NUTRITIONAL STRATEGIES TO MITIGATE THE NEGATIVE EFFECTS OF NECROTIC ENTERITIS IN BROILER CHICKENS

by

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(Under the Direction of Todd Applegate)

ABSTRACT

The objective of this work was to evaluate the effects of supplementing dietary sodium butyrate (SB), essential oils (EO), and zinc (Zn) for broilers and their ability to modulate the intestinal microbiota and immune-system with and without enteric challenges. The general hypotheses of these studies were that these nutritional interventions would improve growth performance by modulating the intestinal microbiota and immune-system of broiler chickens challenged and unchallenged with coccidia plus *C. perfringens*. In the first study, the effect of different challenge models to induce necrotic enteritis (NE) was evaluated on the intestinal microbiota of broiler chickens. Subsequently, a study was conducted to evaluate the effect of SB supplementation in broiler chickens fed a diet with reduced concentration of energy and amino acids, and another one to evaluate the effects of SB alone or in combination with EO in broiler chickens challenged with coccidia plus *C. perfringens* on the intestinal microbiota and immune-system. Lastly, two studies were conducted to determine the effects of Zn source on the performance and intestinal physiology, immunology and microbiology of broiler chickens challenged with *Eimeria maxima* or *E. maxima* plus *C. perfringens*. Regardless of the challenge model, the induction of NE negatively impacted the growth performance of the birds. However,

the use of vaccination on d 1 and challenge with *C. perfringens* on d 18-20 is the method of choice to reproduce necrotic enteritis when evaluating its effect on the intestinal microbiota of broilers raised on floor pens with re-used litter. In the supplemented studies, SB and SBEO supplementation to unchallenged and NE-challenged birds contributed to changes in the diversity, composition and predicted functions of the cecal microbiome. Organic Zn increased jejunal villus height, decreased intestinal permeability, and modulated the expression of genes related to the immune response, including IL-8, IL-10, IgA, TLR-2, A20, and iNOS. In the second study, organic Zn led to lower expression of IL-8 and INF- γ in challenged birds which indicated a lessened inflammatory response. Organic Zn supplementation reduced the frequency of *Lactobacillus* in the ileal microbiota, and the induction to NE increased *Lactobacillus* and enterobacteria in the cecal microbiota.

INDEX WORDS: Broiler chickens, coccidiosis, essential oils, immune system, intestinal microbiota, necrotic enteritis, sodium butyrate, zinc.

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DCOTOR OF PHILOSOPHY

ATHENS, GEORGIA

2018

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December 2018

DEDICATION

I dedicate this dissertation to my loved parents. Without your support throughout my life I would never been able to accomplish this goal. I simply love you and wish one day I could retribute all you have done for me.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Todd Applegate, for the opportunity, support, and guidance throughout my doctoral studies. Thank you also for your constant availability, advices and support in all aspects of my professional and personal life. In addition, I would like to thank my committee members, Dr. Charles Hofacre, Dr. Woo-Kyun Kim, Dr. Brian Jordan, and Dr. Roselina Angel for the mentoring during my time at UGA.

I would like to specially express my gratitude to Dr. Kim for opening his lab in a way that I could do my molecular analyses. Dr. Hofacre, Dr. Mathis, Dr. Lumpkins, Chris Tate, and the farm crew for the guidance and help with the *in vivo* experiments. Additionally, I would like to thank Dr. Michael Rothrock from ARS-USDA for assisting with the bioinformatic analyses. I am very thankful for all your advices and help during this time. Dr. Monique França and Susan Williams from PDRC for helping with histology and histopathology analyses.

Thanks to Athens. Thanks to University of Georgia. My life is completely different from what it was when I got here, almost 3 years ago. Gui Verocai you know how special and important you are for me. All the friends and staff from the Department of Poultry Science, my sincere, thank you. In special, Fernanda, Bruno Vieira, Gabi, Manuel, Roshan, Pratima, Ana Maria, Caitlin, Jarred, Jundi, Sean, Jinquan, and many others. From the deepest of my heart, I wanted to embrace all of you and say, you are worth it!

My friends in Athens, in special Fernanda and Marcel, Nicole and Carlos, Grazi, Pablo and Karen, Kelsia and Wycliff. You are part of the family now!

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The advances obtained in poultry production over the last decades is due in part to the improvements in the sanitary management of the birds, specially through the use of antimicrobial growth promoters (AGP) as feed supplements. Several active compounds, when used in low doses, prevent the colonization and proliferation of enteric pathogens in the gastrointestinal tract (GIT), control enteric diseases, and improve feed efficiency and the growth performance of animals.

However, the European Union (EU) restricted, from January 2006, the use of any AGP in animal production (Castanon, 2007), and the United States restricted, from January 2017, the use of medically important antibiotics as growth promoters, in the diets of broiler chickens, only allowing for therapeutic and disease prevention purposes. Consequently, consumer perception is that antibiotic-free produced poultry is superior to conventionally raised poultry in spite of a lack of supporting scientific data. A widely accepted definition of antibiotic-free poultry in the United States is that there is “no use of antibiotics (including ionophore anticoccidials) at the farm” (Cervantes, 2015) meaning that coccidiosis and necrotic enteritis (NE) prevention must rely on synthetic or “chemical” anticoccidials, or on live coccidiosis vaccines, or on rotations between drugs and vaccines (Cervantes, 2015).

After the restriction of AGP by the EU, the use of coccidiostat ionophores, that has effects against *Eimeria* and some Gram-positive bacteria such as *Clostridium perfringens*, has increased significantly in the production of broiler chickens, and it is seen as the most effective option to control NE (Abildgaard et al., 2010). However, there is also an increasing pressure to restrict the use of ionophores, mainly in the no antibiotic ever (NAE) production systems, and to find suitable ways to control the proliferation of *C. perfringens* and the occurrence of NE.

The intestinal microbiota plays a critical role in the animal's productivity because of the strict associations between the microorganisms and the host. These beneficial interactions contribute to maintain the health, integrity of the gut, and the homeostasis of the microbial community in the GIT (Pedroso et al., 2012). The first line of defense against pathogens is the commensal microbiota of the intestine. Many commensal bacteria produce organic acids, such as lactic acid, propionic and butyric, as well as bacteriocins that have effect against gram-positive and gram-negative bacteria (Ding et al., 2017; Kers et al., 2018). Enteric infections can impair performance of the animals, due in part to the dysbiosis caused in the gut. Coccidiosis, a disease caused by the protozoa of the genus *Eimeria*, causes damage to the intestinal epithelia thereby inducing the proliferation of *C. perfringens*, and consequent lower nutrient absorption. Studies have demonstrated that coccidiosis alters the colonization profile of the gut (Hume et al., 2006; Macdonald et al., 2017; Zhou et al., 2017) which can also affect the growth of the animals.

Antibiotic growth promoters are considered the gold standards of performance-enhance feed additives; however, research is focused to find suitable alternatives, including: prebiotics, probiotics, organic acids, enzymes, essential oils, and specific nutrients, such as minerals, that may bring benefits to the animal by modulating the intestinal bacterial community and immune-

system. In spite of this, none of these alternatives have been as efficient as the AGP, and in general, have shown variable results (Niewold, 2007). Therefore, the objective of these projects was to evaluate the effect of a dietary sodium butyrate (SB) supplement, essential oils (EO), and zinc (Zn) in broiler chickens and their ability to modulate the intestinal microbiota and immune-system. The general hypothesis of these studies was that these nutritional interventions would modulate the intestinal microbiota and immune-system of broiler chickens challenged and non-challenged with coccidia plus *C. perfringens*.

Initially, the effect of different challenge models to induce necrotic enteritis on the intestinal microbiota will be discussed in Chapter 2: "Effect of different challenge models to induce necrotic enteritis on the intestinal microbiota of broiler chickens".

Subsequently, a study was conducted to evaluate the effect of SB supplementation in broiler chickens fed a diet with reduced concentration of energy and amino acids which is shown in Chapter 3: "Sodium butyrate improved performance while modulating the cecal microbiota and regulating the expression of intestinal immune-related genes of broiler chickens".

Additionally, a study was conducted to evaluate the effects of SB alone or in combination with EO in broiler chickens challenged with coccidia plus *C. perfringens*. The results are shown in Chapter 4: "Effects of supplementation of protected sodium butyrate alone or in combination with essential oils on the cecal microbiota of broiler chickens challenged with coccidia and *Clostridium perfringens*", and Chapter 5: "Expression of tight junction and immune-related genes of broiler chickens supplemented with sodium butyrate alone or in combination with essential oils under coccidia and *Clostridium perfringens* challenge".

Lastly, two studies were conducted to build on prior work by determining the effects of Zn source on the performance and intestinal physiology, immunology and microbiology of broiler chickens challenged with *Eimeria maxima* or *E. maxima* plus *C. perfringens*. These results are shown in Chapter 6: "Zinc source modulates intestinal inflammation and intestinal integrity of broiler chickens challenged with coccidia and *Clostridium perfringens*", and Chapter 7: "Can dietary zinc diminish the impact of necrotic enteritis on the growth performance by modulating intestinal physiology, immunology, and microbiology of broiler chickens?".

LITERATURE REVIEW

Coccidiosis and Necrotic Enteritis: disturbance of intestinal function

Coccidiosis is the most important parasitic disease in commercial poultry production systems (Allen and Fetterer, 2002). In chickens, coccidiosis is caused by up to seven Eimeria species, including *E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. praecox*, *E. necatrix*, and *E. tenella*. After being ingested, sporulated oocysts release the sporocysts that invade epithelial cells (Allen and Fetterer, 2002) and stimulate mucus production by goblet cells as a response to eliminate the coccidia. Coccidiosis is considered a primary predisposing factor for the development of NE, due in part to this increased mucogenesis (Dahiya et al., 2006; Collier et al., 2008). In addition to creating a more anaerobic environment, which is essential for the growth of *Clostridium perfringens* (commensal and causative agent of NE), the over production of mucus provides a substrate for the proliferation of this bacterium. Furthermore, the lesions caused by *Eimeria* increase leakage of plasma proteins into the intestinal lumen (Prescott et al., 2016) and decrease nutrient digestion and absorption, providing additional substrates that can lead to bacterial imbalance (Pedroso et al., 2012). Since the primary location of NE is in the duodenum and jejunum, it is reasonable to ague that *E. acervulina* and *E. maxima* would be the primary species causing NE (Hofacre et al., 2018). Mathis and Hofacre (2005) conducted a study comparing the different species of *Eimeria* and their effects in predisposing the birds to develop NE and observed that *E. maxima* had the largest effect.

Two main components are responsible for maintaining the intestinal mucosa integrity. The first is the mucosal layer, which serves as a barrier between luminal contents and

enterocytes lining the intestine. Mucin-type glycoproteins that make up the mucosal layer can aggregate different bacterial species, and in some cases, prevent the attachment of pathogens to the intestinal epithelium (Star et al., 2012). The intestinal mucosal layer is a key component of the innate immune response, and the innate and adaptive intestinal immune systems exhibit different spatio-temporal development in broiler chickens (Zhang et al., 2015). These authors observed that expression of the MUC2 gene, the primary secreting muco-protein, rapidly increases from embryonic d 14 to post-hatch d 1 in the ileum and cecum of broiler chickens. On the other hand, the enteric gene expression of immunoglobulin A (IgA), the primary antibody in mucosal secretions that prevents the entry of commensal or pathogenic bacteria into subepithelial areas (Brisbin et al., 2008), had slower development from embryonic d 14 to post-hatch d 5 followed by rapid development up to d 21 and 14 in the ileum and cecum, respectively (Zhang et al., 2015). However, enteric challenges such as coccidiosis or NE may alter the development of the immune response, and certain nutrients and feed additives may become limiting factors for appropriate immune function.

The second barrier component of the intestine is the enterocyte layer. The intestinal epithelium of the chicken is constantly renewed as proliferating cells in the mucosal crypts differentiate, predominantly to enterocytes, and migrate to the upper part of the villus, where they are eventually lost through desquamation (Uni et al., 2006). As described by Fernando and McCraw (1973), this turnover rate increases considerably during a coccidial challenge, and the magnitude and duration of this response can vary among intestinal regions. For example, these authors reported that in *Eimeria acervulina* infected-chickens the villus height of the duodenum reached its minimum value at 6 d post-infection, tending to return to normal afterwards, but still below that of non-challenged birds. On the other hand, the impact of the infection on villus

height in the jejunum was less pronounced, and after d 6, the villus height was 40 and 50% higher, in the middle and distal portions, respectively, in challenged when compared to non-challenged birds (~ 900 and 700 µm in challenged birds vs. 550 and 350 µm in control birds). Even though the performance of the birds was not reported in this study, this may suggest that in the recovery phase previously infected birds tend to have better performance due to the higher epithelial cell proliferation, as observed by Bortoluzzi et al. (2015).

Intestinal inflammation in coccidiosis-infected broilers results in villus necrosis, characterized by decreased villus:crypt ratio, crypt dilatation and goblet cell depletion, as well as increased expression of inflammatory genes (iNOS, IL-1β, IL-8 and MyD88; Tan et al., 2014). Furthermore, the co-infection of *Eimeria* with *C. perfringens* induces higher inflammation when compared with birds infected with *Eimeria* or *C. perfringens* alone (Collier et al., 2008), supporting the hypothesis that the host inflammatory response to eliminate coccidia provides a growth advantage for *C. perfringens*. Even though the immunopathology of NE in chickens is still unclear (Oh and Lillehoj, 2016), several studies have tried to explain the main changes that occur in the intestinal immune cells following NE infection (Park et al., 2008; Collier et al., 2008; Kim et al., 2014).

The activation of the immune system with moderate inflammation is essential for the survival of the birds following an infection, which can require subsequent tissue repair (Tan et al., 2014). In a recent study by Kim et al. (2014) on the immune response to NE, 1,049 genes were differentially expressed in intraepithelial lymphocytes of infected chickens, in which 601 were up and 448 were down-regulated. The totality of these genes (1,049), were grouped into five primary biological functions, which were all related with the immune response. This type of study generates a huge amount of data that must be carefully interpreted; however, once we

can predict the sequence of genes, and timing of expression, different nutritional strategies may be developed with the objective to optimize the immune response to facilitate pathogen clearance, minimize tissue damage, and maximize growth rate.

Shifts in the intestinal microbiota are also associated with NE (Stanley et al., 2012; Stanley et al., 2014; Antonissen et al., 2016). Even though *C. perfringens* is known to be the major causative agent in NE, other microorganisms can also contribute to the establishment and progression of the disease (Stanley et al., 2012). Using 16S rRNA sequencing, Stanley et al. (2012) showed that in *C. perfringens* infected chickens the structure of the cecal microbiota drastically changed, affecting mainly members of the orders *Clostridiales* and *Lactobacillales*. Additionally, some short-chain fatty acid (SCFA)-producing bacteria were decreased due to NE challenge, showing that not only *C. perfringens* proliferation but also the overall dysbiosis that it causes may be related to the pathogenesis of the disease. Nutrient availability as well as cross-feeding mechanisms among different bacterial groups in the gut may also be affected by shifts in the microbiota induced by *C. perfringens* (Stanley et al., 2014). Thus, it is reasonable to argue that different dietary nutrients and feed additives, in NE infected flocks, may indirectly optimize immunity through the selection of commensal bacteria that play important roles in modulating the host immune system, or vice-versa.

Nutritional interventions to reduce the risks of necrotic enteritis

In addition to its aforementioned barrier role, the primary function of the GIT is efficient digestion and absorption of nutrients. To support these functions, nutritionists must understand the intestinal immune system and its relationship with the microbial community as a distinct organ with specific nutrient needs. In practice, nutrient profiles used in feed formulations for

broiler chickens are typically based on economically important production outcomes such as weight gain, feed intake, feed conversion ratio and carcass yield (Kidd, 2004). The nutrient and energy need for immunity or disease resistance, however, may be understudied. Enteric challenges such as coccidiosis or NE may alter the development of the immune response, and certain nutrients and feed additives may become limiting factors to produce key proteins required for appropriate immune function.

The restrictions in the use of AGP in the diets of broiler chickens has been driven by regulations in many countries and consumer pressure to reduce the use of AGP. There has been an increasing number of studies looking for AGP replacements that are claimed to aid in performance, mitigate symptoms associated with a particular insult, or both (Applegate et al., 2010). The list includes a diverse range of products such as enzymes, plant extracts, probiotics, organic acids, antimicrobial peptides, among many others (Applegate et al., 2010). Butyrate and phytogenics are potential candidates for promoting intestinal health mainly due to their effects on the epithelial barrier function and immune-modulating properties (Ahsan et al., 2016; Du et al., 2016; Bortoluzzi et al., 2017; Sikandar et al., 2017) prior or after an injury. As we have demonstrated previously, SB modulates the expression of immune-related genes in the jejunum of disease-free broiler chickens (Bortoluzzi et al., 2017) and inhibits the expression of cytokines in chicken macrophages stimulated by LPS (Zhou et al., 2014). Moreover, it has been reported that SB upregulates the expression of TJ protein encoding genes such as claudin-1 and 4, occludin and zonula occludens-1 after challenge to induce to NE, with additional benefits to the intestinal morphology (Song et al., 2017). On the other hand, essential oils (thymol and carvacrol), have been shown to alleviate the severity of lesions caused by *C. perfringens*

challenge, but were not able to recover the expression of TJ protein encoding genes in its totality (Du et al., 2016).

Additionally, Zn is an essential trace mineral possessing unique chemical properties, which allows it to serve structural and catalytic roles for proteins and enzymatic reactions, acting mainly as an important cofactor (Troche et al., 2015). Zinc is a cofactor in antioxidant enzymes as well as a mediator of T cell development, and modulator of cytokine production (Prasad et al., 2011). Higher concentrations of Zn are rationalized based on literature wherein Zn metabolism changes with coccidial and bacterial challenges. Specifically during these challenges, plasma Zn greatly decreases (Turk and Stephens, 1966, 1967; Southern and Baker, 1983; Turk, 1986; Richards and Augustine, 1988) while hepatic Zn greatly increases as it is bound through up-regulation of metallothionein (MT) during an acute phase response to these challenges (Richards and Augustine, 1988). Therefore, this review will further focus on the roles of SB, EO, and Zn and their ability to reduce the risk of NE in broiler chickens; also, the importance of the intestinal microbiota in broiler chickens will be discussed.

Sodium butyrate use in diets of broiler chickens

Short-chain fatty acids (SCFA) are a group of molecules that contain from one to seven carbons produced within the intestinal lumen by bacterial fermentation of plant materials such as celluloses, fibers, starches and sugars for which animals lack the necessary enzymes to break these compounds down (Guilloteau et al., 2010). The SCFA with higher abundance in the GIT are acetate, propionate, and butyrate (Bedford and Gong, 2018). These acids have several effects for animals. For example, acetic and propionic acids are used as acidifiers in piglets' diets to help overcome enteric issues, such as post-weaning diarrhea. Additionally, butyrate has

multiple effects on the intestine. It serves as an energy source for epithelial cells, stimulates mucus production, controls the intestinal barrier function, promotes pathogen control, and modulates the immune-system (Guilloteau et al., 2010).

Butyrate is typically supplied as a feed supplement as Na, K, Mg or Ca salts which are odorless and easier to handle (Guilloteau et al., 2010). Sodium butyrate is the sodium salt of butyric acid which contains sodium atom in place of hydrogen of -OH group (Figure 1; Ahsan et a., 2016). Butyric acid has received particular attention as a feed supplement in the diet of broiler chickens. While butyrate is naturally produced by fermentation in the cecum, the production of SCFA in the small intestine is limited (Levy et al., 2015), thereby suggesting that its dietary supplementation would be beneficial, mainly when targeting different regions of the small intestine. Synthetic sources of butyrate have been the focus of numerous studies in poultry (Leeson et al., 2005; Hu and Guo, 2007; Timbermont et al., 2010; Sunkara et al., 2011; Qaisrani et al., 2015). However, it has been described that uncoated butyrate could be absorbed or metabolized before reaching the distal portions of the small intestine (van der Wielen et al., 2002). Dietary supplementation with a protected source of butyrate may delay the release of the substance along the gastrointestinal tract, thereby having plausible functional effects on the lower GIT. Besides the location of the GIT where butyrate is released, the dose of butyrate is also a factor that should be considered when using it as a feed supplement.

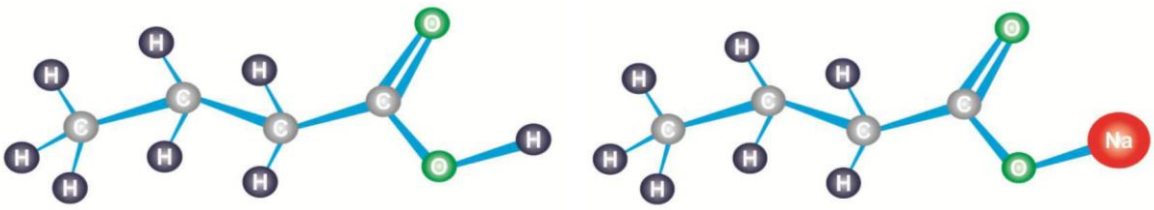

Figure 1.1: Structure of butyric acid (A) and sodium butyrate (B). Adapted from Ahsan et al. (2016)

The efficacy in which SB will be absorbed and used depends on the pH of the GIT location (crop, proventriculus, gizzard, small intestine). With a pH lower than 4.82 (pKa of butyric acid), most of the molecules of butyric acid remain un-dissociated which is the desirable form for higher antimicrobial activity (Ahsan et al., 2016). Sodium butyrate is converted into butyric acid after ingestion due to the acidic pH found in the proximal regions of the GIT. Thereafter, when it reaches the small intestine (alkaline pH) butyric acid is dissociated into butyrate and hydrogen ions, and butyrate is absorbed as a source of energy (Ahsan et al., 2016), as well as exerting many other functions on the host (Zhou et al., 2014; Bortoluzzi et al., 2017; Sikandar et al., 2017; Song et al., 2017). In a previous study (Bortoluzzi et al., 2017), SB supplementation partially counteracted the impairment in performance of broilers fed a diet formulated with reduced energy and amino acid concentrations, modulated the immune system and cecal microbiota.

