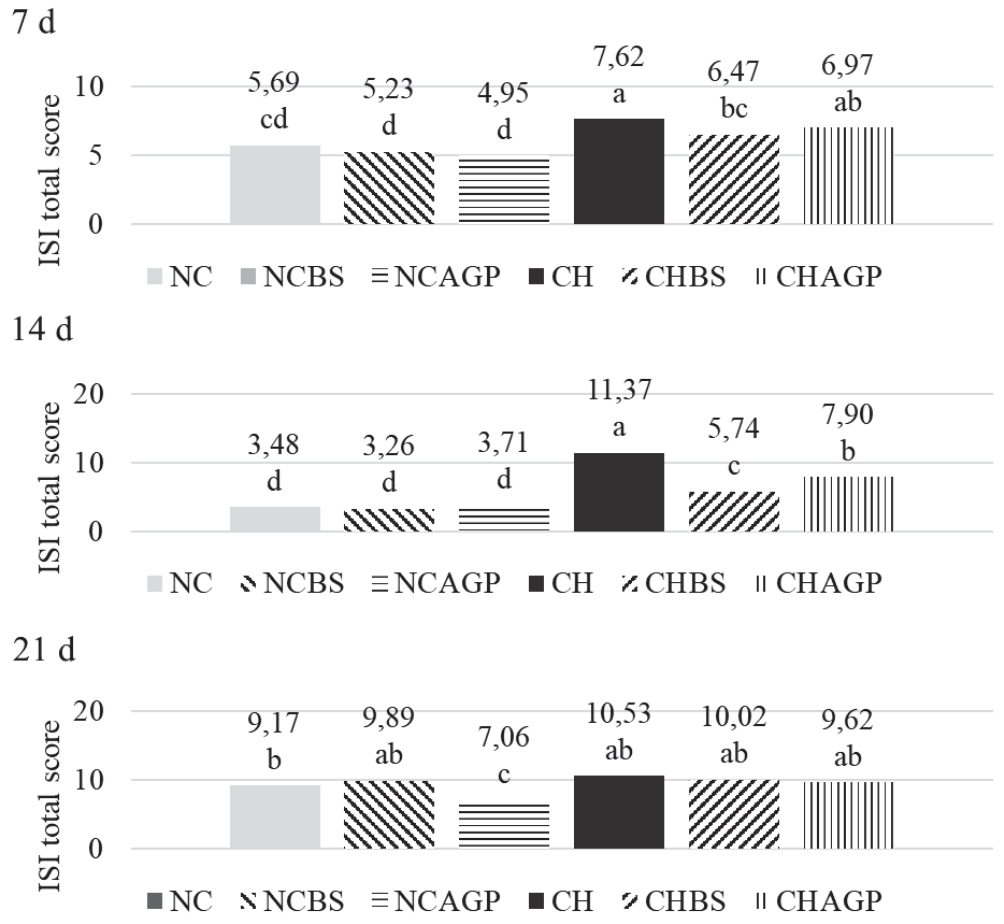
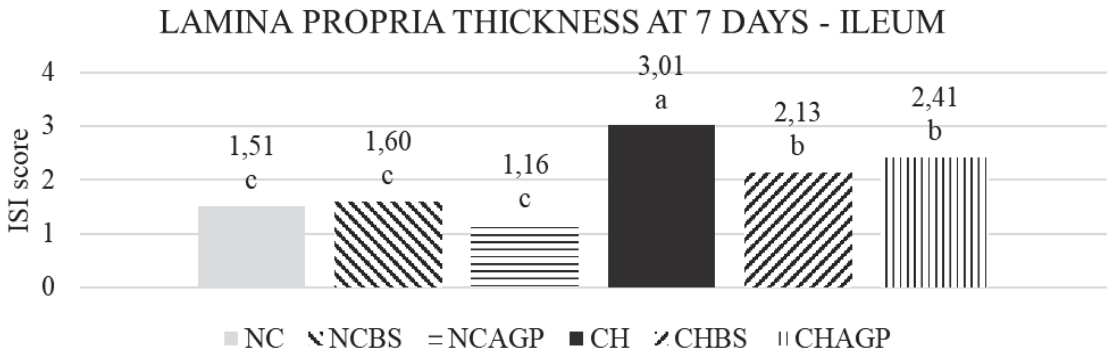



**FIGURE 3.** ISI total score in the ileum at each period. NC: negative control, NCBS: non-challenged and B. subtilis 29784 added; NCAGP: non-challenged and Enramycin added; CH: positive control; CHBS: challenged and B. subtilis 29784 added; CHAGP: challenged and Enramycin added. Different superscript letters indicate significant difference ($p<0.05$).