It has been demonstrated that butyrate is important to maintaining the intestinal barrier function (Wang et al., 2012; Song et al., 2017), as well as for the appropriate development of the GIT (Guilloteau et al., 2010; Sikandar et al., 2017). Wang et al. (2012) showed that butyrate upregulated the expression of claudin 1, and redistributed zonula occludens-1 and occludin in the cellular membrane. Yet, Song et al. (2017) showed that SB upregulated the expression of claudin 1 and 4, ZO-1, and occludin in broiler chickens induced to NE. Even though the expression of genes that encode for TJ proteins is an important indicator of the integrity of the intestinal barrier, these results would have to be confirmed with other measures of intestinal permeability, such as passage of substances from the intestinal lumen to the blood.

Butyrate may regulate the production of inflammatory cytokines by modulating the intestinal immune cells, such as lymphocytes and macrophages (Guilloteau et al., 2010). Despite this knowledge, the mechanism by which butyrate exerts its anti-inflammatory effects remains to be determined (Chang et al., 2014). However, butyrate seems to have an anti-inflammatory effect mediated by signaling pathways (Meijer et al., 2010), such as the modulation of pro-inflammatory cytokines via impairment in NF-kB activation (Guilloteau et al., 2010). Butyrate leads to epigenetic adaptations, changes in the gene function without changing the DNA sequence, due to its histone deacetylase inhibitory effects, which can result in hyperacetylation of histones, and can change the expression of a large number of genes (Marks et al., 2000). This epigenetic effect caused by butyrate may be responsible for the changes in the expression of genes involved in the inflammatory process, reducing pro-inflammatory cytokine expression and upregulation of anti-inflammatory ones (Meijer et al., 2010; Vinolo et al., 2011; Fung et al., 2012).

Besides its effects on immune-modulation, butyrate serves as an energy source for enterocytes and colonocytes, stimulates mucus synthesis, promotes intestinal cell proliferation, differentiation and maturation, controls intestinal barrier function, decreases apoptosis of normal cells, and has antimicrobial effects against pathogenic bacteria (Guilloteau et al., 2010). In fact, the immune-modulation promoted by butyrate may be due to its indirect effect on the microbial population of the gut. Some studies have shown the effects of butyrate in controlling pathogens in poultry, such as *Salmonella*, *Clostridium perfringens*, and modulating the *Lactobacillus* population (Van Immerseel et al., 2005; Hu and Guo, 2007; Timbermont et al., 2010; Namkung et al., 2011). Furthermore, a question remains on what effect butyrate may elicit on the cecal microbiota, and what translational effects it elicits on the host.

In a study conducted by Yang et al. (2018) it was observed that butyrate glycerides did not change the diversity of the microbiota but changed its composition. There was a decrease in potential pathogenic bacteria and increase in the abundance and diversity of bacteria belonging to the genus *Bifidobacterium*, which has a wide spectrum of benefits through the production of several metabolites. For example, it has been observed that serum choline, dimethylamine, trimethylamine, and succinate, particularly derived from *Bifidobacterium*, were increased in birds supplemented with butyrate (Yang et al., 2018), suggesting that energy and metabolism lipids may also be regulated by bacterial metabolites. Indeed, butyrate modulated the energy and lipids metabolism by upregulating genes involved in the reduction of synthesis, storage, transportation, and secretion of lipids in the jejunum, and enhancement of the oxidation of ingested lipids and fatty acids in the liver (Yin et al., 2016; Yang et al., 2018).

Additional studies have been conducted to investigate the effect of butyrate supplementation in the diets of broiler in the presence and absence of disease challenge. For instance, Bortoluzzi et al. (2017) observed that dietary inclusion of SB *per se* did not have a significant impact on the cecal microbiota of broiler chickens. However, when the birds were fed a nutritionally-reduced diet, the composition and predicted function of the cecal bacteria community varied drastically, and the supplementation of SB reduced these variations. Similar findings have been observed by Zhou et al. (2017) wherein coated SB had no significant effect on the cecal microbiota of healthy chickens but balanced the shifts of microbial composition caused by *E. tenella* infection, and decreased cecal colonization of *Salmonella* after experimental infection (Van Immerseel et al., 2004). Enteric diseases that affect the proper digestion and absorption of nutrients in the foregut will most likely have an impact on the microbial communities in the hindgut (Castillo et al., 2008). Therefore, besides the direct

effects that butyrate has on the function and metabolism of the intestine itself, microencapsulated sources of butyrate that are released throughout the GIT can diminish the impact of these pathogens in the lower intestine.

Phytogenics use in diets of broiler chickens

Phytogenic feed additives constitute a complex mixture of volatile substances, which are usually lipophilic (Hashemi and Davoodi, 2012), such as terpene hydrocarbons, simple alcohols, aldehydes, ketones, phenols, esters, and fixed organic acids, in different concentrations, in which a pharmacologically active compound is most prevalent. There is considerable variations associated to their chemical composition, depending mostly on the weather, season of harvest, location or storage conditions which are responsible for the differences in efficacy of these compounds (Applegate et al., 2010). These secondary metabolites of plants have no direct roles on photosynthetic, respiration, protein synthesis, nutrient assimilation, and many other cellular processes, and are generally found in only one plant species or related group of species (Hashemi and Davoodi, 2012). They are generated only in specific developmental stages of the plant, and commonly have anti-feeding and anti-infecting chemicals that are effective against microbes (Hashemi and Davoodi, 2012).

Remmal et al. (2011) explained the need to look for substances with reduced possibility to develop antimicrobial resistance, besides the capacity of leaving no residues in the final product. For example, these authors investigated the anticoccidial effect, in vitro, of 10 different phytogenics, and verified that all of them have action against *Eimeria* oocysts. However, essential oils from *Artemisia absinthium*, Tea tree, *Thymus vulgaris* (thyme) and *Syzygium aromaticum* (clove oil) were more effective than salinomycin in reducing the number of viable

oocysts. On the other hand, in vivo studies have demonstrated beneficial effects of the bio-active plant compounds against coccidiosis (Allen et al., 1997; Giannenas et al., 2003; Naidoo et al., 2008; Lee et al., 2011; Almeida et al., 2012; Kim et al., 2013). *Artemisia annua* (Allen et al., 1997) and carvacrol (Giannenas et al., 2003) promoted effect against *E. tenella*. *Tulbaghia violacea* reduced the oocyst shedding and prevented the loss in performance of broiler chickens (Naidoo et al., 2008). Lee et al. (2011) obtained better performance and immune response of broilers challenged with *E. tenella* and supplemented with phytogenics. Substances that have capacity to generate oxidative stress in the parasite (*Artemisia annua*) are more effective against *E. Tenella,* while those with antioxidant properties (curcumin) will have more effect against *E. acervulina* and *E. maxima* (Allen and Ferreter, 2002). Therefore, feed additives with action against *Eimeria* will also help reduce the effects of NE since coccidiosis is the most important predisposing factor to develop NE.

Enteric diseases lead to loss in productivity, reduce the well-being of the birds, and increase the risk of carcass contamination (Dahiya et al., 2006). Coccidiosis can increase mucus production by goblet cells and induce the proliferation of *C. perfringens* (Baba et al., 1997). Besides damaging the intestinal epithelium, *Eimeria* infection can increase the availability of undigested feed which can be used as a substrate by the pathogenic bacteria, causing dysbiosis (Pedroso et al., 2012). The normal population of *C. perfringens* is low in the intestine, but changes in the normal microbiota can rapidly increase its density and the occurrence of NE (Dahiya et al., 2006). Necrotic enteritis is a multifactorial disease and a number of predisposing factors are needed for its development, such as *Eimeria* infection, removal of coccidiostats and AGP, poor environmental and management conditions, stress, immunosuppression, and diet (Dahiya et al., 2006).

A growing number of publications have demonstrated that coccidiosis and/or NE alters the microbial colonization profile of the gut (Hume et al., 2006; Kim et al., 2015; Zhou et al., 2017; Macdonald et al., 2017), but a blend of EO reduced these variations (Oviedo-Rondon et al., 2006). Furthermore, *Capsicum* and *Curcuma longa* oleoresins modulated the gut microbiota of broilers induced to NE by increasing *Candidatus Arthromitus* and *Lactobacillus*, besides reducing the negative consequences of NE on body weight gain and intestinal lesions score (Kim et al., 2015). *Candidatus Arthromitus* is a segmented filamentous bacteria (SFB) and has a close relationship with epithelial cells in the ileum and is paramount for the modulation of the immune system mainly by inducing regulatory T cells (Lillehoj et al., 2018). Even though additional studies concerning the use of phytogenics on gut microbiota and host-pathogen interactions are necessary (Lillehoj et al., 2018), the use of EO is becoming a common practice primarily due to the improvements of gut functions, which include the stabilization of the microbiota and better nutrient utilization and absorption (Diaz-Sanchez et al., 2015).

Although the exact mechanism of action of phytogenics is highly different depending on the active compound, it is believed to include: disruption of the cellular membrane of pathogens, modification of cells affecting the virulence capacity of the microorganism, stimulation of the immune system, protection against pathogen binding to the intestinal mucosa (Diaz-Sanchez et al., 2015). Among the phytogenics, EO have been subject of many studies mainly due to their antimicrobial and growth promoter effects. The mechanism of action of EO is based on its chemical composition, and on the location of the hydroxyl group; for example, thymol and carvacrol are isomeric molecules and possess similar antimicrobial effects (Diaz-Sanchez et al., 2015). Additionally, these plant metabolites have also been shown to exert immunomodulatory effects on the host, including induction of heat shock proteins, induction of

Toll-like receptors, and induction of proliferation and maturation of T-Helper cell (Th-1 and Th-2) to maintain a balance between cellular and humoral immune response (Hashemi and Davoodi, 2012).

Hashemi and Davoodi (2012) reviewed the chemical compounds of some phytogenics and their effects on the immune system. For instance, most of these activities rely on the stimulation or modulation of cytokines production, stimulation of macrophage production and action, among others. Similarly, Diaz-Sanchez et al. (2015) reviewed the effects of several chemical plant compounds and their antimicrobial effects and actions on the growth performance of the birds. Most of the studies focus on the effects of thymol, capsaicin, and carvacrol and show a reduction of *E. coli*, *Salmonella*, and *C. perfringens* with the use of these substances in the diets of chickens (Diaz-Sanchez et al., 2015). Additionally, Bortoluzzi et al. (2014) observed that dietary supplementation of 30 mg/kg of beta-acids isolated from hops lead to the same feed efficiency as zinc bacitracin in broilers fed diets with 5% of poultry bio-product meal and wheat bran. Yet, this same compound modulated the microbial population in the intestine (Bortoluzzi et al., 2015), and cytokines production (Bortoluzzi et al., 2016). Therefore, different systems and measurements should be evaluated when conducting studies with these feed additives due to their broad range of effects, and possible mechanisms of action.

When looking for AGP replacements one should keep in mind that a combination of products to address several components of the gut health may bring more benefits than using just a single approach (Lillehoj et al., 2018). This was the case in a study conducted by Jerzsele et al. (2012) wherein it was shown that a blend of ginger oils and carvacrol combined with SB reduced the gross lesions caused by NE and had beneficial effects on the intestinal morphology, even though the intestinal microbiota was not studied. Necrotic enteritis is a multi-factorial

disease, and therefore, a single alternative may not be able to promote all the effects needed to overcome its effects. For instance, the combination of EO with SB seems to have synergistic effects due to their broad mechanisms of action on the host; yet, the combination of these feed additives with probiotics, organic minerals, enzymes and others is yet to be determined.

Use of Zinc for controlling enteric diseases in broilers

Zinc is an essential micromineral required for growth, and influences intestinal development and/or regeneration during and after enteric diseases (MacDonald, 2000). The indispensability of Zn in the diets of animals has been recognized for years (Salim et al., 2008). Zinc is a hydrophilic ion and cannot cross cellular membranes by simple diffusion, which requires specialized mechanisms for its cellular uptake. Integral membrane transport proteins are used to move Zn across the lipid bilayer of the plasma membrane (Tako et al., 2005). Over the years, inorganic sources of Zn have been used as oxides and sulfates to supplement the diets of broiler chickens above the NRC (1994) recommended concentrations (Lesson, 2005).

When inorganic trace minerals are fed and reach the upper parts of the GIT they tend to dissociate due to the low pH and interact with other minerals or dietary compounds (Mwangi et al., 2017), decreasing their bioavailability. Thus, it is reasonable to argue that when the absorptive capacity of the intestine is impaired due to an enteric infection, such as coccidiosis and NE, a more available source of Zn may be needed. The use of organic Zn to supplement broiler diets is becoming a more common practice when looking to enhance mineral uptake, improved growth performance, and reduce mineral excretion (Burrell et al., 2004; Yan and Waldroup, 2006; Nollet et al., 2007; Mwangi et al., 2017). Zinc nutrition has become an active area of research, mainly in broilers. Adequate Zn intake and absorption is essential for many

metabolic and biological functions, including growth, reproduction, meat quality, and immune response against pathogen challenge (Salim et al., 2008).

Zinc is paramount for adequate functioning of heterophils, mononuclear phagocytes and T lymphocytes (Kidd et al., 1996). Furthermore, studies have shown the impact of Zn on growth performance and antioxidant system (Mwangi et al., 2017); immune defense and inflammation (Prasad et al., 2011; Li et al., 2015), and intestinal permeability (Zhang and Guo, 2009). It has been demonstrated that dietary Zn concentrations higher than the 40 mg/kg recommended by NRC (1994) lowers the impact of coccidiosis in broilers (Bafundo et al., 1984; Troche, 2012). Yet, *Eimeria acervulina* infection has been shown to reduce the effect of Zn toxicity in chickens when added to diets in high concentrations (2,000 and 4,000 mg/kg), most likely due to the impaired absorption rate (Southern and Baker, 1983). More recently, Troche (2012) showed that the Zn concentration required for maximum body weight gain in chickens went from 45 mg/kg in uninfected chickens to 75 mg/kg in coccidiosis infected birds. Therefore, enteric infections impair the proper absorption of Zn which may become deficient to promote adequate growth and functioning of important systems.

The effects of Zn on intestinal immunology and permeability have been observed previously (Prasad et al., 2011; Troche, 2012; Li et al., 2015). Organic Zn induced higher expression of A20, an anti-inflammatory regulator, downregulated the expression of inflammatory inducers, including NF-kB p65 (Prasad et al., 2011; Li et al., 2015), and promoted MUC2 and IgA production, when compared to its inorganic counterpart (Prasad et al., 2011). Epigenetic mechanisms alter gene expression without changes in DNA sequence and can explain the effects of Zn on the cell. The higher expression of A20 promoted by organic Zn is most likely due to an epigenetic effect by lowering DNA methylation (Li et al., 2015).

Changes in the intestinal permeability may be influenced by modulation (down or up-regulation) and/or functionality of TJ proteins, in which bacterial derived proteases may cause its degradation (Awad et al., 2017) by a broad range of mechanisms. However, it has been demonstrated that *C. perfringens* enterotoxins are able to attach to the cell surface by binding to the TJ proteins, especially to the claudin family proteins (Eichner et al., 2017). With the objective of understanding the molecular basis of the reduction of the intestinal permeability promoted by Zn supplementation, Zhang and Guo (2009) evaluated the gene expression of TJ proteins in weaning piglets as well as protein expression by Western blots. They observed that Zn enhanced intestinal permeability, by upregulating the expression of occludin and zonula occludens- 1 at the mRNA and protein levels. In another study, Zhang et al. (2012) observed that supplemental Zn upregulated the expression of occludin and claudin-1 in the ileum of chickens challenged with *Salmonella* Typhimurium.

Despite to the fact that Zn is widely used in animal nutrition in high concentrations with the objective of promoting growth, there is a lack of studies available on the effects of Zn on the intestinal bacterial community (Starke et al., 2014). Starke et al. (2014) evaluated the effect of high dietary Zn (2,425 mg/kg) in the diet of weaned piglets on the intestinal microbiota and their metabolic activity and observed that the diet had a large impact on three species of *Lactobacillus* mainly. *Lactobacillus amylovorus* was the most impacted species, wherein its decrease coincided with lower lactic acid concentrations due to the high dietary Zn. Furthermore, it seems that high dietary Zn concentrations have no effect (Broom et al., 2006) or increase the number of enterobacteria in the gut (Hojberg et al., 2005), which may suggest that these bacteria possess a mechanism to counteract high Zn concentrations (Starke et al., 2014). Yet, bacterial metabolites in the small and large intestines were lower in the high dietary Zn

group, mainly because of a decrease in propionate concentration, and partially due to lower n-butyrate concentrations. On the other hand, Zn supplementation (120 mg/kg) restored the cecal microbial community of *Salmonella* Typhimurium infected chickens by increasing the number of total bacteria and Lactobacillus, and reducing *Salmonella* colonization (Shao et al., 2014). Therefore, it seems that the effect of Zn supplementation relies on the concentration that it is used in the diet and the presence, absence, and/or severity of a challenge.

Importance of intestinal microbiota in broiler chickens

Chicks hatch with the GIT nearly devoid of microorganisms mainly due to the absence of contact with the hen during incubation and after hatching. Some strategies can be adopted to speed the initial colonization of the GIT with beneficial bacteria, including spraying the surface of the eggs with a selection of good bacteria at the moment of transfer of the eggs to the hatcher, in ovo inoculation of probiotics, amongst other strategies. For example, Pedroso et al. (2016) inoculated a competitive-exclusion product into eggs at d 18 of incubation and observed that the inoculation increased diversity and affected the composition of the intestinal microbiota, even though this effect was transient. In another study, cecal microbiota from good and poor feed efficiency birds was used to spray the eggs surface prior to the hatching and to study the initial colonization profile of the GIT (Donaldson et al., 2017). These authors observed that even though the performance of the donors was not transferred to recipient birds, the cecal treatment reduced bird-to-bird variation, which may lead to a better uniformity in terms of performance to the treated flocks.

In the last few years, many publications have elaborated on factors affecting the intestinal microbiota, mainly because of the large influence that these microorganisms may

have on the GIT. It has been estimated that the intestinal microbial community is composed for more than 800 species of bacteria, with significant impact on the health and nutritional status of the host (Laparra and Sanz, 2010). In broiler chickens, dietary composition, host characteristics (breed, age, sex), and external environment factors affect microbial communities in the GIT (Kers et al., 2018). Contaminated litter and other management conditions influence the composition of the intestinal microbiota, directly as a source of bacteria, or indirectly by its effect on the physical barrier and defense of the intestine (Apajalahti et al., 2004). Yet, the luminal microbiota may be regulated by the influx of nutrient from the diet, by the rate of passage of the intestinal content, and level and activity of antimicrobial substances (Kautsos and Arias, 2006). Laparra and Sanz (2010) also stated that the dietary compounds are metabolized during their passage through the GIT, before being absorbed, serving as a substrate to different microbial communities. Indeed, nutrients that are not used by the animal may be used by the microbiota and lead to a state of dysbiosis.

In coordination with the intestinal mucosa, the intestinal microbiota is responsible for the first line of defense in an animal and works by regulating cellular permeability, altering the expression of genes in goblet cells for increased mucus production and stimulating secretion of antimicrobial peptides (Laparra and Sanz, 2010). As such, a well-established intestinal microbiota brings benefits to the host due to production of vitamins, immune modulation, and inhibition of pathogens, whereas microbial imbalance may contribute to the development of metabolic and immunologic diseases (Jeurissen et al., 2002) and increase competition for nutrients with the host (Yang et al., 2009). In addition to directly causing morphological damage, diseases such as coccidiosis and NE can decrease the abundance of desirable groups of

bacteria, particularly segmented filamentous bacteria, that play a role in the modulation of the host immune system (Antonissen et al., 2016).

According to Gabriel et al. (2006), the sites with the most bacterial activity and highest concentration in the digesta in broiler chickens are the crop and cecum. The small intestine has a lower number of microorganisms due to the unfavorable conditions that are not ideal for microbial proliferation. In the duodenum, for example, there is high secretion of digestive enzymes, high oxygen pressure, high concentrations of antimicrobial substances (bile salts), as well as constant movement of digesta from the gizzard and jejunum, which constantly changes the duodenal micro-conditions. On the other hand, in the distal portion of the small intestine the microorganisms may have more adequate conditions to grow, but still with a lower diversity than the cecum; at this site, due to the lower movement of content the number and diversity of microorganism increase considerably, with predominance of strict anaerobic populations of bacteria.

Lu et al. (2003) reported that it is necessary to understand the dynamic of the intestinal microbiota of broilers fed simple vegetable-based diets before testing the effects of antimicrobial feed additives. For instance, these authors observed that chickens fed diets based on corn and soybean meal, without an animal protein source, antibiotic or coccidiostat, the ileal digesta was dominated by bacteria belonging to the genus *Lactobacillus,* followed by members of the family *Clostridiaceae,* genera *Streptococcus* and *Enterococcus*. On the other hand, the cecal digesta showed more frequency of members of the family *Clostridiaceae*, followed by the genera *Fusobacterium, Lactobacillus* and *Bacteroides*. Nevertheless, the intestinal microbiota may vary according to the feed efficiency of the birds (Stanley et al., 2012), or, animals with

better feed efficiency may be studied and used as biomarkers for modulation and early establishment of the microbiota (Pedroso et al., 2012).

The digestive tract of birds also hosts several different pathogenic bacteria, such as *Salmonella* and *Escherichia coli*. Gram-negative bacteria have in the composition of their cell wall lipopolysaccharides (endotoxins) that are released during the lyse of the bacterial cell. These toxins act in the thermoregulation center of the hypothalamus. Other toxins may affect the intestinal motility, leading to diarrhea (Gabriel et al., 2006). Through competitive exclusion, the beneficial microbiota can bind to receptors on the intestinal epithelial cells and prevent the binding of pathogens, which will be excreted.

The intestinal microbial community may be difficult to be cultivated mainly because some bacterial species need specific nutritional conditions that are not known and not supplied by the standard culture media (Pedroso et al., 2012). These authors also reported that some bacteria grow easily in the presence of others, which is known as a cross-feeding mechanism, wherein metabolites produced by one species are used by another species to grow; yet, culture media are specific for each bacterial group. Therefore, the study of intestinal ecology through molecular techniques is efficient to understand the dynamics of the gut microbiota (Pedroso et al., 2012).

Despite the benefits that the microbiota can bring to the host, one should keep in mind that these microorganisms may consume and compete for nutrients with the host (Yang et al., 2009). The costs associated with the benefits of the microbiota may include: competition for nutrients, stimulation of the epithelial cell turnover, secretion of toxic compounds, and induction of the inflammatory response (Yegani and Korver, 2008). Therefore, an adequate

microbial balance is essential not only to maintain the health of the flock, but also to avoid the excessive expenditure of dietary nutrients and energy.

Moreover, most of the times the increase in *Lactobacillus* is associated with better intestinal health. However, Torok et al. (2011) reported that the presence of three species of *Lactobacillus* (*L. salivarius*, *L. aviarius* and *L. crispatus*) in the ileum of broiler chickens was correlated with worse performance, which can explain why broilers with lower frequency of *Lactobacillus* showed better growth performance. *Lactobacillus salivarius* causes deconjugation of biliary salts in the GIT of broiler chickens (Guban et al., 2006); this fact should be taken into consideration mainly in young chicks, due to the lower capacity to digest and absorb lipids (Knarreborg et al., 2002a). Yet, it has been noticed that *C. perfringens* can also lead to deconjugation of biliary salts (Knarreborg et al., 2002b).

The understanding and monitoring of the intestinal microbial ecosystem are paramount to develop strategies and interventions to modulate the microbiota and reduce the occurrence of enteric diseases. Most of the research has been focused on the interactions between nutrition and cecal microbiota, but the events that occur in the crop and small intestine are also important (Gabriel, 2006; Oviedo-Rondon, 2009). AGP added to the feed of broiler chickens have the function to maintain the balance of the intestinal microbiota and avoid excessive proliferation of bacteria with pathogenic potential (Brisbin et al., 2008). These substances reduce the microbial load in the GIT, leading to higher availability of nutrients to the host and lower activation of the inflammatory response.

Coccidiosis is one of the most important predisposing factors for the development of NE in chickens (Prescott el al., 2016). The increased mucus production that occurs due to the *Eimeria* infection (Collier et al., 2008), and leakage of plasma proteins into the intestinal lumen

(Prescott et al., 2016) increase the proliferation of *C. perfringens*, the causative agent of NE. Besides increasing the susceptibility to NE, coccidiosis also leads to changes in the overall structure of the intestinal microbiota (Stanley et al., 2014; Wu et al., 2014; Zhou et al., 2017). Wu et al. (2014) demonstrated a reduction in the cecal microbial diversity following *Eimeria* infection, and reduction of many members of the family *Ruminococcaceae*. Yet, *Eimeria* infection caused changes in short-chain fatty acids (SCFA) produced in the ceca of chickens (Stanley et al., 2014).

Clostridium perfringens infection is also associated with shifts in the intestinal microbiota (Stanley et al., 2012; Stanley et al., 2014; Antonissen et al., 2016). *C. perfringens* can interact and compete with other microorganisms in the gut, which may alter its proliferation, production of the toxins and the severity of the disease (Antonissen et al., 2016). Stanley et al. (2012) reported that NE infection changed the abundance of important bacterial families in the gut, such as *Clostridiales* and *Lactobacillales*. Additionally, SCFA-producing bacteria as well as segmented filamentous bacteria decreased due to NE challenge, demonstrating that the overall dysbiosis may also be related to the pathogenesis of the disease (Stanley et al., 2014). Thus, it is reasonable to hypothesize that different dietary supplements to NE infected flocks may directly influence the diversity and composition of the intestinal microbiota, mainly through growth and proliferation of commensal bacteria that play important roles on the general physiology of the host.

Sodium butyrate and phytogenic feed additives can help control the excessive proliferation of bacteria and subclinical diseases. Studies have shown the potential of plant compounds in the diets of broiler chickens to control intestinal pathogens and to maintain the beneficial microbiota (Tiihonen et al., 2010; Mountsouris et al., 2011; Abdel-Wareth et al.,

2012; Hashemi et al., 2012; Kim et al., 2013). For instance, plant extracts are able to reduce *E. coli* and *Lactobacillus* in the cecum of broiler chickens (Tiihonen et al., 2010), *E. Coli* (Hashemi et al., 2012), or *C. perfringens* (Abildgaard et al., 2010). Furthermore, when used in conjunction, feed additives may have synergistic effects on the host which has been shown by Jerzsele et al. (2012), wherein a blend of ginger oils and carvacrol plus SB reduced the impact of NE in chickens.

Butyrate itself has a broad range of mechanisms of action and multiple effects on the intestine. It serves as an energy source for epithelial cells, stimulates mucus production, controls the intestinal barrier function, promotes pathogen control, and modulates the immune-system (Guilloteau et al., 2010). Therefore, it is believed that butyrate may alleviate the negative effects of NE in chickens by modulating the intestinal microbiota, immune-system, and the intestinal barrier function. It has been recently shown that dietary supplementation of chickens with a protected source of SB modulated the diversity, composition, and predictive function of the cecal microbiota (Bortoluzzi et al., 2017). Indeed, SB may directly modulate the intestinal microbiome, through its bactericidal effect, or indirectly by stimulating the growth of beneficial lactic acid bacteria (Ahsan et al., 2016).

The dominance of beneficial microorganisms over pathogens is essential to maintain gut homeostasis and is directly related to the adequate absorption of nutrients by the intestine, with better performance and lower incidence of enteric disease. The objective in studding the microbiota should not only reflect its composition, but the relationship between microorganisms and the host, and the functions that these microorganisms are exerting on the gut. For instance, it is known that the GIT of germ-free animals has lower maintenance cost when compared to conventional animals, and lower immunologic activation, and therefore, more nutrients can go

towards the growth of the animals. However, farm raised broilers are in constant contact with microorganisms which has a significant impact on the nutritional costs and health of the birds.

Although the interactions between commensal bacteria and host immune system are not fully understood, the intestinal immune system is responsible for initiating and propagating responses to commensal and pathogenic microorganisms. As such, the understanding of these interactions, in normal and disease situations, will allow the formulation of diets with different feed additives, that will be able to totally, or at least partially, attenuate the negative effects of enteric diseases. In this regard, we still do not know whether the potential benefits of butyrate, EO, and Zn are through "reprograming" the tissue before the challenge, lessening the invasiveness and propagation of the pathogen, improving clearance of the pathogen, and/or improving the healing capacity after an injury. Furthermore, the immunomodulation and selection of a beneficial microbiota through dietary interventions will likely aide the bird for coping with pathogen challenges.

The understanding is that healthy animals develop a balanced microbiota without overstimulating the immune system that is costly in terms of nutrients allocation, but necessary to eliminate pathogens (Applegate et al., 2010). However, we do not know much beyond changes in microbial structure within different intestinal regions, i.e., information is missing with regards to microbial-cross talk, modulation of pathogenicity factors by nutrients and feed additives, and endogenous losses associated with microbial dysbiosis and immune response (Applegate et al., 2010). Therefore, this dissertation sought to address the effects of sodium butyrate, essential oils based on ginger and carvacrol oils, and zinc on the intestinal physiology, immunology, and microbiology of broiler chickens raised without challenge or under coccidia and *C. perfringens* challenge.

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CHAPTER 2

EFFECT OF DIFFERENT CHALLENGE MODELS TO INDUCE NECROTIC ENTERITIS ON THE INTESTINAL MICROBIOTA OF BROILER CHICKENS[1]

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[1]To be submitted to *Poultry Science*.

ABSTRACT

The objective of this study was to evaluate the diversity, composition, and predicted function of the intestinal microbiota of broiler chickens raised under three different conditions and methods to induce NE. Three experiments were conducted in completed randomized designs to compared non-challenged vs. challenged birds with eight replicates per treatment. The birds were fed a non-medicated diet based on corn and soybean meal and had *ad libitum* access to feed and water throughout the study. The chicks in experiment 1 and 2 were vaccinated against coccidiosis on d 1. Experiment 1: non-challenged and challenged birds were raised on floor pens with new litter and 58 birds/pen. The challenge consisted of *Eimeria maxima* inoculation on d 14, and *Clostridium perfringens* via water on d 18-19. Cecal microbiota was evaluated on d 18, 21, and 28. Experiment 2: non-challenged and challenged birds were raised on floor pens with recycled litter and 50 birds/pen. The challenge consisted of challenged with *C. perfringens* via feed from d 18-20. Ileal and cecal microbiota were evaluated on d 21. In experiment 3, non-challenged and challenged birds were raised in battery cages with 8 birds/cage. Challenged birds were inoculated with *E. maxima* on d 14, and *C. perfringens* on d 19-21. Ileal and cecal microbiota were evaluated on d 21. In the three experiments DNA was isolated from the ileal and/or cecal digesta, and the microbiota analyzed through 16S rRNA sequencing. Diversity (α and β), composition, and predicted function were assessed in all the experiments. The performance of the birds was impaired in the three studies, regardless of the method used to induce NE. In experiment 1, the microbiota did not significantly change across ages. In experiment 2, α-diversity indices were lower in challenged vs. non-challenged birds in both ileal and cecal microbiota. The cecal microbiota composition and function was more affected than the ileal microbiota. In experiment 3, Chao index (α-diversity) increased in

challenged vs. non-challenged birds. The composition of the ileal and cecal microbiota was not significantly affected, and only the predicted function of the cecal microbiota changed between non-challenged and challenged birds. In conclusion, growth performance of the birds was impaired, regardless of the challenge model used. The largest effect on the microbiota was observed in experiment 2, when birds were raised on floor pens with reused litter, vaccinated against coccidiosis, and challenged with *C. perfringens* on d 19-21. There seems to be a reduction in some species of short-chain fatty acids producing bacteria, such as *Blautia*, in the intestine of challenged chickens raised on floor pens, but not in battery cages.
Keywords: Broilers, microbiota, necrotic enteritis

INTRODUCTION

Necrotic enteritis (NE) is an enteric disease of poultry caused by *Clostridium perfringens* (Prescott et al., 2016). This bacterium is widely distributed, and on poultry farms is mainly isolated from soil, birds' excreta, litter, feed, etc (Lee et al., 2011). Necrotic enteritis leads to important economic losses, estimated to be over $6 billion annually, mainly due to the poor performance and veterinary costs (Wade and Keyburn, 2015). Traditionally, the control of NE has been done through sub-therapeutic use of antibiotics supplemented to the diets of broiler chickens. However, there is an increasing pressure to restrict the use of AGP and ionophores, mainly in the no antibiotic ever (NAE) production systems. Therefore, there is a need to understand the molecular events that happen in the birds after an infection to further develop nutritional or management strategies to control the disease.

There are a growing number of studies evaluating different methods to induce NE and their effects on the intestinal health of broiler chickens (Stanley et al., 2012; Stanley et al.,

2014; Antonissen et al., 2014). A recent publication by Wilson et al. (2018) showed that a predisposing factor such as coccidiosis was needed to develop lesions characteristic of NE, impairment in performance, and clinical signs. Coccidiosis is considered one of the most important predisposing factors to NE due to the lesions caused in the intestine by the *Eimeria* (Prescott et al., 2016). The stimulation of the innate immune-response by the coccidia, i.e., increased mucus production, will facilitate the proliferation of *C. perfringens* (Collier et al., 2008). Yet, other predisposing factors have been evaluated such as inclusion of dietary fish meal (Stanley et al., 2014), mycotoxins (Antonissen et al., 2014), and combination of *Salmonella* plus *Eimeria* (Latorre et al., 2018).

Additionally, studies have shown that the *Eimeria* and/or *C. perfringens* infection may change the structure of the intestinal microbiota (Stanley et al., 2012; Stanley et al., 2014; Zhou et al., 2017). However, it is still unclear if the dysbiosis generated is another predisposing factor for NE, or if it is a consequence of the proliferation of *C. perfringens* and consequent necrosis (Antonissen et al., 2016). It has been shown that *C. perfringens* infection affects mainly members of the order *Clostridiales* and *Lactobacillales* and reduced short chain fatty acid (SCFA)-producing bacteria (Stanley et al., 2012).

The dominance of beneficial microorganisms over pathogens is essential to maintain gut homeostasis and is directly related to the adequate absorption of nutrients by the intestine, leading to better performance and lower incidence of enteric diseases. The objective in studying the microbiota should not only reflect its composition, but the relationship between microorganisms and the host, and the functions that these microorganisms are exerting on the gut. The understanding is that healthy animals develop a balanced microbiota without overstimulating the immune system that is costly in terms of nutrients allocation, but necessary

to eliminate pathogens (Applegate et al., 2010). However, we do not know much beyond changes in microbial structure over time and within different intestinal regions. Therefore, we hypothesized that the microbiota of different sections of the intestine of broiler chickens would be differently affected by different challenge models to induce NE. The objective of this study was to evaluate the diversity, composition, and predicted function of the intestinal microbiota of broiler chickens raised under three different conditions and methods to induce NE in broiler chickens.

MATERIAL AND METHODS

The animal care and use procedures of the three experiments followed the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) under supervision of a licensed poultry veterinarian. The diets were formulated based on corn and soybean meal to meet or exceed the nutrient requirements according to NRC (1994). The feed was not supplemented with antibiotics or anticoccidials and consisted of a starter, grower and finisher, in experiments 1 and 2, and only a starter diet in experiment 3.

Experiment 1

Housing, birds and treatments

Nine hundred twenty-eight (928) one-day-old Ross 708 chicks were used in this study. Chicks were weighed individually and by pen for equal weight distribution and placed on 16 floor pens with new litter in a completely randomized design (12 birds/m^2, 58 birds/pen, 8 pens/treatment). The treatments were: non-challenged control group (Control) and a challenged group (Challenged control). Feed and water were available *ad libitum* throughout the trial. Birds

were provided a lighting program as per the primary breeder recommendations. The challenge model consisted of coccidial vaccine (Coccivac®-B52) at the normal recommended dosage on d 0, ~ 5,000 sporulated oocysts of *E. maxima*, kindly donated by Dr. Lorraine Fuller (Department of Poultry Science, University of Georgia) was orally gavaged to each bird on d 14 to the chickens in the challenged group. On days 19, and 20, the same birds were challenged with *C. perfringens*, as follows: feed was withdrawn for four hours and water was withdrawn for two hours prior to administration of *C. perfringens*. A measured amount of water (~200 ml of water containing ~1x10^8 cfu/mL of *C. perfringens*) that was consumed within 30 minutes was used for each pen. Once the challenge water was consumed, treatment feed and clean water were returned to pen.

Sample collection and analysis performed on days 18, 21, and 28

At d 18, 21, and 28 two birds per experimental unit (pen) were randomly selected and euthanized by cervical dislocation. The caeca were collected and placed into a Ziploc bag, immediately put in ice, and taken to the lab. Thus, the cecal content of the two birds was gently squeezed, pooled, and homogenized for microbiota analysis.

Experiment 2

Housing, birds and treatments

Eight-hundred (800) one-day-old Cobb 500 chicks were used in this trial. Upon arrival to the farm all birds were vaccinated (by spray cabinet) with a commercially approved coccidial vaccine (Coccivac®-B52) at the normal recommended dosage. Chicks were weighed individually and allocated to pens for similar weight distribution and placed on 16 floor pens

with recycled litter in a completely randomized design (12 birds/m^2, 50 birds/pen and 8 pens/treatment). The treatments were: non-challenged control group (Control) and a challenged group (Challenged control). Feed and water were available *ad libitum* throughout the experiment. Birds were provided a lighting program as per the primary breeder recommendations. Therefore, the challenge model consisted of a coccidial vaccine on d 0, and on d 18 to 20, birds were gavaged daily with *C. perfringens* via feed, as follow: feed and water were withdrawn for four hours prior to administration of *C. perfringens*. A measured amount of feed was sprayed with a solution containing *C. perfringens* culture (~$1x10^8$ cfu/mL), thoroughly mixed and given to birds in each challenged pen. Once the challenge feed was consumed, treatment feed and water were returned to pen.

Sample collection and analysis performed on day 21

On d 21, two birds per experimental unit were randomly selected, euthanized by cervical dislocation, and the entire GIT was collected into a Ziploc bag, immediately refrigerated in ice, and taken to the lab. The ileal and cecal contents from the two birds were separated (gently squeezed) and pooled for later analysis of the ileal and cecal microbiota.

Experiment 3

Housing, birds and treatments

Three hundred-eighty-four one-day-old male Cobb 500 were used in the experiment. Chicks were weighed individually and by pen for equal weight distribution and placed into 16 battery cages (8 birds/cage and 8 cages/treatment), in a completely randomized design. Chickens had a*d libitum* access to water and feed during the entire experimental period. The

treatments were: non-challenged control group (Control) and a challenged group (Challenged control). On d 14, all birds in the challenged treatment were orally gavaged with ~5,000 sporulated oocysts of *E. maxima*, kindly donated by Dr. Lorraine Fuller from the Department of Poultry Science, University of Georgia. A broth culture containing *C. perfringens* (~10^8 cfu/mL) was orally gavaged to challenged birds for 3 days from d 19 to 21.

Sample collection and analysis performed

On d 21, two birds per experimental unit were randomly selected, euthanized by cervical dislocation, and the entire GIT was collected into a Ziploc bag, immediately refrigerated in ice, and taken to the lab. The ileal and cecal contents from the two birds were collected (gently squeezed) and frozen at -20^{o}C for further analysis of the ileal and cecal microbiota.

Analysis of the ileal and cecal microbiota

The sample preparation was done in accordance with Bortoluzzi et al. (2018) with a slight modification when using ileal digesta. Cecal digesta (Experiment 1) and ileal and cecal digesta (Experiment 2 and 3) were diluted in a 1:10 sterile PBS solution; 5 mL of mixed (ileal) and 1mL of the mixed solution (cecal) were transferred to an adequate size tube, centrifuged for 3 m at 3,270 g, the supernatant discarded, and 200 µg of the content used for DNA isolation.

DNA isolation of the ileal and cecal digesta, and PCR amplification and sequencing followed a standardized protocol used routinely by our lab (Bortoluzzi et al., 2017; Bortoluzzi et al., 2018). The 16S rRNA was sequenced using the Illumina Miseq platform. The bioinformatic analysis was done according to Bortoluzzi et al. (2018). Briefly, sequences were paired-end and quality trimmed using Geneious (Newark, NJ). Operational taxonomic units

(OTUs) were assigned at a 97% identity using SILVA database. Alpha (Chao 1, Observed species, Phylogenetic diversity (PD) of the Whole Tree, and Shannon indexes) and beta diversity indexes were calculated using QIIME v1.9.1. The nonparametric statistical tests PERMANOVA was used to compare categories, using the weighted (quantitative) UniFrac metric measure. Principal Coordinates Analysis (PCoA) was used to visualize the data. PICRUSt was used to analyze the predictive function of the microbiota using the Kyoto Encyclopedia of Genes and Genomes (KEGG; Uji, Kyoto, Japan) pathways (Kanehisa and Soto, 2000).

Statistical Analysis

Performance data were analyzed as a one-way ANOVA using the GLM procedure of the SAS system (9.4, SAS Institute Inc., Cary, NC). All data were tested for normality and homogeneity of variances. The frequency of the main bacterial groups observed was submitted to a non-parametric one-way ANOVA (Kruskal-Wallis test) and, in case of significant difference ($p \leq 0.05$), means were separated by Dunn test. Welch's T-test was applied to compare KEGG pathways ($P \leq 0.05$) using STAMP (Halifax, Nova Scotia, Canada) version 2.1.3 (Parks et al., 2014).

RESULTS

Overall performance

In Experiment 1, it was observed that the challenge with *E. maxima* on d 14, and *C. perfringens* on d 18 and 19 did not affect growth performance of the birds from d 1 to 21 (Table 2.1). There was a trend ($P = 0.06$), however, towards decreased FI by the challenged birds.

Considering the whole experimental period (1 to 41 d of age), the challenge increased FI (P = 0.04) and impaired the FCR (P = 0.007) when compared to unchallenged birds by 5 and 5.2%, respectively.

In Experiment 2 (Table 2.1), from d 1 to 28, it was observed that the challenge with *C. perfringens* decreased BW gain (P = 0.02) and worsened FCR (P < 0.0001). Considering the entire experimental period (d 1 to 42), BW gain and FCR were negatively affected (P < 0.0001) by the challenge. Feed intake, however, was not affected by the challenge in this challenged model.

Similar to Experiment 2, BW gain and FCR were affected by the challenge in Experiment 3 by 37.3 and 30.1%, respectively (Table 2.1). In this study the birds were raised in battery cages and challenged with *E. maxima* on d 14 and *C. perfringens* on d 19-21. From d 1 – 21, BW gain decreased (P = 0.001) and the FCR was impaired (P = 0.003) due to the challenge. From d 1 to 28, a similar effect was observed, without effect (P > 0.05) on the FI.