**A)**




**B)**

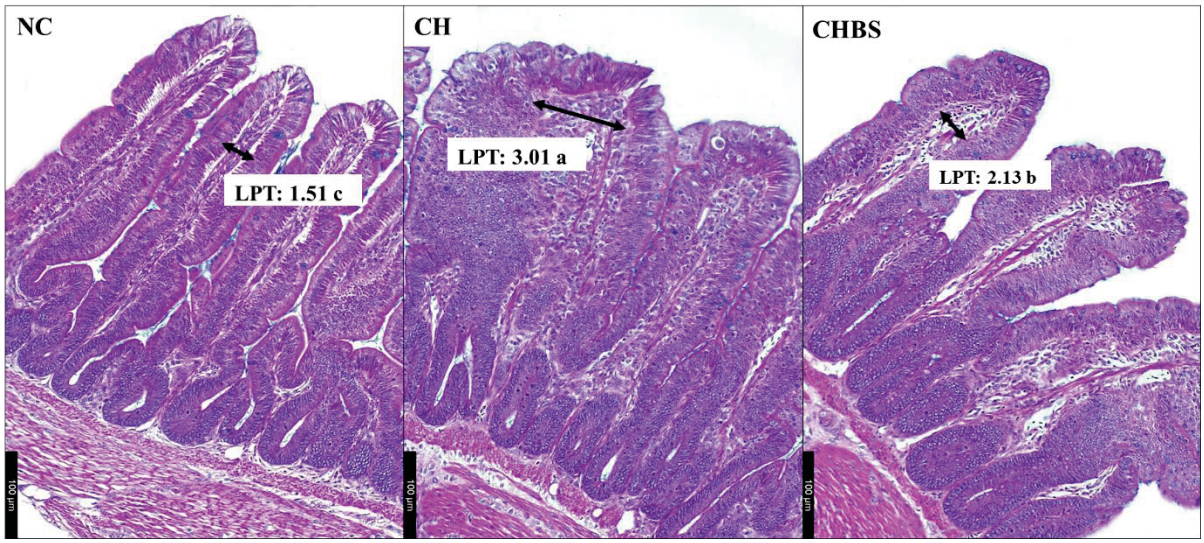
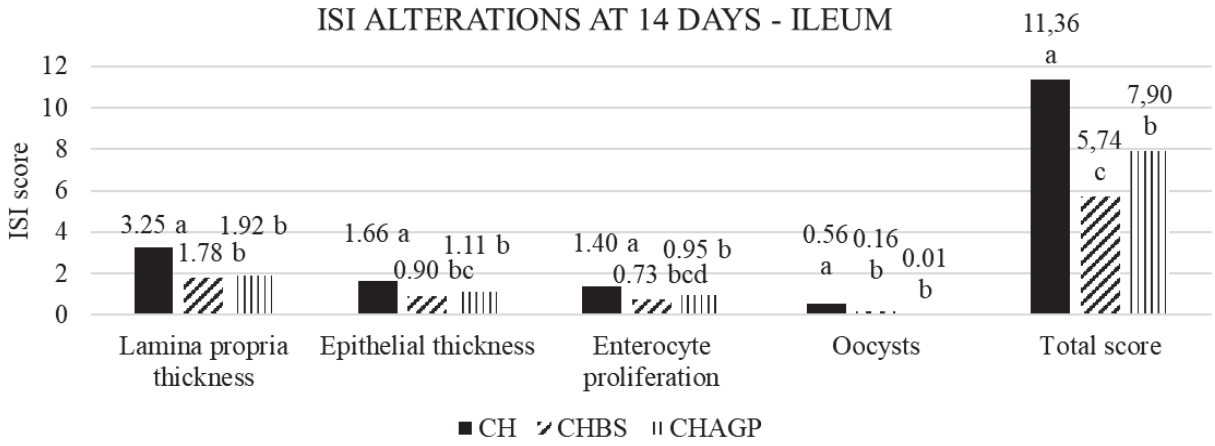



**FIGURE 4. A)** Lamina propria thickness score among all treatments at 7 days of age. NC: negative control, NCBS: non-challenged and *B. subtilis 29784* added; NCAGP: non-challenged and Enramycin added; CH: positive control; CHBS: challenged and *B. subtilis 29784* added; CHAGP: challenged and Enramycin added. Different superscript letters indicate significant difference (p=0.001). **B)** Photomicrographs of hematoxylin and eosin-stained chicken ileum sections at 7 days. Alcian Blue was used to stain the goblet cells. Lesions found in CH and CHBS in the ileum at 7 days. Lesions found: LPT: lamina propria thickness (200X).

**A)**




**B)**

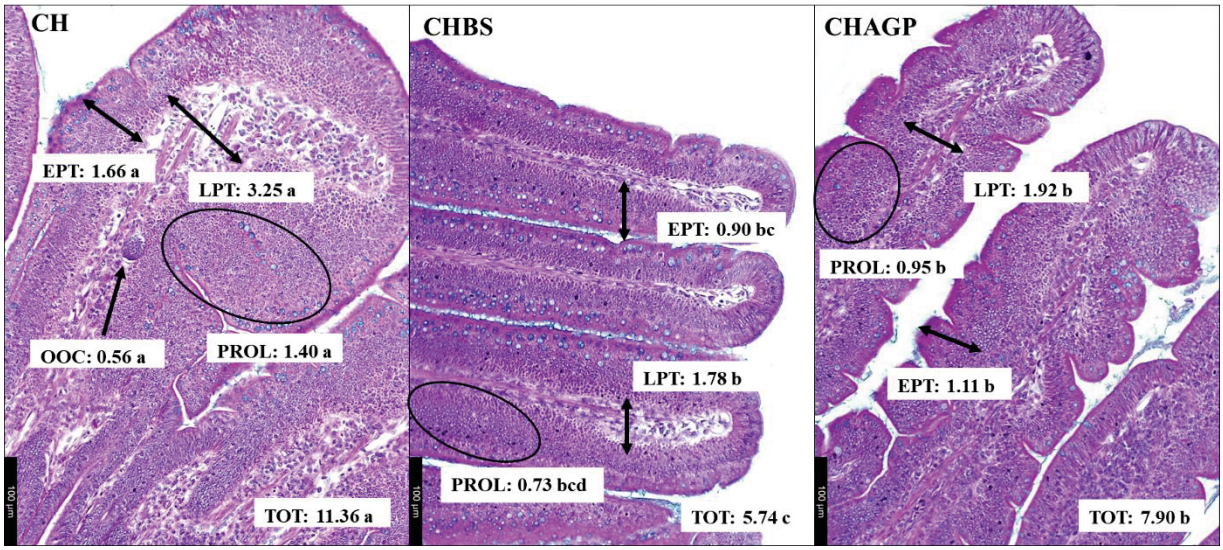
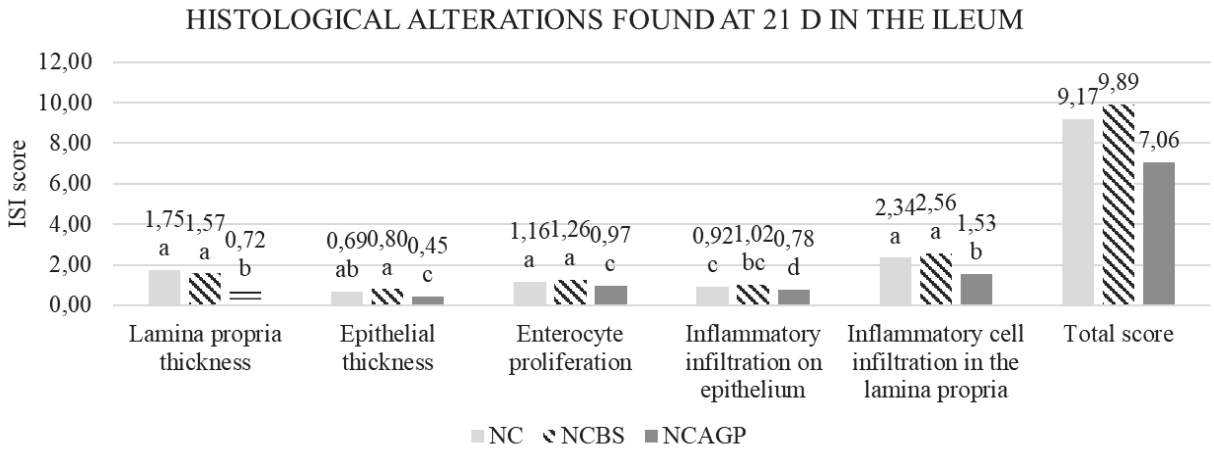