Regarding the lesion score characteristic of NE, the challenge increased its severity in the three experiments (P < 0.0001; Table 2.1). In a score from 0 to 3, (0: normal, 1: slight mucus covering the small intestine, 2: necrotic small intestine mucosa, and 3: sloughed and blood in the small intestinal mucosa and contents; Hofacre et al., 1998), the Experiments 1, 2, and 3 showed a score of 0.5, 1.2, and 1.29, respectively.

Experiment 1

Alpha and beta-diversity – cecal microbiota

There was no effect of the challenge on the α-diversity indices (within sample variability; Table 2.2) of the cecal microbiota. PERMANOVA analysis showed that there was

no effect of the challenge on the β-diversity (between sample variability) on any of the ages evaluated (Figure 2.1).

Composition of the cecal microbiota

The relative abundance of the main phyla present in the cecal microbiota of the birds is presented in Table 2.3. *Firmicutes, Bacteroidetes*, and *Proteobacteria* were most abundant phyla, without effect of challenge and regardless of age. *Ruminococcus*, *Lactobacillus*, and *Bacteroides* were the most frequent genera found in the cecal microbiota of chickens, followed by the family *Ruminococcaceae* and order *Clostridiales*, regardless of the treatment and age (Table 2.4). On d 18, there was no difference between non-challenged and challenged on any the bacterial groups analyzed. On d 21, there was a trend (P = 0.09) toward decreased frequency of *Blautia*, and on 28 the genus *Faecalibacterium* tended (P = 0.06) to be in lower in challenged birds vs. non-challenged.

Predicted function

The predicted functions that were changed according to the treatment across ages are shown in Figure 2.2. On d 18, it was observed that the category "Streptomycin biosynthesis" was enriched in non-challenged birds vs. challenged birds. On d 21, however, 11 categories were enriched in challenged birds vs. non-challenged, mainly related to DNA, protein, and nucleotide metabolism. On d 28, there was only one function related to aminobenzoate degradation enriched in challenged vs. non-challenged birds.

Experiment 2

Alpha and beta-diversity – ileal and cecal microbiota

Different from Experiment 1, in Experiment 2 the challenge impacted the α-diversity indices (Table 2.5). The challenge with *C. perfringens* decreased the diversity indices of the ileal microbiota (Chao, P = 0.02; observed species, P = 0.007; PD whole tree, P = 0.03; and Shannon index, P = 0.06). In the cecal microbiota, a similar effect was observed wherein the challenged decreased Shannon index (P = 0.009) and tended to decrease Chao (P = 0.09), number of observed species (P = 0.10), and PD whole tree (P = 0.07).

Regarding the β-diversity (between sample variability), PERMANOVA analysis showed that the challenge changed the structure of the cecal microbiota (P = 0.03), but not the ileal microbiota, i.e., the cecal microbiota of challenged birds had a microbiota with higher variability vs. non-challenged birds. This effect is shown in PCoA plots (Figure 2.3).

Composition of the ileal and cecal microbiota

The main phyla present in the ileal and cecal digesta microbiota of the birds in Experiment 2 is shown in Table 2.6. It was observed that *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the most frequent phyla, regardless the treatment or intestinal section. The cecal microbiota presented a lower proportion of *Firmicutes* and higher abundance of *Bacteroidetes* when compared with the ileal microbiota (Table 2.6). The challenge decreased the frequency of *Firmicutes* (P = 0.005), and increased *Bacteroidetes* (P = 0.003) in the cecal microbiota.

The composition of the ileal and cecal microbiota in Experiment 2, notably changed due to the infection (Table 2.7). In the ileal microbiota, *Enterococcus* decreased (P = 0.05) due to the challenge. Yet, the challenge tended to increase *Lactobacillus* (P = 0.09), decrease *Candidatus* Arthromitus (P = 0.06), *Ruminococcus* (P = 0.07), *Streptococcus* (P = 0.07), and

Blautia (P = 0.06). In the cecal microbiota, the challenge increased *Bacteroides* (P = 0.01), decreased members of the family *Ruminococcaceae* (P = 0.01), and genus *Ruminococcus* (P = 0.03), and tended to decrease *Blautia* (P = 0.06).

Predicted function

The predicted functions of the ileal and cecal microbiota are shown in Figure 2.4. While the ileal microbiota had only 6 different categories, the cecal microbiota showed 50 different functions that changed due to the challenge. In the ileal microbiota, the only function that was enriched when the birds were challenged was "Ion channel". In the cecal microbiota most of the functions (38) were enriched when the birds were challenged with *C. perfringens*.

Experiment 3

Alpha and beta-diversity – ileal and cecal microbiota

The α-diversity indices are shown in Table 2.8. The Chao index increased (P = 0.05) and the number of observed species tended to increase (P = 0.10) in the ileal microbiota of challenged vs. non-challenged birds. The same pattern was observed in the cecal microbiota. The β-diversity of the ileal microbiota was not different between treatments; however, the PERMANOVA analysis showed that the structure of the cecal microbiota changed with treatment (P = 0.01; Figure 2.5).

Composition of the ileal and cecal microbiota

The composition of the ileal and cecal microbiota in Experiment 3 is shown in Table 2.9. *Firmicutes, Proteobacteria* and *Tenericutes* were the most abundant phyla in the ileal microbiota. On a lower taxonomic ranking, the ileal microbiota was dominated by *Lactobacillus*, *Streptococcus*, *Enterococcus*, and the family *Enterobacteriaceae*; with no differences between non-challenged and challenged birds. The cecal microbiota had a composition similar to the ileal microbiota; however, *Proteobacteria* and the family *Enterobacteriaceae* tended to increase (P = 0.08) in the cecal microbiota of challenged birds.

Predicted function

The predicted functions of the ileal microbiota were not affected by the challenge in Experiment 3 (Figure 2.6). In the cecal microbiota, however, there were 30 predicted functions that were enriched in the challenged birds vs. non-challenged birds.

DISCUSSION

Necrotic enteritis is a multi-factorial disease which requires pre-disposing factors to propitiate the overgrowth of *C. perfringens*, the production of its toxins, and the occurrence of the disease. Factors that increase the prevalence of this disease include mainly management, health and nutritional causes (Latorre et al., 2018). In the present study, we observed that regardless of the challenge model used, the performance of the birds was severely affected by the challenge. Regarding the intestinal microbiota, birds that were raised on floor pens, vaccinated against coccidiosis at the moment of arrival to the farm, and challenged with *C. perfringens* on d 18-20 suffered the largest impact on the structure of the ileal and cecal microbiota. It is important to note that birds in Experiment 2 were raised on reused litter which is a big contributor for the

establishment of the intestinal microbiota (Kers et al., 2018). The use of reused litter may have benefited the birds, as the mortality due to NE in Experiment 2 was lower than in Experiment 1 (8.5% vs 12.7%). On the other hand, the severity of the gross lesion characteristic of NE of birds in Experiment 2 was greater than the birds in Experiment 1. These findings suggest that birds in Experiment 1 developed a clinical NE with higher mortality while birds in Experiment 2 developed a milder NE, with lower mortality, but larger disturbance of the diversity and composition of the intestinal microbiota.

In a report by Latorre et al. (2018), *Salmonella* Thyphimurium was used to immunosuppress chicks on d 1, and then *E. maxima* on d 18, and *C. perfringens* was orally gavaged on d 23-24 to induce NE. These authors observed that the challenge decreased the abundance of butyric acid-forming bacteria and increased the frequency of *Proteobacteria* related bacteria which contains mainly opportunistic pathogens such as *Escherichia*, *Salmonella* and *Proteus* (Latorre et al., 2018). In terms of diversity, the challenge did not affect α-diversity indices, but changed the β-diversity, i.e., increased the variability between samples (Latorre et al., 2018). The results from Experiment 1 presented herein partially agree with Latorre et al. (2018) as both α- and β-diversity did not change due to the challenge.

On the other hand, using a different challenge method (*C. perfringens* daily from d 14 -20), Zhang et al. (2018) showed that the α-diversity indices increased after the challenge, as in Experiment 3 of the present study. Coincidently, the study conducted by Zhang et al. (2018) and our 3th study were performed in battery cages and showed similar results in terms of α-diversity indices. Pedroso et al. (2006) showed that the environment where the birds are raised (floor pens vs. battery cages) may alter the structure and composition of the intestinal microbiota. Indeed, we found in Experiment 2 (birds raised on floor pens with recycled litter)

that the α-diversity indices decreased after challenge in both ileal and cecal digesta. Lower diversity of the microbiota has been linked with poorer general health (Kers et al., 2018) which would help explain the negative effects of NE in broilers.

Not only the diversity but also microbial composition of the intestine suffers changes following *C. perfringens* infection (Stanley et al., 2012; Stanley et al., 2014; Antonissen et al., 2016; Latorre et el., 2018). It has been demonstrated that NE has a large influence mainly on the abundance of members belonging to the order *Clostridiales* and *Lactobacillales* (Stanley et al., 2012). More recently, Latorre et al. (2018) demonstrated that induction of NE decreased the frequency of the family *Clostridiaceae* and increased *Enterobacteriaceae* and *Lactobacillaceae.* In the studies presented herein, it was observed that the challenge reduced SCFA-producing bacteria (Experiment 1), reduced *Firmicutes*, mainly *Ruminococcaceae* members, and increased *Bacteroidetes* (Experiment 2), and increased *Enterobacteriaceae* (Experiment 3). Therefore, it is evident that different challenge models lead to changes in the microbial structure of the gut which may potentially increase the expenditure of nutrients and energy by the microbiota, reducing their availability to the bird.

The understanding and monitoring of the intestinal microbial ecosystem are paramount to develop strategies and interventions to modulate the microbiota and reduce the occurrence of enteric diseases. The understanding is that healthy animals develop a balanced microbiota without overstimulating the immune system that is costly in terms of nutrient allocation, but necessary to eliminate pathogens (Applegate et al., 2010). However, not much is known beyond changes in microbial structure within different intestinal regions, i.e., information is missing regarding microbial changes and their influence on the host. In order to obtain more information on the functions performed by the microorganisms in the intestine, the microbial functions were

predicted using PICRUSt comparing the microbiota of non-challenged vs. challenged birds in each study. Our results showed that the different models to reproduce NE affected functions mainly of cecal microorganisms in Experiments 2 and 3. Yet, in Experiment 1, most of the changes were observed on d 21, i.e., after the inoculation of *C. perfringens*, wherein functions related to DNA replication and repair were enriched in the microbiota of challenged birds. In Experiment 2, the cecal microbiota of challenged birds was enriched with functions related to metabolism of amino acids and amino acids related enzymes. Therefore, regardless of the challenge method, the predictive functions performed by the microbiota changed after the challenge, suggesting that the changes of these functions may also be related to the pathogenesis of NE.

CONCLUSION

Regardless of the challenge model, the induction to NE negatively impacted bird performance. Birds that were raised on floor pens with new litter, and challenged with *E. maxima* on d 14, and *C. perfringens* on d 18-19 had the highest mortality; however, these birds showed the lowest severity of lesions characteristic of NE. Birds that were raised on floor pens with reused litter, vaccinated on d 1, and challenged with *C. perfringens* on d 18-20 had the largest variations in terms of diversity and composition of the ileal and cecal microbiota. Yet, the α-diversity indices decreased due to the challenge. However, the changes in the microbiota of birds raised in battery cages and challenged with *E. maxima* on d 14 and *C. perfringens* on d 19-21 were the opposite in terms of α-diversity, with higher diversity after challenge. Therefore, the challenged model used in Experiment 2 would be the method of choice when looking to develop a milder NE with large effects on the intestinal microbiota.

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Table 2.1: Performance of broiler chickens raised under different conditions and challenge models to induce necrotic enteritis.

Experiment 1							
Treatment	1 to 21 d			1 to 41 d			
	BW gain, g	FI, g	FCR	BW gain, g	FI, g	FCR	Lesion score[1], 21 d
Control	667	896	1.347	2,331	3,576 [b]	1.535 [b]	0.00 [b]
ChaControl	633	854	1.381	2,291	3,764 [a]	1.620 [a]	0.50 [a]
SEM	14.8	18.2	0.03	20.9	74.7	0.03	0.12
P Value	0.31	0.06	0.30	0.41	0.04	0.007	<0.0001
Experiment 2							
	1 – 28 d			1 – 42 d			
	BW gain, g	FI, g	FCR	BW gain, g	FI, g	FCR	Lesion score, 21 d
Control	1,107 [a]	1,717	1.552 [b]	2,464 [a]	4,357	1.769 [c]	0.00 [b]
ChaControl	975 [b]	1,645	1.689 [a]	2,187 [c]	4,351	1.990 [a]	1.20 [a]
SEM	60.0	96.3	0.034	74.4	180	0.049	0.30
Probability	0.02	0.37	<0.001	<0.001	0.69	<0.001	<0.001
Experiment 3							
	1 – 21 d			1 – 28 d			
	BW gain, g	FI, g	FCR	BW gain, g	FI, g	FCR	Lesion score, 21 d
Control	493	726	1.468 [b]	855 [a]	1261	1.493 [b]	0.00 [b]
ChaControl	337	660	1.998 [a]	536 [b]	1113	2.137 [a]	1.29 [a]
SEM	36.1	39.3	0.112	77.9	94.0	0.132	0.24
Probability	0.001	0.34	0.003	0.003	0.26	<0.001	<0.001

[a–b] Means with different superscripts in a column differ significantly ($P < 0.05$). Values are means ± SEM of 8 replicates (58, 50, and 8 birds/replicate, respectively), except for lesion score (3 birds/replicate). BW gain: body weight gain; FI: feed intake; FCR: feed conversion ratio. ChaControl: challenged control group. [1]The scoring was based on a 0 to 3 score, with 0 being normal and 3 being the most severe.

Table 2.2: Alpha diversity indices of the cecal microbiota of broiler chickens at 18, 21, and 28 d of age of broiler chickens challenged (ChaControl) or not with *E. maxima* and *C. perfringens* and raised on floor pens: Experiment 1.

	Chao	OS	PD Whole tree	Shannon
18 days				
Control	323.2	258.9	18.5	5.19
ChaControl	322.0	261.5	18.6	5.32
	Probability			
Challenge	0.92	0.88	0.93	0.65
21 days				
Control	297.7	234.5	17.3	5.04
ChaControl	311.4	250.7	17.9	5.09
	Probability			
Challenge	0.48	0.48	0.66	0.90
28 days				
Control	355.4	283.3	21.3	5.26
ChaControl	323.9	253.5	19.4	4.88
	Probability			
Challenge	0.22	0.22	0.13	0.20

OS: Observed species; PD whole tree: Phylogenetic diversity of the whole tree. ChaControl: challenged control group. Values are means of 8 replicates (pool of 2 birds/replicate).

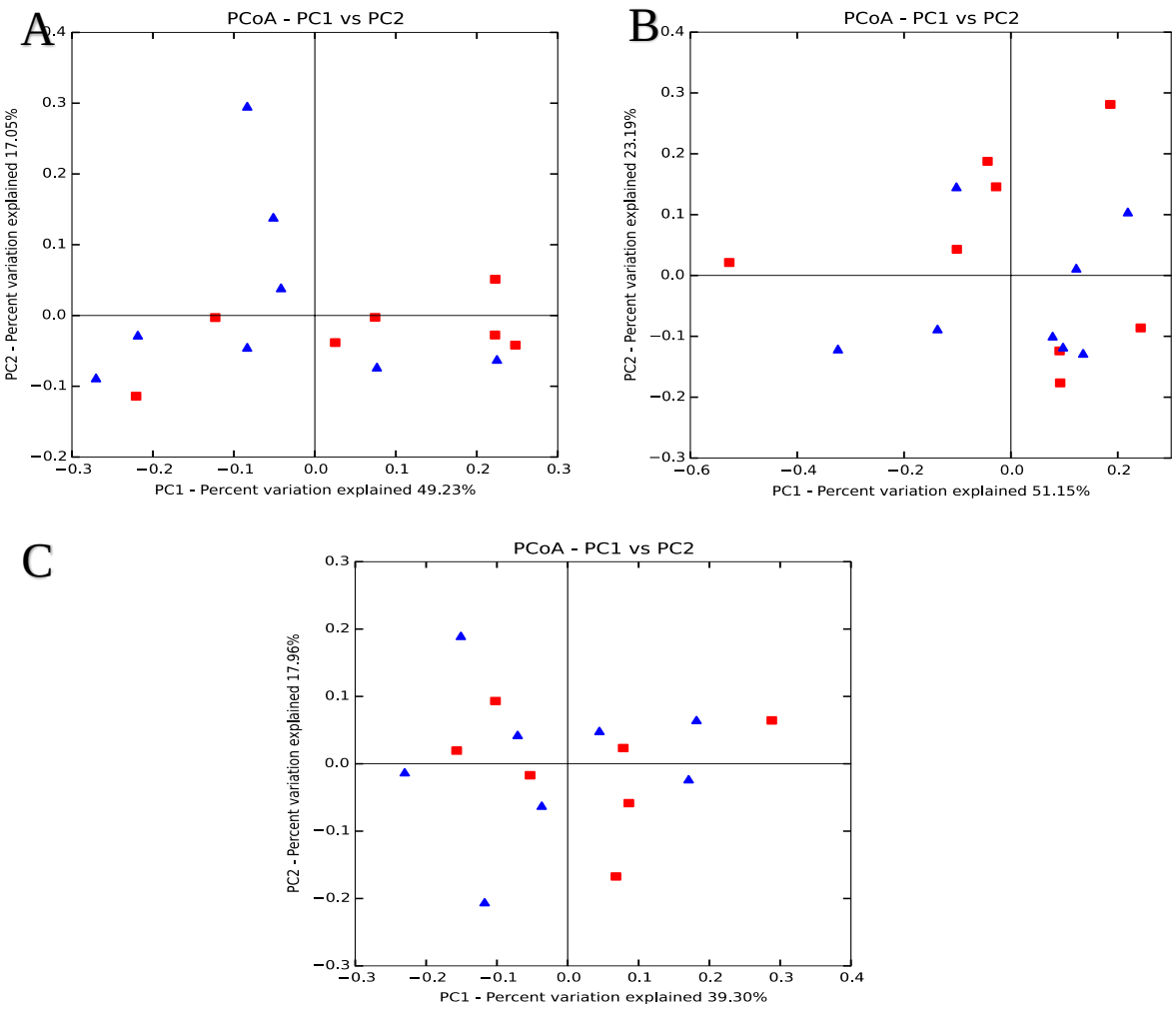


Figure 2.1: Weighted UniFrac PCoA of the cecal microbiota at 18 d (A), 21 d (B), and 28 d (C) of age of broiler chickens challenged or not with *E. maxima* and *C. perfringens* and raised on floor pens: Experiment 1. Red squares are non-challenge and blue triangles are challenged birds. Values are means of 8 replicates (pool of 2 birds/replicate).

Table 2.3: Relative abundance (%) of the main phyla present in the cecal microbiota of broiler chickens at 18, 21, and 28 d of age of broiler chickens challenged (ChaControl) or not with *E. maxima* and *C. perfringens* and raised on floor pens: Experiment 1.

		18 days	
Treatment	*Firmicutes*	*Bacteroidetes*	*Proteobacteria*
Control	78.8	18.8	1.50
ChaControl	86.7	10.9	1.70
SEM	2.80	2.70	0.30
Probability	0.13	0.11	0.56
		21 days	
Treatment	*Firmicutes*	*Bacteroidetes*	*Proteobacteria*
Control	71.7	25.5	0.89
ChaControl	73.8	23.9	1.06
SEM	4.50	4.20	0.10
Probability	0.83	0.67	0.50
		28 days	
Treatment	*Firmicutes*	*Bacteroidetes*	*Proteobacteria*
Control	65.7	31.2	1.41
ChaControl	60.6	34.9	1.39
SEM	3.40	3.20	0.30
Probability	0.42	0.42	0.82

Values are means of 8 replicates and a pool of 2 birds/replicate. ChaControl: challenged control group; SEM: standard error of mean. Values are means ± SEM of 8 replicates (pool of 2 birds/replicate).

Table 2.4: Relative abundance (%) of the main bacterial groups present in the cecal microbiota of broiler chickens at 18, 21, and 28 d of age of broiler chickens challenged (ChaControl) or not with *E. maxima* and *C. perfringens* and raised on floor pens: Experiment 1.

18 days								
Treatment	*Ruminococcus*	*Lactobacillus*	*Bacteroides*	*Ruminococcaceae*	*Clostridiales*	*Blautia*	*Faecalibacterium*	*Lachnospiraceae*
Control	27.7	18.5	12.1	7.6	4.7	4.3	5.1 b	2.0
ChaControl	27.2	23.9	4.2	9.1	6.4	2.9	3.4 b	2.0
SEM	3.00	3.70	2.50	1.10	1.10	0.05	1.10	0.04
Probability	0.73	0.42	0.30	0.64	0.75	0.17	0.48	0.82
21 days								
Treatment	*Ruminococcus*	*Lactobacillus*	*Bacteroides*	*Ruminococcaceae*	*Clostridiales*	*Blautia*	*Coprococcus*	*Lachnospiraceae*
Control	13.3	22.9	17.5	8.0	6.3	5.6	4.3	2.8
ChaControl	13.0	31.7	15.4	6.9	5.6	2.7	4.4	2.3
SEM	1.50	2.70	4.20	1.30	0.90	0.08	0.08	0.07
Probability	0.75	0.93	0.83	0.75	0.83	0.09	0.92	0.75
28 days								
Treatment	*Ruminococcus*	*Lactobacillus*	*Bacteroides*	*Ruminococcaceae*	*Clostridiales*	*Coprococcus*	*Faecalibacterium*	*Lachnospiraceae*
Control	14.3	18.6	23.0	6.3	9.1	1.8	5.5	6.5
ChaControl	14.4	25.2	26.1	4.3	6.2	0.9	2.5	4.0
SEM	1.80	2.50	2.60	0.08	1.00	0.03	1.00	0.08
Probability	0.19	0.91	0.36	0.30	0.11	0.13	0.06	0.17

Values are means of 8 replicates and a pool of 2 birds/replicate. ChaControl: challenged control group; SEM: standard error of mean. Values are means ± SEM of 8 replicates (pool of 2 birds/replicate).