**FIGURE 5. A)** ISI alteration scores in the ileum of challenged groups at 14 days of age. CH: positive control; CHBS: challenged and *B. subtilis 29784* added; CHAGP: challenged and Enramycin added. Different superscript letters indicate significant difference ($p<0.05$). **B)** Photomicrographs of hematoxylin and eosin-stained chicken ileum sections at 14 days among challenged groups. Alcian Blue was used to stain the goblet cells. Lesions found: LPT: lamina propria thickness, EPT: epithelial thickness, PROL: proliferation of enterocytes, OOC: presence of oocysts and TOT: total score (200X).

**A)**




**B)**

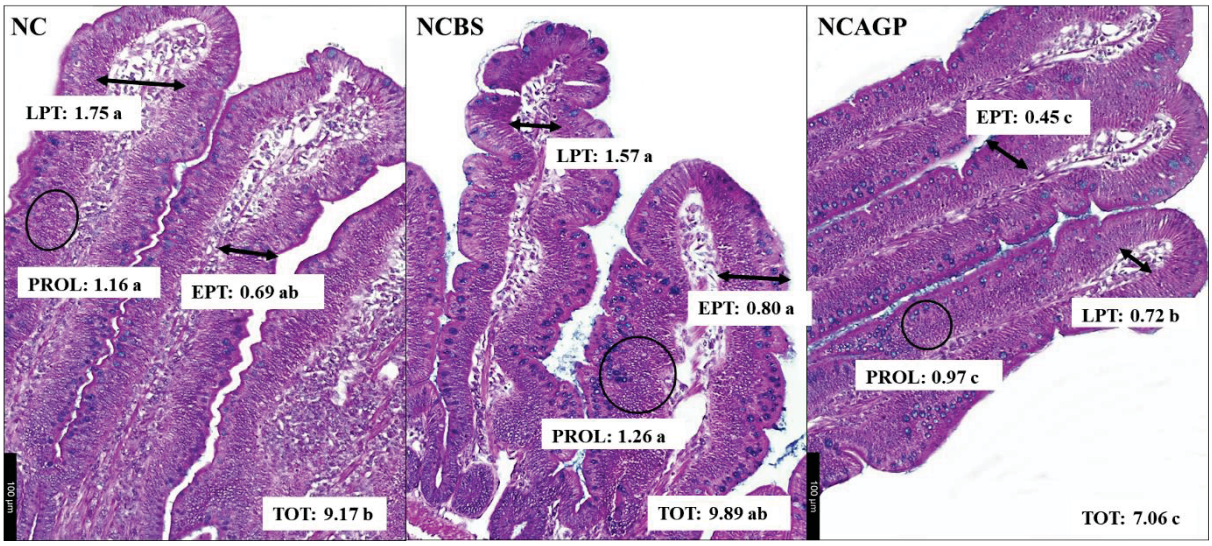



**FIGURE 6. A)** ISI alteration scores in the ileum among all treatments at 21 days of age. NC: negative control, NCBS: non-challenged and *B. subtilis 29784* added; NCAGP: non-challenged and Enramycin added; CH: positive control; CHBS: challenged and *B. subtilis 29784* added; CHAGP: challenged and Enramycin added. Different superscript letters indicate significant difference ($p<0.05$). **B)** Photomicrographs of hematoxylin and eosin-stained chicken ileum sections at 14 days among challenged groups. Alcian Blue was used to stain the goblet cells. Lesions found: LPT: lamina propria thickness, PROL: proliferation of enterocytes and TOT: total score (200X).

At 14 days, birds fed with the probiotic (CHBS group) presented the lowest ($p<0.001$) ISI total score in comparison to the other challenged groups, followed by the birds supplemented with AGP (CHAGP group) (figure 3). The better condition of the ileum mucosa in the CHBS group was due to reduced scores of lamina propria thickness, epithelial thickness, enterocyte proliferation and presence of oocysts (figure 5). No difference was observed among the challenged groups at 21 days or among the non-challenged groups at 7 and 14 days. However, the NCAGP group presented the lowest ($p<0.001$) ISI total score in comparison to the other non-challenged animals at

21 days (figure 3). The improved condition of ileum mucosa in the NCAGP group at this age as due to lower scores ($p<0.001$) of lamina propria and epithelial thickness, enterocyte proliferation and inflammatory infiltration in the epithelium and lamina propria found in the bird's histology (figure 6).

The immunochemistry demonstrated that CD4+, CD8+ and macrophage cells counts in the ileum were statistically increased ($p=0.001$) in challenged birds when compared to non-challenged animals, at all ages (figure 7).

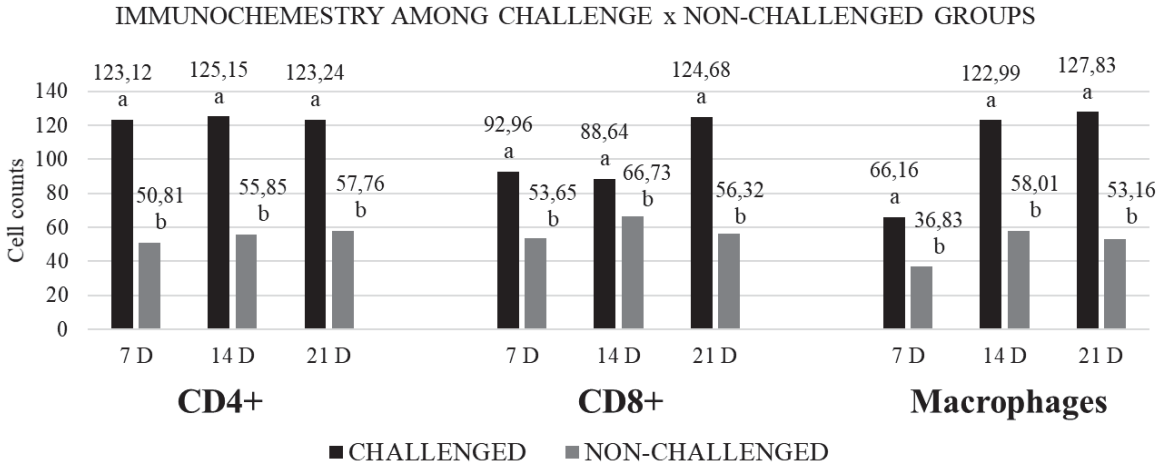



**FIGURE 7.** CD4+, CD8+ T lymphocytes and macrophage cell counts of non-challenged birds and challenged with *C. perfringens* and *Eimeria* spp. at 7, 14 and 21 days of age.

Among the challenged animals (figure 8), the AGP supplementation (CHAGP group) presented statistically lower CD4+ cell count at 7 days when compared to the CH and CHBS groups. At 21 days, the CHAGP group presented a lower count of CD4+ cells in comparison to the CH animals, however, no significant difference was verified among birds fed with *Bacillus* and the other groups. The count of CD8+ cells among the challenged birds was significantly ($p<0.001$) affected by the probiotic at 7 days, when the CHBS group presented the lowest quantity of these cells among the two other groups. At 21 days, no difference was found in the count of CD8+ cells among the birds fed with either *Bacillus subtilis* or enramycin (CHBS and CHAGP groups, respectively), however, both products were effective in reducing ($p<0.001$) the count of these cells in comparison to the untreated challenged birds (CH group).

While no difference was verified in the counts of CD4+ and CD8+ cells among challenged groups at 14 days (figure 8), that was the only age where the count of macrophages was affected significantly among the challenged groups.