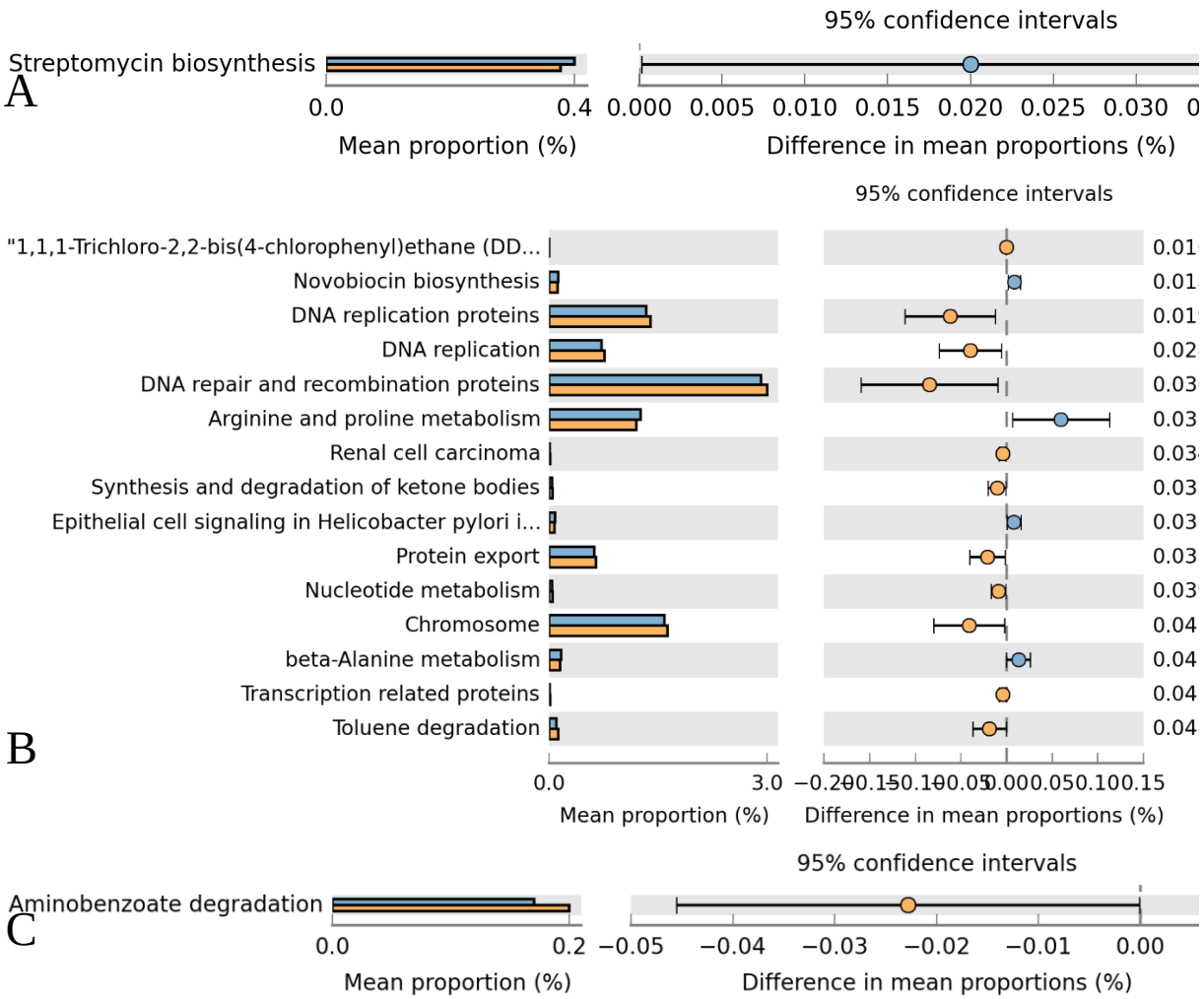


Figure 2.2: Predicted functions of the cecal microbiota at 18 (A), 21 (B), and 28 (C) d of age of broiler chickens non-challenged (blue bars) or challenged (orange bars) with *E. maxima* and *C. perfringens* and raised on floor pens: Experiment 1. Values are means of 8 replicates (pool of 2 birds/replicate).

Table 2.5: Alpha diversity indices of the ileal and cecal microbiota of broiler chickens challenged with *Clostridium perfringens* and raised on floor pens: Experiment 2.

	Ileum			
	Chao	OS	PD whole tree	Shannon
Control	198.5 [a]	156.6 [a]	9.60 [a]	2.92
ChaControl	150.5 [b]	118.4 [b]	6.40 [b]	2.45
	Probability			
Challenge	0.02	0.007	0.03	0.06
	Cecum			
	Chao	OS	PD whole tree	Shannon
Control	380.9	315.4	23.0	5.90 [a]
ChaControl	328.9	270.5	19.4	5.30 [b]
	Probability			
Challenge	0.09	0.10	0.07	0.009

OS: Observed species; PD whole tree: Phylogenetic diversity of the whole tree. ChaControl: challenged control group. Values are means of 8 replicates (pool of 2 birds/replicate).

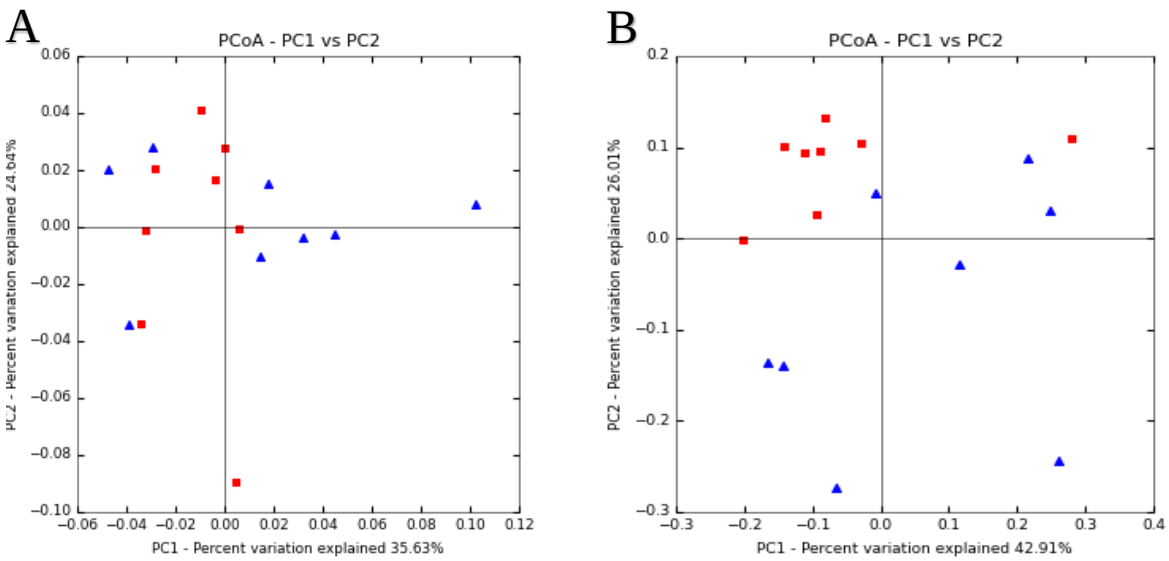


Figure 2.3. Weighted UniFrac PCoA of the ileal (A), and cecal microbiota (B) of broiler chickens challenged with *Clostridium perfringens* and raised on floor pens: Experiment 2. Red is non-challenged, and blue is challenged. Values are means of 8 replicates (pool of 2 birds/replicate).

Table 2.6: Relative abundance (%) of the main phyla present in the ileal and cecal microbiota of broiler chickens challenged (ChaControl) or not with *Clostridium perfringens* and raised on floor pens: Experiment 2.

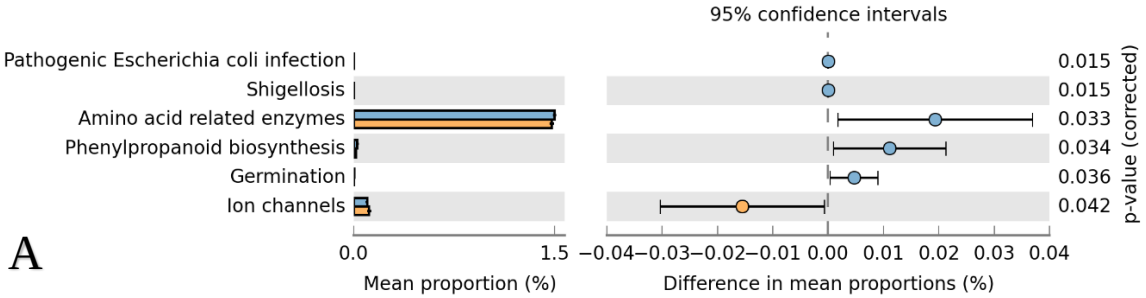
Ileal microbiota – 21 d			
Treatment	*Firmicutes*	*Bacteroidetes*	*Proteobacteria*
Control	99.7	0.06	0.13
ChaControl	99.5	0.03	0.06
SEM	0.18	0.02	0.03
Probability	0.24	0.83	0.12
Cecal microbiota – 21 d			
Treatment	*Firmicutes*	*Bacteroidetes*	*Proteobacteria*
Control	60.3 [a]	35.8 [b]	2.1
ChaControl	47.5 [b]	49.8 [a]	0.7
SEM	2.19	2.36	0.47
Probability	0.005	0.003	0.09

ChaControl: challenged control group; SEM: standard error of mean. Values are means of 8 replicates (pool of 2 birds/replicate).

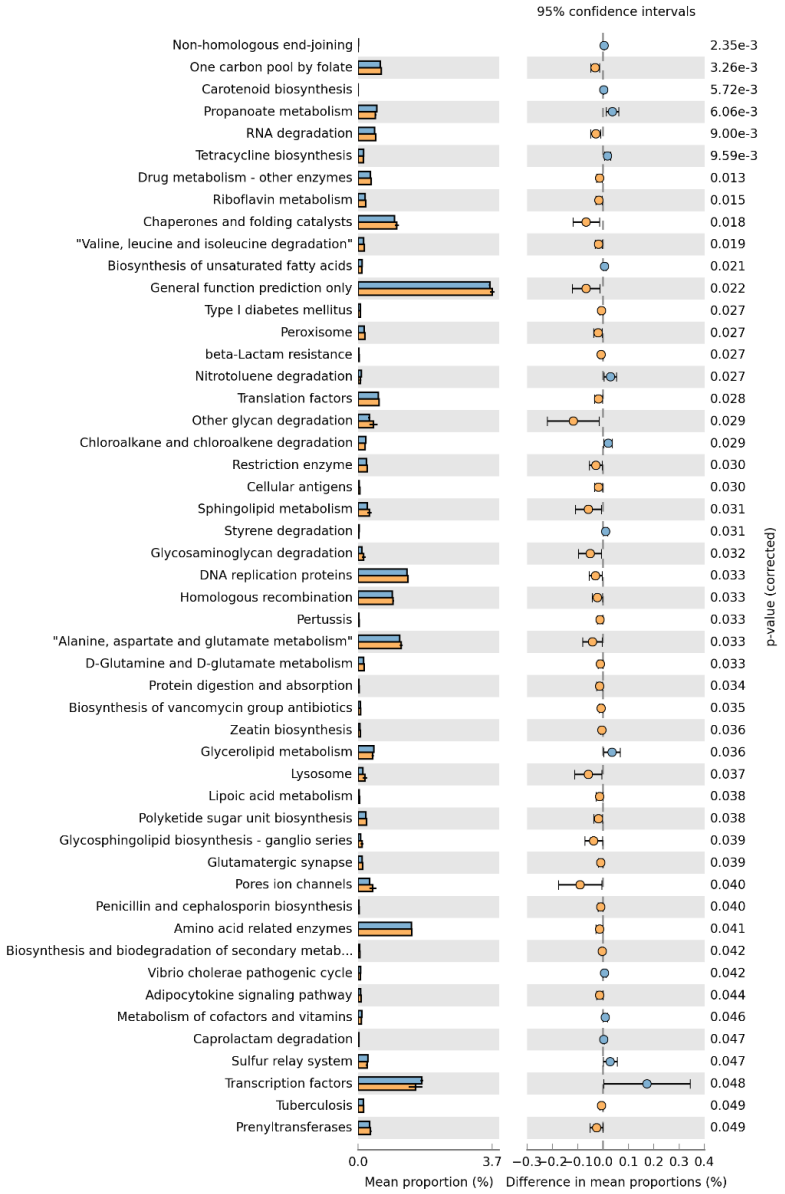
Table 2.7: Relative abundance (%) of the main bacterial groups present in the ileal and cecal microbiota of broiler chickens challenged (ChaControl) or not with *Clostridium perfringens* and raised on floor pens: Experiment 2.

Ileal microbiota – 21 d							
Treatment	*Lactobacillus*	*C. Arthromitus*	*Ruminococcus*	*Clostridiales*	*Enterococcus*	*Streptococcus*	*Blautia*
Control	93.07	3.71	0.51	0.09	0.33 [a]	0.33	0.14
ChaControl	96.94	1.23	0.15	0.06	0.13 [b]	0.04	0.03
SEM	1.16	0.85	0.12	0.02	0.07	0.11	0.05
Probability	0.09	0.06	0.07	0.53	0.05	0.07	0.06
Cecal microbiota – 21 d							
Treatment	*Bacteroides*	*Lactobacillus*	*Ruminococcaceae*	*Ruminococcus*	*Faecalibacterium*	*Lachnospiraceae*	*Blautia*
Control	21.46 [b]	11.29	7.88 [a]	14.30 [a]	3.67	1.65	2.15
ChaControl	35.03 [a]	14.69	3.46 [b]	7.72 [b]	3.64	0.83	0.99
SEM	2.45	2.38	0.80	0.32	0.82	0.25	0.29
Probability	0.01	0.83	0.01	0.03	0.53	0.12	0.06

Values are means of 8 replicates and a pool of 2 birds/replicate. ChaControl: challenged control group; SEM: standard error of mean.



A



B

Figure 2.4. Predicted functions of the ileal (A) and cecal (B) microbiota of chickens non-challenged (blue bar) or challenged (orange bar) with *Clostridium perfringens* and raised on floor pens: Experiment 2. Values are means of 8 replicates (pool of 2 birds/replicate).

Table 2.8. Alpha diversity indexes of the ileal and cecal microbiota on d 21 of broiler chickens challenged (ChaControl) or not with *E. maxima* and *Clostridium perfringens* and raised in battery cages: Experiment 3.

	Chao	OS	PD	Shannon
Ileal microbiota				
Control	152 [b]	122	4.36	2.59
ChaControl	179 [a]	141	4.93	2.88
	Probability			
Challenge	0.05	0.10	0.22	0.31
Cecal microbiota				
Control	149 [b]	114	6.86	3.41
ChaControl	172 [a]	133	6.38	3.51
	Probability			
Challenge	0.03	0.08	0.40	0.34

OS: Observed species; PD whole tree: Phylogenetic diversity of the whole tree. ChaControl: challenged control group. Values are means of 8 replicates (pool of 2 birds/replicate).

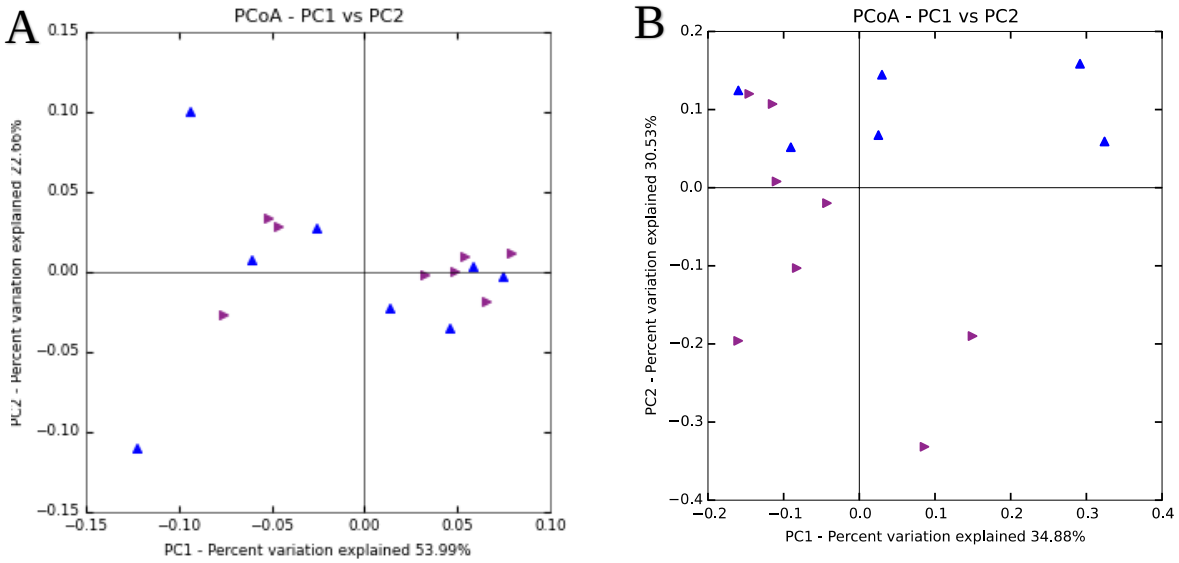
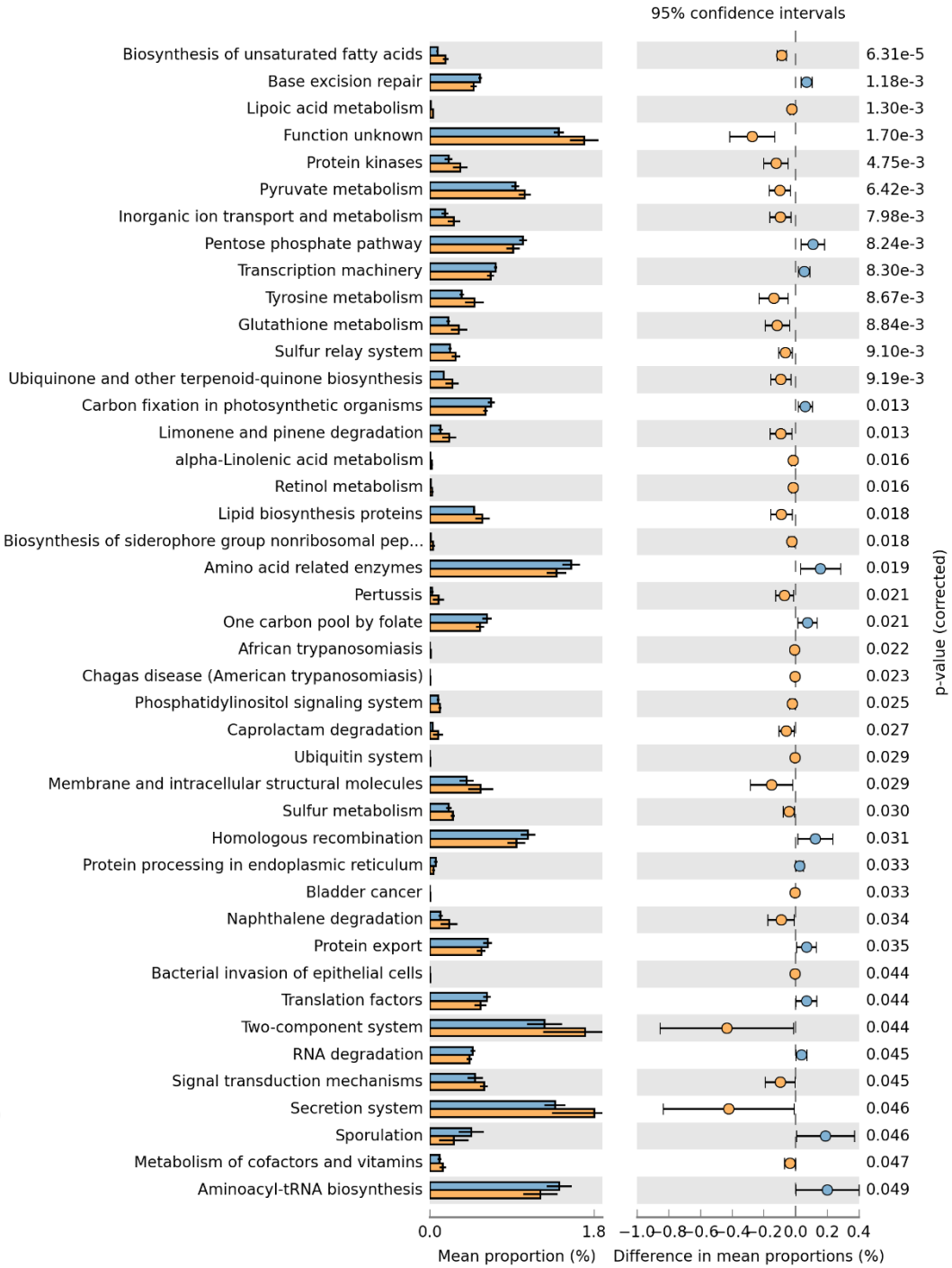


Figure 2.5: Weighted UniFrac PCoA of the ileal (A) and cecal (B) microbiota on d 21 of broiler chickens challenged with *E. maxima* and *C. perfringens* and raised in battery cages: Experiment 3. Blue triangles are not challenged, and purple arrows are challenged. Values are means of 8 replicates (pool of 2 birds/replicate).

Table 2.9. Relative abundance (%) of the main bacterial group present in the ileal and cecal microbiome of broiler chickens challenged (ChaControl) or not with *E. maxima* and *Clostridium perfringens* and raised in battery cages: Experiment 3.

	Ileal microbiota						
	Firmicutes	*Proteobacteria*	*Tenericutes*	*Lactobacillus*	*Streptococcus*	*Enterococcus*	*Enterobacteriaceae*
Control	99.0	0.56	0.04	94.2	3.19	0.57	0.37
ChaControl	99.1	0.60	0.04	96.0	1.01	0.72	0.45
SEM	0.22	0.20	0.02	1.50	1.35	0.26	0.16
Probability	0.67	0.29	0.82	0.83	0.46	0.34	0.12
	Cecal microbiota						
	Firmicutes	*Proteobacteria*	*Tenericutes*	*Lactobacillus*	*Clostridiales*	*Coprobacillus*	*Enterobacteriaceae*
Control	79.0	12.85	7.28	28.5	0.07	0.07	5.70
ChaControl	63.9	31.82	3.47	37.4	1.33	0.54	21.9
SEM	4.83	4.89	1.72	7.25	0.45	0.20	4.06
Probability	0.13	0.08	0.42	0.35	0.91	0.14	0.08

Values are means of 8 replicates (pool of 2 birds/replicate). ChaControl: challenged control group; SEM: standard error of mean.



B

Figure 2.6. Predicted functions of the cecal microbiota of broiler chickens non-challenged (blue bar) or challenged (orange bar) with *E. maxima* and *C. perfringens* and raised in battery cages. Experiment 3. Values are means of 8 replicates (pool of 2 birds/replicate).

CHAPTER 3

SODIUM BUTYRATE IMPROVED PERFORMANCE WHILE MODULATING THE CECAL MICROBIOTA AND REGULATING THE EXPRESSION OF INTESTINAL IMMUNE-RELATED GENES OF BROILER CHICKENS[1]

Bortoluzzi, C., A. A. Pedroso, J. J. Mallo., M. Puyalto, W. K. Kim, and T. J. Applegate. 2017. *Poultry Science*. 96: 3981-3993.

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ABSTRACT

This study evaluated the effect of sodium butyrate (SB) on performance, expression of immune-related genes in the cecal tonsils, and cecal microbiota of broiler chickens when dietary energy and amino acids concentrations were reduced. Day-old male Ross 708 broiler chicks were fed dietary treatments in a 3 x 2 factorial design (8 pens per treatment) with 3 dietary formulations (control diet; reduction of 2.3% of amino acids and 60 kcal/kg; and reduction of 4.6% of amino acids and 120 kcal/kg) with or without the inclusion of 0.1% of SB. Feed intake (FI), body weight gain (BW gain), and feed conversion ratio (FCR) were recorded until 28d of age. From 14 to 28 d, there was an interaction of nutrient density by SB ($P = 0.003$) wherein BW gain of birds fed SB was impaired less by the energy/amino acids reduction than unsupplemented birds. A similar result was obtained from 1 to 28 d ($P = 0.004$). No interaction ($P < 0.05$) between nutrient density by SB was observed for FCR. Nutritional density of the diets and SB modified the structure, composition and predicted function of the cecal microbiota. The nutritionally reduced diet altered the imputed function performed by the microbiota, and the SB supplementation reduced these variations, keeping the microbial function similar to that observed in chickens fed a control diet. The frequency of bacterial species presenting the butyryl-CoA:acetate CoA-transferase gene increased in the microbiota of chickens fed a nutritionally reduced diet without SB supplementation and was not changed by nutrient density of the diet when supplemented with SB (interaction; $P = 0.01$). SB modulated the expression of immune related genes in the cecal tonsils; wherein SB upregulated the expression of A20 in broilers fed control diets ($P < 0.05$) and increased IL-6 expression ($P < 0.05$). These results show that SB had positive effects on the productive performance of broilers fed nutritionally

reduced diets, partially by modulating the cecal microbiota, and exerting immune-modulatory effects.

Keywords: Broiler, immune system, intestinal microbiota, sodium butyrate.

INTRODUCTION

Intestinal bacteria produce short-chain fatty acids (SCFA), which are derived from cecal fermentation of compounds that cannot be digested by the animals, such as cellulose, fiber, starch and sugar (Guilloteau et al., 2010). Butyrate, a SCFA, can be used as an energy source by intestinal cells, can also positively influence intestinal cell proliferation, differentiation, maturation, and could positively alter the intestinal barrier, among other functions (Onrust et al., 2015).

While butyrate is naturally produced by fermentation in the cecum, the production of SCFA in the small intestine is limited (Levy et al., 2015). Synthetic sources of butyrate have been the focus of numerous studies in poultry (Leeson et al., 2005; Hu and Guo, 2007; Timbermont et al., 2010; Sunkara et al., 2011; Qaisrani et al., 2015). However, it has been described that uncoated butyrate could be absorbed before reaching the distal portions of the small intestine (van der Wielen et al., 2002). Dietary supplementation with a protected source of butyrate may delay the release of the substance along the gastrointestinal tract, thereby having plausible functional effects on the lower gastrointestinal tract (GIT). Besides the location of the GIT where butyrate is released, the dose of butyrate is also a factor that should be considered when aiming to decrease the variability of results across studies; for example, Hu and Guo (2007) used up to 2,000 mg/kg of butyrate, while Timbermont et al. (2010) used a maximum of 330 mg/kg. Barcelo et al. (2000) showed that 100 mM of butyrate may be toxic for colonic

goblet cells, and decrease the secretion of mucus, demonstrating that high concentrations of butyrate may also have deleterious effects.

Butyrate may regulate the production of inflammatory cytokines by modulating the intestinal immune cells, such as lymphocytes and macrophages (Guilloteau et al., 2010). Despite this knowledge, the mechanism by which butyrate exerts its anti-inflammatory effects remains to be determined (Chang et al., 2014). However, butyrate seems to have an anti-inflammatory effect mediated by signaling pathways (Meijer et al., 2010), such as the modulation of pro-inflammatory cytokines via impairment in NF-kB activation (Guilloteau et al., 2010). On the other hand, some studies have shown the effects of butyrate in controlling pathogens in poultry, such as *Salmonella*, *Clostridium perfringens*, and modulating the *Lactobacillus* population (Van Immerseel et al., 2005; Hu and Guo, 2007; Timbermont et al., 2010; Namkung et al., 2011). Furthermore, a question remains on what effect butyrate may elicit on the cecal microbiota, and what translational effects it elicits on the host.

To our knowledge, there is no study examining the effects of protected sources of dietary butyrate on the cecal microbiota of broiler chickens and its association with the intestinal immune system. Therefore, dietary sodium butyrate was hypothesized to improve the performance of broilers fed a nutritionally-reduced diet, by modulating the expression of immune-related genes and modifying the cecal microbiota of broiler chickens. The objective of this study was to evaluate the effect of a coated sodium butyrate-based additive on performance, expression of immune-related genes in the cecal tonsils, and the composition and predicted function of the cecal microbiota of broiler chickens when dietary energy and amino acids concentrations were reduced.

MATERIAL AND METHODS

Housing, birds, and treatments

The animal care and use protocol was reviewed and approved by the Purdue University Animal and Use Committee. One-day-old male Ross 708 broiler chicks (2,208) were used in the experiment. Chicks were weighed individually and allocated by pen such that mean pen BW were not different (48 pens with 46 birds/pen and 8 replicates/treatment), in a completely randomized design. The nutritional program consisted of two diets; a starter diet (0 to 14 d), and a grower diet (15 to 28 d) fed from 1 to 28 d of age. Birds had *ad libitum* access to water and feed in mash form during the entire experimental period. For each phase, a basal diet was formulated with corn and soybean meal to meet or exceed the nutrient specifications (with the exception of dietary treatment reductions in energy and amino acids) of chicks to meet or exceed NRC (1994; Table 3.1). The grower feed samples were analyzed for nutrient content (Table 3.1); however, the starter feed samples were not properly stored, and the nutrient content could not be determined. Amino acid profile (method 982.30; AOAC International, 2006) was determined at the University of Missouri Experiment Station Field Laboratory (Columbia, MO). Crude fiber, and crude protein (methods 962.09, and 990.03, respectively; AOAC International 2006) were determined by Dairy One (Ithaca, NY).

The dietary treatments were arranged in a 3 x 2 factorial design (6 treatments) with 3 dietary formulations (control diet, a diet formulated with a reduction of 2.3% of amino acids and 60 kcal, and a diet formulated with a reduction of 4.6% of amino acids and 120 kcal) with or without the inclusion of 0.1% of sodium butyrate (Norel S.A., Madrid, Spain). The sodium butyrate (SB) additive, that contained 70% sodium butyrate and 30% sodium salts of palm fatty

acid distillate, was supplemented to replace Sulkafloc (purified cellulose) that was used to dilute the energy and amino acid content of the diets.

Sample collection and analysis performed

Birds and feed were weighed weekly by pen and the mortality recorded daily. Average feed intake (FI) and body weight gain (BW gain) were corrected for mortality when calculating feed conversion ratio (FCR) for each pen. At 28 d of age, one bird per pen, totaling eight birds per treatment, was selected, euthanized by CO_2 exposure, and the cecal tonsils and cecum content were collected and frozen at −80 °C and −20 °C, respectively, for subsequent analysis. Based on the productive performance results, the gene expression and cecal microbiota analyses were performed only in four treatments (control diet and diet formulated with a reduction of 2.3% of amino acids and 60 kcal with or without the supplementation of SB; i.e., a 2 x 2 factorial design).

DNA extraction of the cecal microbiota

The DNA isolation was conducted following the manufacturer recommendations (PowerViral Environmental RNA/DNA Isolation Kit – Mo Bio; QIAGEN, Carlsbad, CA, USA). Briefly, bacterial cells were lysed using beads, phenol:chloroform:isoamyl alcohol and solution 1 of the Mo Bio DNA extraction kit by vortexing at maximum speed for 10 min in the Mo Bio Vortex Adapter (QIAGEN, Carlsbad, CA). After this step, the upper aqueous layer was transferred to a clean collection tube, the solution 2 was added, incubated at 4^{o} C for 5 min, centrifuged, and the supernatant transferred to a new tube. The solutions 3 and 4 were

added and the lysate was filtered. The filter was then washed and the DNA recovered. The presence of DNA was verified by agarose gel electrophoresis (1.5%).

PCR amplification and sequencing

The V3-V4 hypervariable region of the 16S rRNA gene was amplified using the primer FwOvAd_341f and ReOvAd_785r as previously described (Klindworth et al., 2013). Each PCR reaction contained DNA template (10 ng), 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM), 12.5 µl 2X Kapa HiFi Hotstart ready mix (Anachem, Dublin, Ireland), and water to a final volume of 25µl. The DNA was subjected to initial denaturation at 95°C for 3 min. Amplification was then achieved by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. Final extension was at 72°C for 5 min. PCR products were cleaned using AMPure XP magnetic beads (Labplan, Dublin, Ireland) and submitted to another PCR to incorporate indexes (Illumina Nextera XT indexing primers, Illumina, Sweden) to the samples. Each PCR reaction contained 5µl of each index primer, 25µl 2x Kapa HiFi Hot Start Ready mix (Anachem, Dublin, Ireland), and 10µl water. PCRs were completed as described above, but only 8 amplification cycles were completed instead of 25. PCR products were cleaned, and pooled and paired ends were sequenced at a read length of 300 nucleotides on a MiSeq platform (Illumina, Inc., San Diego, CA).

Bioinformatics

All sequence processing was performed using MOTHUR software (Ann Arbor, MI) version 1.37.1 (Schloss et al., 2009). Sequences were paired-end and quality trimmed. Sequences containing more than 8 homopolymers nucleotides, and mismatched or ambiguous

bases were removed. High-quality sequences were aligned against the SILVA database (Ribocon GmbH, Bremen, Germany) release 119 (Pruesse et al., 2007). UCHIME software (Tiburon, CA) was used to identify and remove chimeric sequences (Edgar et al., 2011). Number of sequences per sample was normalized based on the sample with the lowest number of reads for statistical comparison (Gihring et al., 2012). Operational taxonomic units (OTUs) were assigned at a 97% identity using the average neighbor algorithm, and taxonomic assignments were made using the Ribosomal Database Project taxonomy (RDP; East Lansing, MI) as described by Cole et al. (2009). Diversity indexes were calculated using MOTHUR. Representative sequences of each OTU were classified using BLASTN (Bethesda, MD) as described (Altschul et al., 1990). Predictive functions of the cecal communities were performed using Picrust software (Boston, MA) online galaxy version (Langille et al., 2013; Afgan et al., 2016). A reference OTU table was generated using Greengenes (Berkeley, CA) core set database (DeSantis et al., 2006). A closed-reference OTU table was normalized by the 16S rDNA copy number, the metagenome was predicted, and they were categorized by function based on Kyoto Encyclopedia of Genes and Genomes (KEGG; Uji, Kyoto, Japan) pathway (Kanehisa and Goto, 2000). The obtained biome file was processed by STAMP (Halifax, Nova Scotia, Canada) version 2.1.3 (Parks et al., 2014).

Determination of butyryl-CoA:acetate CoA-transferase gene by quantitative PCR

Bacterial genomic DNA isolated from the cecal microbiota of the broilers was amplified with 16S rDNA universal and butyryl-CoA:acetate CoA-transferase primers (Table 3.2). Standard template DNA was prepared from *Roseburia* sp. strain A2-183 as described previously (Louis et al., 2004) and standard curves were prepared with five standard

concentrations of 10^7 to 10^3 gene copies/µl. A pooled sample was made from 8 replicates from the same treatment and qPCR was performed in triplicate in a 20 µl total reaction using 10 µl SYBR Green PCR Master Mix (Bio-Rad, Foster City, CA), 10nM final primer concentration, and 8µl of DNA (5 nM/µL). The reaction program consisted of 1 cycle at 95°C for 3 min followed by 40 cycles of 30 s at 95°C, 30 s at 60°C for the 16s rDNA gene, and 53°C for butyryl-CoA:acetate CoA-transferase gene, and 30 s at 72°C. Data are expressed as the relative frequency of butyryl-CoA:acetate CoA-transferase genes detected per 16S rRNA gene (Louis and Flint, 2007).

Determination of the gene expression in cecal tonsils

The preparation of the samples for the qPCR analyses was performed as described by Horn et al. (2014). Briefly, total RNA was isolated from 50 mg of the cecal tonsils tissue using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The precipitated RNA was suspended in 20 µL of RNase free water and stored at -80°C. RNA quantity was assessed by UV spectrophotometer and then treated with DNAse (Invitrogen, China). The first-strand cDNA was synthesized from 5 µL of total RNA using oligodT primers and Superscript II reverse transcriptase, according to the manufacturer's instructions (Invitrogen, Shanghai, China). Synthesized cDNA was diluted (5X) with sterile water and stored at -20°C. The real-time PCR amplification was performed in a 25 µL reaction mixture containing 5 µL of diluted cDNA, 12.5 µL of 2x SYBR Green PCR Master Mix (Bio-Rad, Foster City, CA), 2.5 µL of each primer (Table 2), and 3 µL of PCR-grade water. The PCR procedure for A20 (ubiquitin-editing enzyme A20), interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 10 (IL-10), γ interferon (γ INF) and nuclear factor kappa B (NF-kB) consisted of

heating the reaction mixture to 95ºC for 10 min followed by 40 cycles of 95ºC for 15 s and 57ºC, 57ºC, 50ºC, 55ºC, 53ºC, 54ºC for 20 s for each primer, respectively, and 72ºC for 15 s. The relative standard curve method was used to quantify the mRNA concentrations of each gene in relation to the reference gene (GAPDH). The mRNA relative abundance was calculated (Livak and Schmittgen, 2001). All samples were analyzed in duplicate.

Statistical analysis

The growth performance and gene expression data were analyzed as a 2-way ANOVA using the GLM procedure of the SAS system (SAS Institute, 2011). The model included the main effect of diet, SB, and their interaction. The pen was considered as the experimental unit. The means showing significant ($P \leq 0.05$) treatment differences in the ANOVA were then compared using Tukey's test. All data were tested for normality and homogeneity of variances, using the UNIVARIATE procedure and Bartlett test of SAS system (SAS Institute, 2011), respectively.

Procedure GLM was utilized to analyze differences in microbial diversity indexes and abundance genera. Metastats was used to study if there were OTUs differentially represented between the samples ($P < 0.05$). Unweighted and weighted Unifrac software (Boulder, CO) were adopted to determine differences in presence and absence of OTUs, and abundance, respectively (Lozupone et al., 2011). Welch's T-test was applied to compare the KEGG pathways. Significant OTUs were determined using nonparametric Wilcoxon sum-rank test ($P < 0.05$) followed by linear discriminant analysis ($\log < 2$) that was used to compare differences in the microbiota.

RESULTS AND DISCUSSION

Productive performance

There was no interaction between nutrient density of the diet and SB supplementation in the starter phase (1 to 14 d). However, broilers fed nutritionally-reduced diets had lower BW gain ($P < 0.001$), and SB supplementation increased BW gain by 2.8% ($P < 0.001$; Table 3.3). In the following phase (14 to 28 d), there was an interaction between nutrient density and SB supplementation for BW gain ($P = 0.003$) and FI ($P = 0.01$); wherein BW gain of birds fed SB was impaired less by the energy and amino acid reduction than unsupplemented birds. A similar interaction was observed for the overall experimental period (1 to 28 d), in which the supplementation of 0.1% of SB partially recovered the reduction in BW gain caused by the dietary energy and amino acid reduction ($P = 0.004$). Supplementation of SB did not affect FCR in any of the phases evaluated. Based on these results, the molecular analysis was focused on the control treatment and the treatment fed a diet formulated with a reduction of 2.3% of amino acid and 60 kcal, with or without SB supplementation, totaling four treatments (2 x 2 factorial).

It has been described that butyrate can affect several host functions. For example, butyrate is a common SCFA metabolite of clostridial metabolism (Hold et al., 2003; Duncan et al., 2004; Louis et al., 2004) which can be used as an energy source for epithelial cells (Dalmasso et al., 2008). Further, butyrate has been shown to increase the expression and activity of SGLT1 and GLUT2 transporters in the brush border (Tappenden et al., 1997). Enterocyte surface receptors, such as G protein-coupled receptor (GPR) 43 and GPR41 may function as a sensor of intestinal SCFAs enhancing expression of transporters in cells as they migrate along the villus (Karaki et al., 2008) thereby enhancing feed efficiency (Adil et al., 2010). In addition to the recognized effects on intestinal metabolism, butyrate shows indirect

effects that contribute to the general metabolism of animals (Guilloteau et al., 2010), including microbiota composition and function, which was also the focus of the present study.

Diversity of the cecal microbiota

A total of 253,216 good quality sequences were obtained, 7,913 from each one of the 32 cecal microbiotas analyzed. The calculated sampling coverage of these samples was between 98 and 99%.

The number of observed sequences (OTU), Chao index (number of OTU comprising the microbiota), Shannon index (biodiversity based on sequences uniformity amongst OTU), and Simpson index (richness and evenness) were unaffected by SB and/or the nutritionally-reduced diet ($P > 0.05$; mean OUT, Chao index, Shannon index, and Simpson index were 261, 412, 3.53, and 0.09, respectively). In order to compare the cecal microbiotas of chickens, distance matrices were calculated by weighted and unweighted UniFrac and visualized via principle coordinate analysis (PCA). Unweighted and weighted PCA plots are presented in Figures 3.1A and 3.1B, respectively. UniFrac distances of cecal microbiotas indicated both membership (unweighted), and community structure (weighted) variations. Both analyses revealed differences associated with the presence of SB supplementation and the nutritional level of diets ($P < 0.001$).

Although similar microbial richness was observed among treatments, the community structure was significantly different. Both community membership and structure contributed to differences in cecal microbial communities among diets. Analyzing the presence/absence of OTU (unweighted UniFrac) it is possible to magnify the effects of OTU present in low abundance. When OTU abundance is taken into account (weighted UniFrac), the clusterization

indicates that the most abundant members of a cecal community from chickens were unaffected by dietary treatment.

Composition of the cecal microbiota

Microbial compositions showed high inter-individual variability. Overall, the microbiotas were dominated by the phylum *Firmicutes* (78.1 ± 14.7% in the control group, 70.6 ± 27.2% in the control + SB group, 87.6 ± 7.27 % in the reduced group and 77.5 ± 20.8% in the reduced + SB group; mean ± SD). *Bacteroidetes* was the second most abundant (18.9 ±15.1% in the control group, 26.7 ± 28.0% in the control + SB group, 9.0 ± 7.21% in reduced group and 18.5 ± 22.5% in the reduced + SB group), followed by the *Proteobacteria* phyla (0.08 ± 0.08% in the control group, 0.14 ± 0.18% in the control + SB group, 0.23 ± 0.34% in reduced group and 1.09 ± 2.5% in the reduced + BS group).