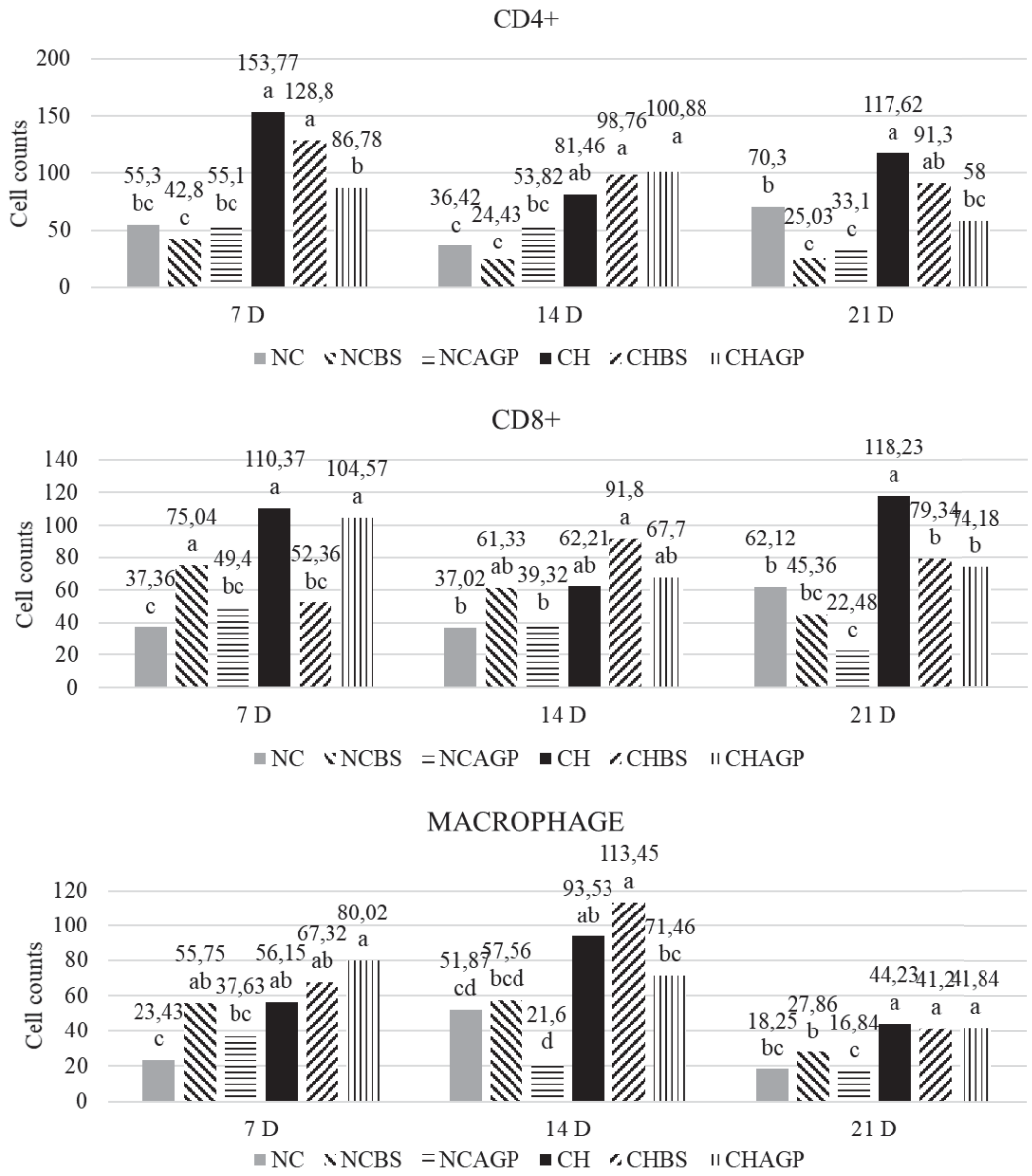



**FIGURE 8.** CD4+, CD8+ T lymphocytes and macrophage cell counts among treatments (NC: negative control, NCBS: non-challenged and *B. subtilis 29784* added; NCAGP: non-challenged and Enramycin added; CH: positive control; CHBS: challenged and *B. subtilis 29784* added; CHAGP: challenged and enramycin added) at 7, 14 and 21 days of age.

At this age, the BS supplementation (CHBS group) mobilized more ($p<0.001$) CD8+ cells to the ileum mucosa in comparison the AGP addition in the feed (CHAGP group). At 21 days, both NCBS and NCAGP groups presented a lower ($p<0.001$) quantity of CD4+ cells when compared to the NC group, however, no difference was verified between the two products. More CD8+ was recruited at 7 days in the birds fed with the *Bacillus subtilis* (NCBS group) in comparison to the two other non-challenged groups. The AGP supplementation reduced ($p<0.001$) the presence of CD8+ cells in

the ileum at 21 days when comparing to the untreated group, however, no significant difference was verified among NCBS group and the others. The NCBS group presented more ($p<0.001$) macrophages in the ileum mucosa at 7 days in comparison to the untreated NC group, however, no significant difference was verified among the birds fed with AGP (NCAGP group) and the two other groups.