At the genus level, the phylum *Firmicutes*, the largest phylum, consisted of *Clostridiales*, *Ruminococcaceae*, *Faecalibacterium*, *Clostridium* VI, *Butyrococcus*, *Lachnospiraceae*, *Clostridium* XIVb, and *Blautia; Bacteroidetes* mainly consisted of *Barnesiella*, *Alistipe*s and *Bacteroides*; and *Proteobacteria* consist mainly of *Enterobacteriaceae* (Figure 3.2). The combination of the nutritional density of the diet and the supplementation of SB affected the distribution of *Ruminococcaceae* in the cecal microbiota (interaction of nutrient density by SB; P = 0.04). Chickens fed a nutritionally reduced diet presented a higher percentage of sequences related to *Ruminococcaceae* (11.9%) in the cecal microbiota than chicken fed a control diet supplemented or not with SB (6.5% and 6.4%, respectively). The amount of *Ruminococcaceae* observed in the cecal microbiota of birds fed a nutritionally-reduced diet supplemented with SB did not differ statistically from the other

groups (9.1%). When only considering nutritional density of the diet, chickens fed a nutritionally-reduced diet tended to present a higher percentage of sequences in the cecal microbiota related to *Butyrococcus* ($P < 0.06$) and *Ruminococcaceae* ($P < 0.07$). On the other hand, chickens fed a control diet had a cecal microbiota enriched for sequences related to *Firmicutes* ($P < 0.05$) and *Clostridiales* ($P < 0.01$).

The phylum *Firmicutes* has been related to the ability to harvest energy from the diet, presenting higher proportion in animals with better feed efficiency (Ley et al., 2005). In the present work, lower nutritional density of the diet reduced the total number of microorganisms related to the phylum *Firmicutes*, which help explain the impairment in BW gain in birds fed this diet; in addition, members of this phylum had their representation modified according to the different dietary treatments. We detected an over-representation of several genera, such groups related to *Ruminococcaceae* and *Butyrococcus* in the microbiota of chickens fed a nutritionally-reduced diet. These groups of bacteria are well known for degradation of complex plant materials, as cellulose and hemicelluloses, being able to secrete xylanase, cellulase and beta-galactosidase and are among the most abundant groups in the cecal content (Ze et al., 2012; Biddle et al., 2013; Wei et al., 2013). A large amount of bacteria specialized in fiber degradation was expected in chickens fed nutritionally-reduced diets due to the high amount of cellulose used to reduce the nutrient density of the diet. The cecal microbiota were likely modified in order to degrade resistant fiber and harvest energy from the lower quality diet.

Due to the similarity in the BW of chickens fed a control diet and chickens fed nutritionally-reduced diet supplemented with SB, a Venn diagram was constructed to identify the shared phylotypes between these groups, after eliminating the phylotypes shared with the group just fed nutritionally-reduced diet (Figure 3.3). Sixty-eight OTU were identified to be

unique, representing 2,380 sequences, 8.8 and 9.0% of the total species observed in the microbiota of birds fed a control or a nutritionally-reduced diet supplemented with SB, respectively. Within these sixty-eight OTU, it was observed the presence of sequences related to *Bacteria* (9 OTU), *Firmicutes* (12 OTU), *Clostridia* (5 OTU), *Clostridiales* (11 OTU), *Lachnospiraceae* (4 OTU), *Ruminococcaceae* (13 OTU), *Erysipelotrichaceae* (1 OTU), *Turibacter* (1 OTU), *Oscilibacter* (2 OTU), *Clostridium* XIVb (1 OTU), *Clostridia* IV (3 OTU), *Flavonifractor* (1 OTU), *Ruminococcus* (1 OTU), and *Bacteroides* (4 OTU) were observed.

Next, the composition of the cecal microbiota was examined using Metastats to identify specific phylotypes associated with the supplementation of SB and the nutritional density of the diet. Just two phylotypes had different abundance in the cecal microbiota of chickens receiving a control diet with or without SB supplementation. The cecal microbiota of chickens receiving a control diet had significantly fewer species related to *Gracilibacter thermotolerans* (OTU92, 88% of similarity to RDP, $P < 0.01$) and more phylotypes related to *Clostridium sufflavum* (OTU124, 87%) than the group receiving a control diet supplemented with SB ($P < 0.004$).

A greater difference in the number of phylotypes was observed comparing the cecal microbiota of birds fed a control diet with that of the birds fed nutritionally-reduced diet. Chickens fed a nutritionally-reduced diet showed a lower percentage of sequences related to *Vallitalea pronyensis* (OTU19, 87%, $P < 0.04$), *G. thermotolerans* (OTU166, 88%, $P < 0.02$), *Oscilibacter valericigenes* (OTU152, 91%, $P < 0.007$), *P. capillosus* (OTU22, 97%, $P < 0.02$) and *Bacteroides thetaiotaomicron* (OTU70, 99%, $P < 0.0009$) and more sequences related to *Clostridium leptum* (OTU31, 92%, $P < 0.03$), *Ruminococcus bromii* (OTU68, 95%, $P < 0.03$), *F. prausnitzii* (OTU105, 93%, $P < 0.02$), *G. thermotolerans* (OTU16, 88%, $P < 0.02$), *Dielma*

fastidiosa (OTU117, 91%, P < 0.01), *Saccharofermentaris acetigenes* (OTU143, 91%, P < 0.01), *Clostridium methylpentosum* (OTU113, 92%, P < 0.04) and *Ruminococcus faecis* (OTU5, 96%, P < 0.007) than the group fed a control diet.

In addition, comparing the cecal microbiota of birds fed a nutritionally-reduced diet with that of birds fed the same diet supplemented with SB, we observed that the frequency of some phylotypes were changed. Chickens fed a nutritionally-reduced diet presented a lower percentage of sequences related to *Faecalibacterium prausnitzii* (OTU69, 92%, P < 0.04), *Clostridium spiroforme* (OTU95, 96%, P < 0.03), *Pseudoflavonifractor capillosus* (OTU104, 89%, P < 0.03), and *Clostridium leptum* (OTU79, 92%, P < 0.02); and more sequences related to *Saccharofermentaris acetigenes* (OTU91, 88%, P < 0.03), *G. thermotolerans* (OTU133, 87%, P < 0.02) and *Odoribacter splanchnicus* (OTU185, 99%, P < 0.0009) than chickens fed the same diet supplemented with SB.

Faecalibacterium prausnitzii is a butyrate-producing bacterium, and species belonging to *Lachnospiraceae* have been related to good feed efficiency (Kameyama and Itoh, 2014), which may contribute to the improved performance observed in birds fed a nutritionally reduced diet supplemented with SB. *F. prausnitzii* is a component of the normal chicken microbiota (Lu et al., 2003; Lu et al., 2008) and a decreased abundance of this microorganism has been associated with inflammatory disease (Sokol et al., 2008; Fujimoto et al., 2013). Similarly, *Subdoligranulum variabile*, a phylogenic closely related to *F. prausnitzii*, has demonstrated the ability to degrade complex carbohydrates and is closely related to several host metabolic pathways (Duncan et al., 2002; Li et al., 2008).

Odoribacter splanchnicus, observed in higher percentage in the microbiota of chickens fed a nutritionally-reduced diet (vs. the same diet supplemented with SB), can ferment

carbohydrates and produce short chain fatty acids. Acetic acid, succinic acid, and butyric acid are important for both microbial and host epithelial cell growth and has been associated with improved performance in chickens (Goker et al., 2011; Asano et al., 2013; Meehan and Beiko, 2014; Li et al., 2016b). Non-pathogenic *Clostridia* species, such as *C. leptum*, that can degrade complex carbohydrates, were observed in higher percentage in the microbiota of chickens fed a nutritionally-reduced diet supplemented with SB (vs. the unsupplemented diet). *C. leptum* has a great impact on the host metabolism and it is sensitive to dietary manipulation (Klein et al., 2016). It is also able to degrade cellulose, produce butyrate and plays an important role in the energy metabolism and development of intestinal epithelial cells (Pryde et al., 2002; Eckburg et al., 2005). *Bacteroides thetaiotaomicron* is known for its contribution to the symbiosis of the microbiota (Xu et al., 2003) and *Blautia* can use carbohydrate as a fermentable substrate and produce acetate and lactate as the major end products of glucose fermentation (Park et al., 2012; Bai et al., 2016).

Finally, the cecal microbiota of birds fed control diet and that of birds fed nutritionally-reduced diet supplemented with SB was compared. Chickens fed a diet containing a nutritionally reduced level of nutrients supplemented with SB presented more sequences related to *Clostridium lactifermantans* (OTU120, 95%, $P < 0.03$), *Subdoligranulum variabile* (OTU56, 99%, $P < 0.03$), *R. bromii* (OTU68, 95%, $P < 0.01$), *O. splanchnicus* (OTU185, 99%, $P < 0.0009$), *Sporobacter termidis* (OTU177, 89%, $P < 0.0009$) and less species related to *Blautia hansenii* (OTU167, 96%, $P < 0.04$), *O. valericigenes* (OTU152, 91%, $P < 0.04$), *G. thermotolerans* (OTU166, 88%, $P < 0.02$), and *Clostridium succinogenes* (OTU128, 87%, $P < 0.02$).

Predicted function of the cecal microbiota

A hypothesis has been proposed that the host and its microbiota have evolved together and that the host genome does not encode for all of the information needed to carry all of the functions (Zaneveld et al., 2008). In the absence of transcriptome data, and since the samples were not stored at -80^{o}C for RNA analysis, PICRUSt was applied to predict the metagenome from 16S data and a reference genome database (Langille et al., 2013). PICRUSt can predict and compare probable functions of a wide range of samples. Few limitations of this approach must be considered: the software does not differentiate among strain level; it cannot analyze gene families if those gene are not included in the imputed database, or if the pathways are not well characterized (Langille et al., 2013); and it also assumes 100% of gene function (if the bacterium is present).

Initially, the predicted function of the cecal microbiota of chickens fed a control diet was compared to that of the group fed a nutritionally reduced diet (Figure 3.4a). The nutritionally reduced diet was predicted to affect ($P < 0.05$) a greater number of pathways in the microbiota, especially related to carbohydrate (pyruvate metabolism) and lipid metabolism (fatty acid biosynthesis, biosynthesis of unsaturated fatty acids, and glycerophospholipid metabolism). However, when the predicted function of the microbiota of chickens fed a control diet and the predicted function of the microbiota of chickens fed a nutritionally reduced diet supplemented with SB were compared, fewer differences in the KEGG pathway were observed (Figure 3.4b). There were 15 different predicted metabolic pathways observed between the first two groups of birds, which showed that the microbiota from birds fed a nutritionally reduced diet was utilizing the cecal content in a dissimilar way since the digesta profile reaching the ceca was distinct between both groups. On the other hand, comparing the predicted function of

the cecal microbiota from broilers fed control diets and the microbiota of chickens fed a nutritionally reduced diet supplemented with SB, only four metabolic pathways were statistically different.

Pyruvate metabolism is well known in the energy process (Turnbaugh et al., 2008) and is described in KEGG database as pathways containing of genes involved in SCFA production (Rampelli et al., 2013). Fiber plays a role in the SCFA production in the gut (Topping and Clifton, 2001; Brouns et al., 2007). Pathways like terpenoid backbone biosynthesis are involved in the metabolism of cofactors and vitamins (Vazquez-Castellanos et al., 2015) and cytoskeleton protein pathways are related to cell motility. The microbiota of chickens fed a control diet presented an increase imputed representation in the glycan biosynthesis and metabolism (N-glycan biosynthesis and other glycan degradation) probably due to the diet composition (Lang et al., 2014). Microbial fermentation transforms N-glycans from diets in SCFA affecting the composition and function of the microbiota (Koropatkin et al., 2012). The identification of the dietary carbohydrate profile could give us insights of the microbial function and validate the predicted metabolic function of the cecal microbiota. In addition, an increased frequency of pathways representing the metabolism of amino acids (phosphonate and phosphinate, and D-arginine and D-ornithine metabolism) was observed, probably due to differences in the amino acid concentration between diets.

Furthermore, we hypothesized that despite the structural changes in the cecal microbiota, butyrate allowed the normalization of the microbial cecal function. Our hypothesis was supported by the observed constancy in the imputed KEGG carbohydrate and lipid pathways in the cecal microbiota of chickens fed a control diet and a nutritionally reduced diet supplemented with SB. Few pathways had a significant distribution, as arachidonic acid

metabolism, that has a role in preventing inflammation (Hyde and Missailidis, 2009), vitamin B_6 metabolism, also involved in the fatty acid metabolism (Horrobin, 1993; Nakamura and Nara, 2004) and bacterial transcription machinery. Unfortunately, a number of OTUs did not match public databases, thus their functions were not imputed.

Frequency of butyryl-CoA:acetate CoA gene in the bacterial community

Butyryl-CoA:acetate CoA-transferase catalyzes the final step of butyrate formation, and is used by several bacterial species in the healthy gut microbiota (Duncan et al., 2004; Onrust et al., 2015). There was a significant interaction between the nutritional density of diets and SB supplementation; wherein the intestinal microbiota of chickens fed a nutritionally-reduced diet without supplementation of SB showed a higher frequency of butyryl-CoA:acetate CoA gene compared to the treatments supplemented with SB ($P = 0.01$; Figure 3.5), and was not affected by nutrient density when diets were supplemented with SB. The higher frequency of butyryl-CoA:acetate CoA-transferase in the cecal microbiota of chickens fed a nutritionally reduced diet without SB, also had a higher frequency of butyrate-producing bacteria, such as *F. prausnitzii* and non-pathogenic *Clostridial* species. Nutritionally-reduced diets were produced by adding cellulose to its composition, and likely the cecal microbiota of chickens fed this diet had a higher frequency of bacteria containing the butyryl-CoA:acetate CoA-transferase gene as a response to the composition of the cecal content. In addition, through a cross-feeding mechanism, lactic acid produced in the small intestine by lactobacilli may be consumed by butyrate-producing bacteria in the cecum (De Maesschalck et al., 2015), which may also explain the higher frequency of the butyryl-CoA:acetate CoA-transferase gene, and consequently butyrate producing bacteria, observed in the microbiota of chickens. Interestingly,

SB decreased the abundance of the butyryl-CoA:acetate CoA-transferase gene in the intestinal microbiota, probably as a result of the dietary butyrate available to the host. It has been shown that the chemical composition of the intestinal ecosystem regulates not only the composition of the microbiota, but the production of butyrate (Dostal et al., 2015).

Expression of immune-related genes

An interaction was observed between nutritional density and SB supplementation for the expression of A20 ($P = 0.04$); wherein A20 was upregulated in chickens fed a nutritionally-reduced diet without SB, but was unaffected by nutritional reduction when supplemented with SB. In addition, SB supplementation upregulated interleukin 6 ($P = 0.007$). The nutritional density of the diet nor the supplementation of SB affected the expression of IL-10, INF γ, NFK-β p65, and IL-1 β ($P > 0.05$; data not shown).

Evidence suggests that a beneficial partnership has evolved between symbiotic bacteria and the immune system. Studies have shown the role of some individual bacteria in suppressing the inflammatory response during an inflammatory disease (Round and Mazmanian, 2009). However, many studies conducted to evaluate the pathogenesis of inflammatory diseases showed an altered immune response against commensal gut microorganisms (Sundin et al., 2015). Few investigations have been conducted to evaluate the effects of immune modulators, such as butyrate, on the normal microbiota of chickens (Zhang et al., 2011). The expression of ubiquitin-editing enzyme A20, a cytoplasmic anti-inflammatory protein able to regulate the inflammatory response and intestinal apoptosis (Vereecke et al., 2009; Catrysse et al., 2014) was upregulated in the cecal tonsils of chickens fed a control diet supplemented with butyrate and in birds fed a nutritionally-reduced diet. Ubiquitin-editing enzyme A20 is related to

intestinal tolerance to lipopolysaccharide (Wang et al., 2009). Immuno-regulatory bacterial strains and butyrate producers can lead to an upregulation of A20, and beneficially modulate the Toll-like receptors 4 (TLR4) activation by reducing the activation of mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) pathways and the production of proinflammatory cytokines (Song et al., 2012; Tomosada et al., 2013). An upregulation of interleukin-6 (IL-6) was observed in the cecal tonsils in the presence of SB; IL-6 is a potent, pleiotropic, inflammatory cytokine that mediates a plethora of physiological functions, including cell survival and amelioration of apoptotic signals (Kamimura et al., 2003).

CONCLUSION

Overall, it was observed that the nutritional reduction of energy and amino acids impaired the performance of broiler chickens, but the supplementation of SB could partially counteract this effect. The cecal microbiota of chickens showed a large amount of fiber degraders and SCFA producers, especially in the groups fed a nutritionally reduced diet supplemented with SB. The nutritional reduction changed the predicted function performed by the microbiota, and the SB supplementation reduced these variations, keeping the imputed microbial function more similar to that of the control diet fed broilers. The frequency of bacterial species presenting the butyryl-CoA:acetate CoA-transferase gene related to butyrate production was increased in the microbiota of chickens fed a nutritionally reduced diet and reduced with SB supplementation. Additionally, SB supplementation was able to modulate the immune response. Butyrate is a bacterial metabolite critical to intestinal health and host performance. Based on the results herein, the use of synthetic sources of butyrate may bring benefits in terms of performance and intestinal function of broiler chickens.

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Table 3.1. Composition of the experimental diets and calculated and determined nutrient composition.

	1-14d			15-28d		
Ingredient, %	Control	Reduced	Reduced 2	Control	Reduced	Reduced 2
Corn	57.12	55.96	54.81	58.72	57.40	55.84
Soybean meal, 47.5% CP	35.70	34.87	34.11	33.04	32.49	31.96
Soybean oil	2.300	2.300	2.300	3.614	3.600	3.670
Monocalcium phosphate	1.801	1.814	1.826	1.258	1.268	1.279
Limestone	1.369	1.371	1.372	1.502	1.503	1.503
Sodium chloride	0.457	0.458	0.459	0.407	0.408	0.409
L-lysine HCl	0.321	0.313	0.303	0.174	0.161	0.150
DL-methionine	0.400	0.390	0.380	0.313	0.300	0.290
L-threonine	0.091	0.088	0.085	0.025	0.020	0.016
Vitamin Premix- broilers[1]	0.350	0.350	0.350	0.350	0.350	0.350
Sulka-Floc[2]	0.100	2.083	4.000	0.100	2.004	0.500
Calculated nutrient and energy content						
ME Kcal/Kg	3,050	2,990	2,930	3,150	3,090	3,030
CP, %	22.17	21.67	21.20	20.78	20.40	20.00
Lysine, %	1.430	1.397	1.364	1.240	1.211	1.183
Thr, %	0.940	0.918	0.897	0.830	0.811	0.792
Met+Cys, %	1.070	1.045	1.021	0.950	0.928	0.906
nPP, %	0.500	0.500	0.500	0.380	0.380	0.380
Ca, %	0.950	0.950	0.950	0.900	0.900	0.900
Na, %	0.200	0.200	0.200	0.180	0.180	0.180
Determined nutrient content						
Crude Fiber, %	-	-	-	2.25	3.50	4.00
CP, %	-	-	-	21.29	20.78	20.15
Lysine, %	-	-	-	1.395	1.240	1.190
Thr, %	-	-	-	0.860	0.785	0.750
Met+Cys, %	-	-	-	0.950	0.840	0.810

[1] Supplied the following per kilogram of diet: supplied per kg of diet: vitamin A, 13,233 IU; vitamin D_3, 6636 IU ; vitamin E, 44.1 IU ; vitamin K, 4.5 mg; thiamine, 2.21mg; riboflavin, 6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2mg; pyridoxine, 3.31

mg; folic acid, 1.10 mg; biotin, 0.33 mg; vitamin B_{12}, 24.8 µg; choline, 669.8 mg; iron from ferrous sulfate, 50.1 mg; copper from copper sulfate, 7.7 mg; manganese from manganese oxide, 125.1 mg; zinc from zinc oxide, 125.1 mg; iodine from ethylene diamine dihydroidide, 2.10 mg; selenium from sodium selenite, 0.25 mg.
[2] International Fiber Corporation, North Tonawanda, NY

Table 3.2. Primers used for RT-PCR and qPCR

Target gene	Primer sequence (5′–3′)	Reference
IL-6	F: ATAAATCCCGATGAAGTGG R: CTCACGGTCTTCTCCATAAA	(Gao et al., 2012)
IL-10	F: CAATCCAGGGACGATGAAC R: GCAGGTGAAGAAGCGGTGA	(Gao et al., 2012)
INF- γ	F: TGAGCCAGATTGTTTCGA R: ACGCCATCAGGAAGGTTG	(Gao et al., 2012)
IL-1β	F: AGAAGAAGCCTCGCCTGGAT R: CCGCAGCAGTTTGGTCAT	(Gao et al., 2012)
NF-κB p65	F:GTGTGAAGAAACGGGAACTG R:GGCACGGTTGTCATAGATGG	(Li et al., 2015)
A20	F:GACATCGTGCTAACAGCTTGGA R:AGAAAAGAGGGTATCAGGCACAAC	(Li et al., 2015)
GAPDH	F: ACTGTCAAGGCTGAGAACGG R: CATTTGATGTTGCTGGGGTC	(Gao et al., 2012)
Uni	F: GTGSTGCAYGGYYGTCGTCA R: ACGTCRTCCMCNCCTTCCTC	(Belenguer et al., 2006)
BcoATscr	F: GCIGAICATTTCACITGGAAYWSITGGCAYATG R: CCTGCCTTTGCAATRTCIACRAANGC	(Louis and Flint, 2007)

Table 3.3. Performance of broiler chickens from 1 to 28 days of age, fed diets with different levels of reduction in energy and AA, and supplemented or not with sodium butyrate (SB).