## 4 DISCUSSION

Microbial community can impact the host physiology and, in case of pathogen invasion, can disturb homeostasis triggering an inflammatory response that can negatively impact feed intake, body weight gain and feed conversion rate (KOINTESTINAL and KLASING, 2009; KOINTESTINAL et al., 2018). In this experiment, the challenged groups had lower FI and BWG, and worse FCR at all ages (figure 1), however, no statistical results were found regarding product inclusion. Since there were few repetitions and animals, the focus of our trial was not to evaluate zootechnical performance, but the clinical condition of the animals. Nevertheless, it was already demonstrated that birds supplemented with *Bacillus subtilis* strains 29784 have significant improvement in the performance of broilers, with or without a challenge (RHAYAT et al., 2017; RAJPUT et al., 2013).

In this study, it was observed that *C. perfringens* and *Eimeria* spp. challenge resulted in histological lesions translated into a higher ISI score along with a higher immune cells count when comparing to non-challenged groups at all ages. *Eimeria* spp. oocysts inoculated at day one infiltrated intestinal epithelial cells to perform its reproductive cycle which causes mucosa destruction (SHIRLEY and LILLEHOJ, 2012) and triggers an inflammatory response (HONG et al., 2006) being the cell-mediated immunity the most relevant against coccidiosis, with increased proliferation and infiltration of T lymphocytes (RITZI, 2015). *Clostridum perfringens* inoculation at the 10th, 11th and 12th days of age was expected to interact with the coccidia given at day 1, in fact both agents act synergically contributing for the intestinal dysbiosis of the host, since the coccidia causes intestinal damage, resulting in higher inflammatory cells and plasma outflow into the lumen (VAN IMMERSEEL et al., 2004; PRESCOTT et al., 2016). Improved inflammatory response enhance mucogenesis (COLLIER et al., 2008; RITZI, 2015) and the surpluses of these nutrients are used by *C. perfringens* as a substrate to proliferate (VAN IMMERSEEL et al., 2004; COLLIER et al., 2008).

Considering a challenge with *Eimeria* only, it could be expected a critical phase at the 2nd week after challenge, since maximum oocyst output ranges from 6 to 9 days after infection (ALLEN and FETTERER, 2002). However, the *C. perfringens* challenge given at 10th, 11th and 12th days reflected only at the 3rd week of the experiment, presenting higher ISI scores due to the pathogen's interaction, with an increase of all intestinal parameters. It was also expected more mucus and inflammation process that could be used as substrate by *Clostridium* (BELOTE et al., 2018). Therefore, at 21 days it was the most critical phase of infection of this trial, when ISI total score of the challenged birds was exacerbated due to an increase of all intestinal parameters.

*B. subtilis* 29784 demonstrated lower ISI total score at 7 and 14 days of the challenged group (CHBS) related to minor lamina propria thickness score at both ages, and lower epithelial thickness, proliferation of enterocytes, inflammatory cell infiltration in the lamina propria, proliferation of goblet cells and presence of oocysts at 14 days. Belote et al., (2018) comparing challenged birds supplemented or not with enramycin showed more influence of the AGP in the reduction of lamina propria thickness as it was observed in this study with BS. Although there was no statistical difference in the total score, at 21 days it was observed a lower epithelial thickness in CHBS comparing to CH group.

*B. subtilis* 29784 capacity to bind to the intestinal mucosa might have blocked spots that could be occupied by *C. perfringens* (FULLER, 1975) disfavoring its proliferation and contributing to a balanced microbiota (AL-KHALAIFAH, 2018; PARK et al., 2016; JACQUIER et al., 2019). *B. subtilis* strain 29784 stimulates a greater proliferation of *Ruminococcus, Anaerostipes* and *Lachnospiraceae* (JACQUIER et al., 2019), which are known to produce butyrate, (EECKHAUT et al., 2011; RIDLON et al., 2015) a fermentation product that can reduce pathogen colonization, modulate immunity and suppress inflammation (ZHOU et al., 2014; 2017). Butyrate is also known as an energy source for enterocytes differentiation and proliferation (BEDFORD and GONG, 2018; SIKANDAR et al., 2017) that could result in enhanced surface area and stronger intestinal barrier. In fact, previous trials demonstrated that *B. subtilis* 29784 can improve intestinal morphology of challenged birds by increasing villus height and width (RAJPUT et al., 2013) and decreasing crypts depth (SAMANYA and YAMAUCHI, 2002; MARKOVIĆ et al., 2009; RITZI, 2015). This improvement, besides strengthening intestinal physical barrier, saves metabolism energy of the host that can be used in other physiological activities, such as growing.

*C. perfringens* and *Eimeria* spp. challenge demonstrated, at all ages, a higher release of macrophage, CD4+ and CD8+ T lymphocytes (figure 7) comparing with the non-challenged group, that consequently could have released pro-inflammatory cytokines, which are responsible for harming tight junctions' integrity and recruiting more inflammatory cells (RHAYAT et al., 2019). It can be assumed that when tight junctions are broken, the chances of pathogens to reach blood system and cause inflammation are higher, which then will justify performance alterations.

In this trial it was observed that when *Bacillus subtilis* 29784 was supplemented CD4+ cells were reduced at 7 days, had a peak at 14 days and decreased again at 21 days. CD8+ cell counts also had a peak at 14 days with posterior decrease at 21 days, in birds supplemented with *B. subtilis* (figure 9). These results can be explained by the fact that *B. subtilis* 29784 stimulates cell-mediated immunity through an increase of pro-inflammatory cytokines (MCALINDON; HAWKEY; MAHIDA, 1998; RHAYAT et al., 2019). A study demonstrated that all *B. subtilis* stimulate a minor secretion of IL-8 by intestinal cells primarily (HOSOI et al., 2003; RHAYAT et al. 2019) but the probiotic modulates the intestinal epithelial cells so the next time they get in contact with pro-inflammatory molecules, they will cause a milder inflammation, specially the strain 28794 (RHAYAT et al. 2019). The peak of CD4+ and CD8+ cells in birds fed *B. subtilis* 29784 at 14 days can also be explained by the probiotic ability to increase the expression of toll-like receptors (TLR). TLRs can identify pathogen-associated molecular patterns (PAMP) of damaging or even harmless bacteria (Wan et al., 2016), initiate a pro-inflammatory response and maintain 'a state of awareness' in the host (VIZOSO PINTO et al., 2009; WAN et al., 2016).

Probiotic inclusion was also associated with the release of innate immunity cells, such as macrophages (WAN et al., 2016; AL-KHALAIFAH, 2018), antimicrobial peptides and other (AUVYNET AND ROSENSTEIN, 2009; YANG et al., 2002). In conformity with our results, B. *subtilis* 29784 had a higher macrophage cell counts at 7 days in the non-challenged group (NCBS), that could have been a result of antimicrobial peptide released by the probiotic, which are responsible for recruiting even more macrophages and other immunological cells (AUVYNET and ROSENSTEIN, 2009; YANG et al., 2002; WAN et al., 2016).

Enramycin showed lower ISI total score at all stages specially for a decrease of the lamina propria and enterocyte proliferation at 7 and 14 days, similar results observed by Belote et al, 2018. The antibiotic demonstrated to be more efficient at the most

critical phase of coccidiosis, at 21 days, when it was observed milder alterations of all histological parameters evaluated except for increase of goblet cells. In this experiment it was observed that enramycin had lower presence of the CD4+ cells count at 7 days in the challenged group (CHAGP) and at 21 days in either challenged (CHAGP) or non-challenged group (NCAGP), and CD8+ cells count was decreased at 21 days (figure 9), in accordance to previous studies that suggested that antibiotics have anti-inflammatory properties (NIEWOLD, 2007).

The results of this trial demonstrated that *B. subtilis* strain 29784 feed supplementation had a positive impact on intestinal health of broilers morphology and immunity, confirmed by less intestinal lesions observed in the histology results, increased macrophage activation and decreased inflammatory lymphocytes.

## 5 FINAL CONSIDERATION

In the first chapter it was recognized the economic, well-being and public health impact that enteric diseases have in the chicken production chain and how important it is to have strategies to enhance gut health of production animals, such as using growth-promoter antibiotics and probiotics, for example. In the experiment presented in the second chapter it was possible to notice that the probiotics had a positive effect on gut mucosa, since it decreased the score of lesions and inflammatory parameters but AGPs demonstrated to be more efficient in critical phases of intestinal damage.

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