		1 to 14 d			14 to 28 d			1 to 28 d		
		WG, g	FI, g	FCR	WG, g	FI, g	FCR	WG, g	FI, g	FCR
Without SB	Control	319	409	1.284	1,020 [a]	1,516 [a]	1.482	1,340 [a]	1,931 [a]	1.441
	-2.3% aa/-60 Kcal	307	396	1.290	971 [bcd]	1,457 [ab]	1.496	1,278 [bc]	1,859 [ab]	1.447
	-4.6% aa/-120 Kcal	298	406	1.366	934 [d]	1,440 [b]	1.543	1,231 [d]	1,847 [b]	1.479
With SB	Control	324	406	1.257	992 [abc]	1,466 [ab]	1.479	1,316 [ab]	1,872 [ab]	1.424
	-2.3% aa/-60 Kcal	318	413	1.297	1,002 [ab]	1,490 [ab]	1.487	1,320 [ab]	1,903 [ab]	1.441
	-4.6% aa/-120 Kcal	308	412	1.340	961 [cd]	1,490 [ab]	1.551	1,268 [cd]	1,902 [ab]	1.499
SB										
Absence		308 [b]	403	1.313	975	1,471	1.507	1,283 [b]	1,879	1.456
Presence		317 [a]	410	1.298	985	1,482	1.506	1,301 [a]	1,892	1.454
Diet										
Control		322 [a]	408	1.270 [b]	1,006 [a]	1,491	1.481 [b]	1,328 [a]	1,902	1.433 [b]
-2.3% aa/-60 Kcal		313 [b]	405	1.293 [ab]	987 [a]	1,474	1.492 [b]	1,299 [b]	1,881	1.444 [b]
-4.6% aa/-120 Kcal		303 [c]	409	1.353 [a]	948 [b]	1,465	1.547 [a]	1,250 [c]	1,875	1.489 [a]
SEM		2.9	6.7	0.03	8.1	16.3	0.01	9.7	18.0	0.01
Source of variation					*P Value*					
Diet		<0.001	NS	0.01	<0.001	NS	<0.001	<0.001	NS	<0.001
SB		<0.001	NS	NS	NS	NS	NS	0.04	NS	NS
Diet x SB		NS	NS	NS	0.003	0.01	NS	0.004	0.008	NS

[a–d] Means with different superscripts in a column differ significantly (P < 0.05). Values are means of 8 pens (46 birds/pen).

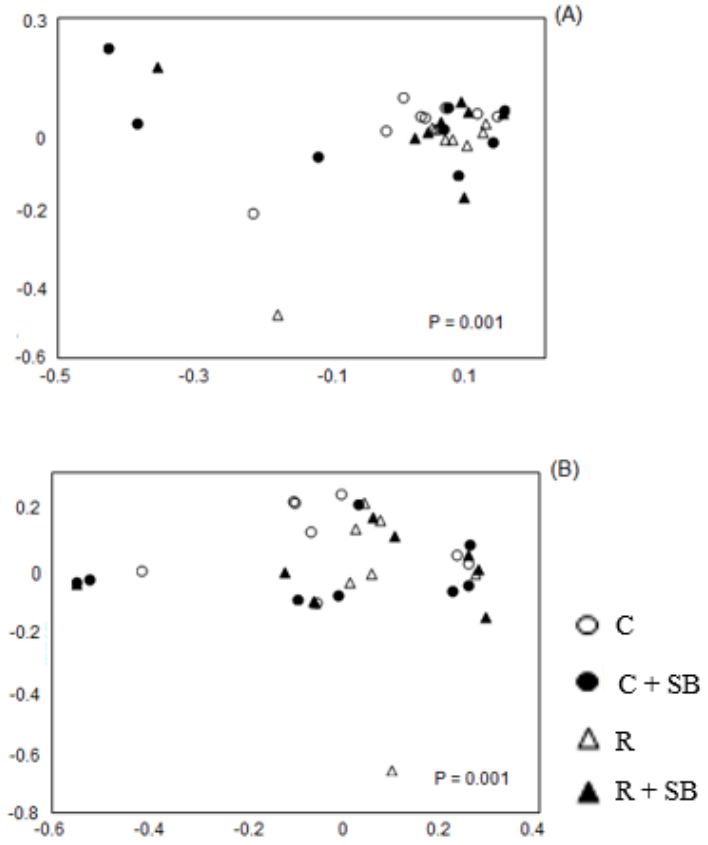

Figure 3.1. Unweighed (A) and weighted (B) UniFrac cecal microbiome visualized via principle coordinate analysis (PCA) from 28 d broiler chickens fed control (C) or nutritionally reduced diet (R) with or without sodium butyrate (SB) supplementation.

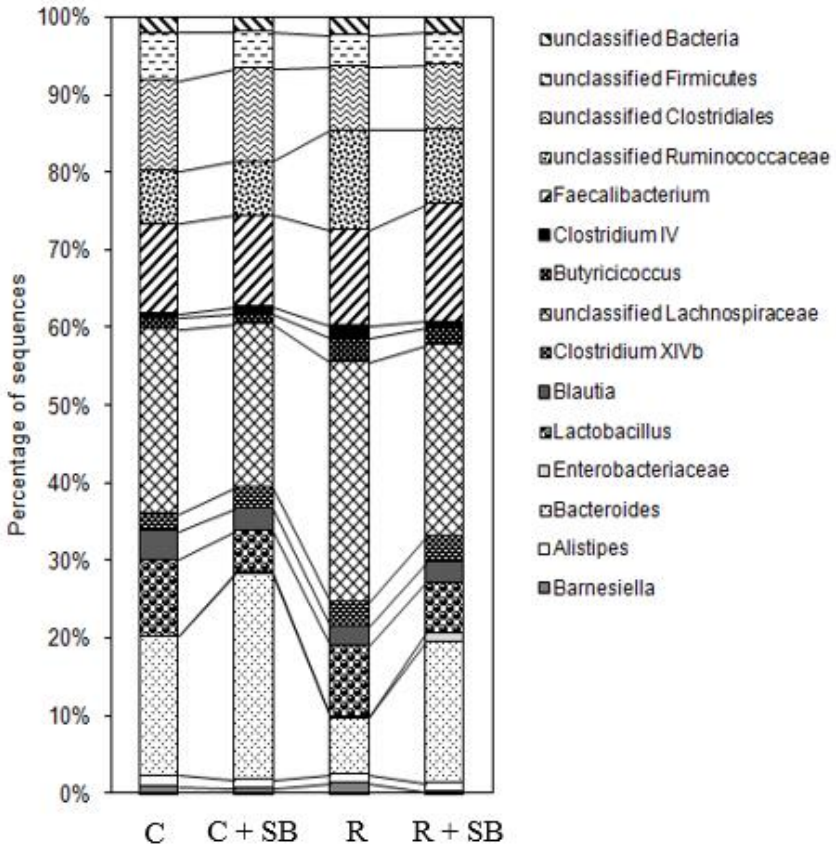

Figure 3.2. Stacked bar charts of the distribution of bacterial species detected in a 16S rDNA pyrosequencing library created by use of cecal contents collected from 28 d broiler chickens fed control (C) or nutritionally reduced diet (R) with or without sodium butyrate (SB) supplementation.

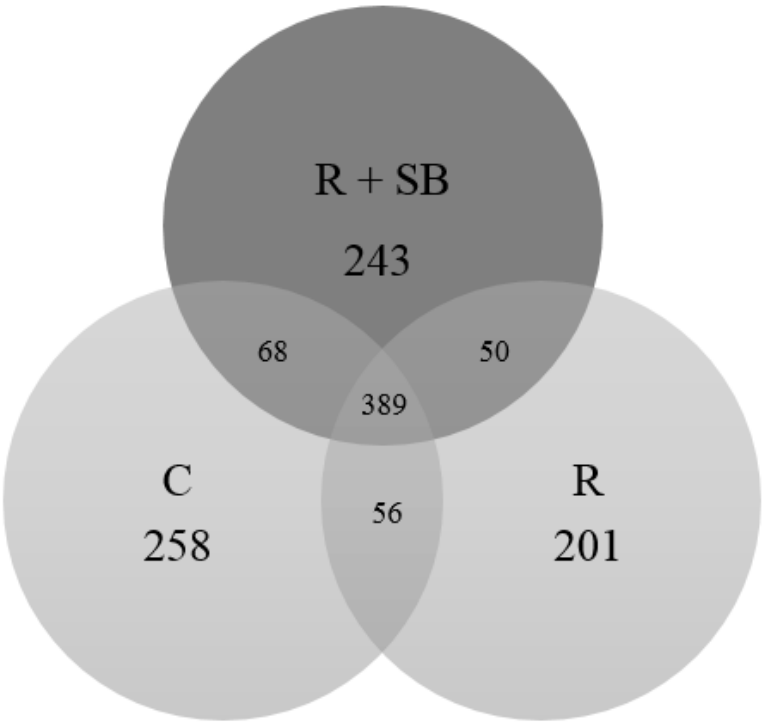

Figure 3.3. Venn diagrams of the shared and unique OTUs (bacterial species) detected in the cecal contents collected from 28 d broiler chickens fed control (C) or nutritionally reduced diet (R) with or without sodium butyrate (SB) supplementation.

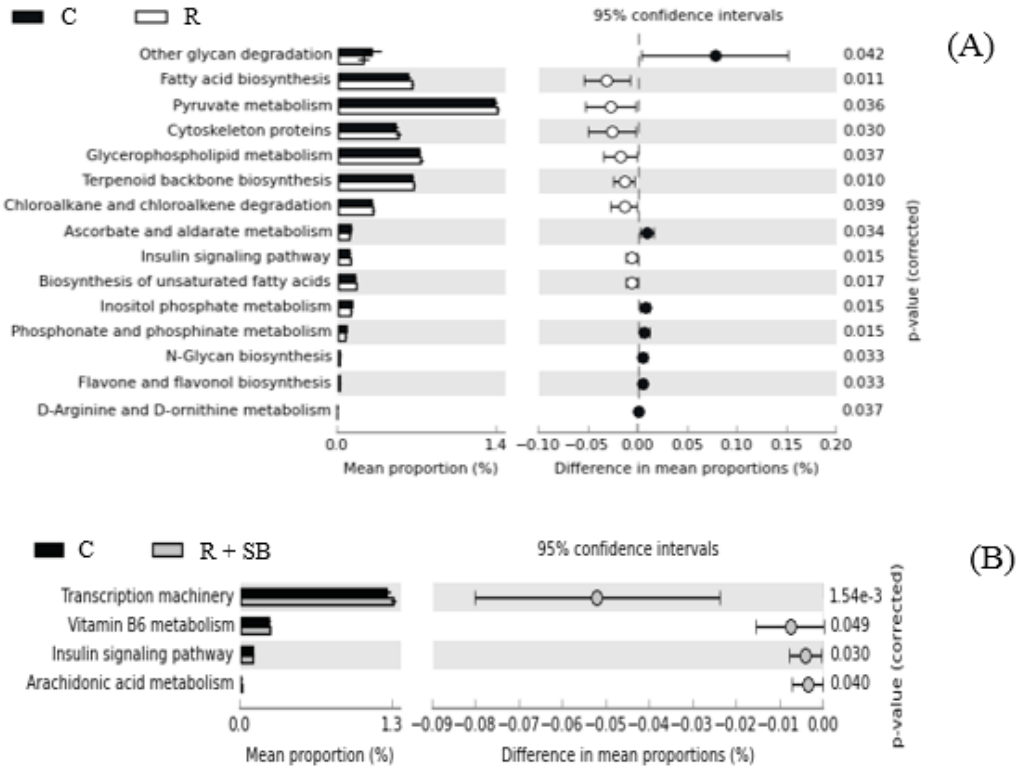

Figure 3.4 Predicted function of the cecal microbiota collected from 28 d broiler chickens fed control (C) or nutritionally reduced diet (R) with sodium butyrate (SB) supplementation.

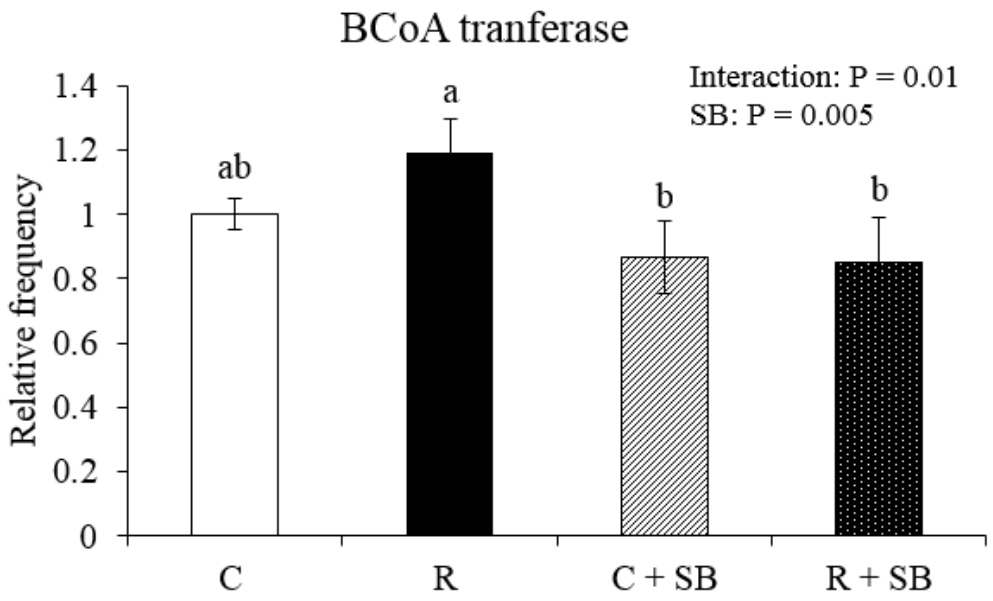

Figure 3.5. Relative frequency of butyryl-CoA:acetate CoA transferase gene in the intestinal microbiome of 28 d broiler chickens fed control (C) or nutritionally reduced diet (R) with or without sodium butyrate (SB) supplementation. Values are means ± SEM(1 bird/pen; 8 birds/treatment). [a–b] Means with different superscripts in a column differ significantly ($P < 0.05$).

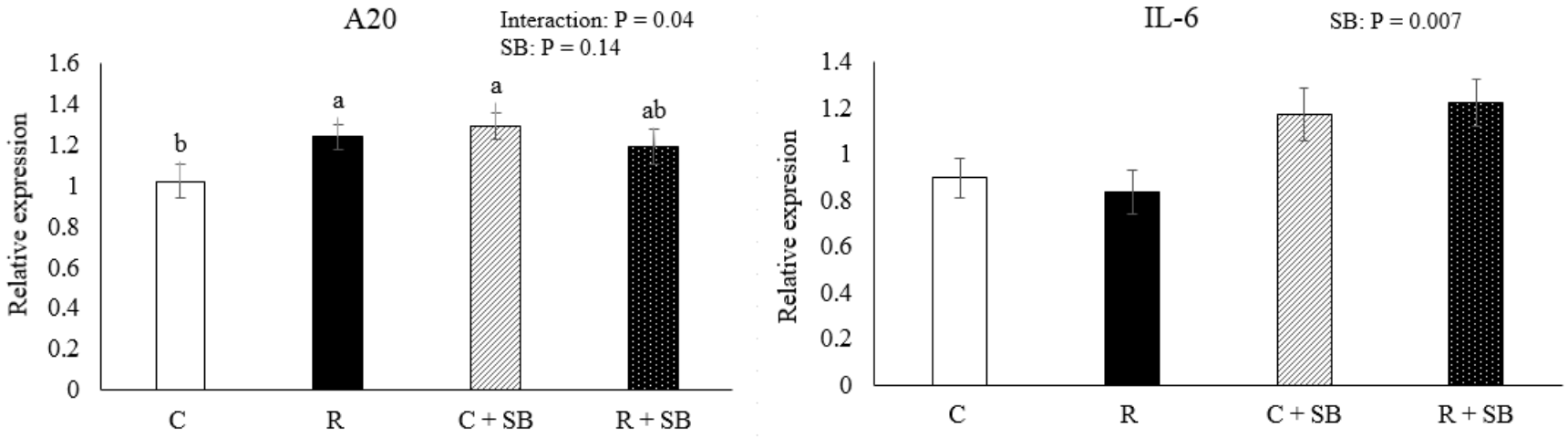

Figure 3.6. Relative gene expression of A20, and Interleukin 6 (IL-6) in the cecal tonsils of 28 d old broiler chickens fed control (C) or nutritionally reduced diet (R) with or without sodium butyrate (SB) supplementation. Values are means ± SEM (1 bird/pen; 8 birds/treatment). [a–b] Means with different superscripts in a column differ significantly ($P < 0.05$).

CHAPTER 4

EFFECTS OF SUPPLEMENTATION OF PROTECTED SODIUM BUTYRATE ALONE OR IN COMBINATION WITH ESSENTIAL OILS ON THE CECAL MICROBIOTA OF BROILER CHICKENS CHALLENGED WITH COCCIDIA AND *CLOSTRIDIUM PERFRINGENS*[1]

Bortoluzzi, C., M. J. Rothrock, B. S. Vieira, J. J. Mallo, M. Puyalto, C. Hofacre, T. J. Applegate. [1]*Front. Sustain. Food Syst. 2:72.*

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ABSTRACT

The objective of this study was to determine the effects of protected sodium butyrate (SB), and protected sodium butyrate plus essential oils (carvacrol and ginger; SBEO) on the cecal microbiota of broilers challenged with *Eimeria maxima* and *Clostridium perfringens*. Birds were assigned to 4 treatments (8 replicates pen of 58 birds/pen): unchallenged control; challenged control; challenged and supplemented with SB; challenged and supplemented with SBEO. On d 13, challenged birds were orally inoculated with ~5,000 *Eimeria maxima* oocysts. On d 18-19, the same birds were exposed to *Clostridium perfringens* via drinking water (~$1x10^8$ cfu/mL). Cecal excreta was collected at d 12, 18, 21, and 28 for microbiome analysis through 16s rRNA sequencing using Illumina MiSeq platform and analyzed using QIIME v. 1.9.1 The cecal microbiota was analyzed over time within each experimental group. The inclusion of SB alone or in combination with EO contributed to larger variations in the cecal microbiota over time than the unsupplemented treatments, as shown by the diversity indices. The community structure and abundance of the cecal microbiota were significantly different across ages, especially in the groups supplemented with SB and SBEO. As shown in the PCoA analysis, the supplementation of SB led to a more stable microbial community and lower between-sample variability over time. In the unchallenged control birds, *Ruminococcus* decreased ($p = 0.006$), whereas *Bacteroides* and *Clostridiales* increased ($p \leq 0.02$) as the birds aged. In the challenged control group, however, the frequency of *Coprococcus* and *Blautia* decreased as birds aged ($p \leq 0.01$), and, *Clostridiales* did not increase. Supplementation of SB, but not SBEO, increased the frequency of *Lactobacillus* ($p = 0.01$) on d 12 compared to d 18 and d 28, and prevented the reduction in the frequency of *Blautia* as the birds aged. Nevertheless, supplementation of SB and SBEO contributed to unique

changes in the predicted functions of the cecal microbiota over time, which was not observed in the unsupplemented birds. SB and SBEO modulated the diversity, composition, and predictive function of the cecal microbiota which may have lowered the negative impact of NE.

Keywords: Butyrate, broilers, essential oils, microbiota, necrotic enteritis

INTRODUCTION

Due to the recent restrictions in the use of antimicrobial growth promoters in the diets of broiler chickens, and move towards a more judicious antibiotic use, the search for gut health feed additives is becoming increasingly important as the incidence of enteric diseases has grown in commercial flocks submitted to antibiotic-free programs (Kaldhusdal et al., 2016; Broom, 2017). Several alternatives have been evaluated and different mechanisms of action have been proposed. However, there is still a lack of knowledge on how different feed additives could at least partially attenuate the impact of enteric diseases, such as coccidiosis and necrotic enteritis (NE) in broiler flocks.

Coccidiosis is one of the most important predisposing factors for the development of NE in chickens (Prescott el al., 2016). The increased mucus production that occurs due to the *Eimeria* infection (Collier et al., 2008), and leakage of plasma proteins into the intestinal lumen (Prescott et al., 2016) increase the proliferation of *Clostridium perfringens*, the causative agent of NE. Besides increasing the susceptibility to NE, coccidiosis also leads to changes in the overall structure of the intestinal microbiota (Stanley et al., 2014; Wu et al., 2014; Zhou et al., 2017). Wu et al. (2014) demonstrated a reduction in the cecal microbial diversity following *Eimeria* infection, and reduction of many members of the family *Ruminococcaceae*. Yet, *Eimeria*

infection caused changes in short chain fatty acids (SCFA) produced in the ceca of chickens (Stanley et al., 2014).

Clostridium perfringens infection is also associated with shifts in the intestinal microbiota (Stanley et al., 2012; Stanley et al., 2014; Antonissen et al., 2016). *C. perfringens* can interact and compete with other microorganisms in the gut, which may alter the proliferation of *C. perfringens*, production of toxins and the severity of the disease (Antonissen et al., 2016). Stanley et al. (2012) reported that NE infection changed the abundance of important bacterial families in the gut, such as *Clostridiales* and *Lactobacillales*. Additionally, SCFA-producing bacteria as well as segmented filamentous bacteria decreased due to NE challenge, demonstrating that the overall dysbiosis may also be related to the pathogenesis of the disease (Stanley et al., 2014). Thus, it is reasonable to hypothesize that different dietary supplements administered to NE infected flocks may directly influence the diversity and composition of the intestinal microbiota, mainly through growth and proliferation of commensal bacteria that play important roles on the general physiology of the host.

Butyrate has multiple effects on the intestine. It serves as an energy source for epithelial cells, stimulates mucus production, controls the intestinal barrier function, promotes pathogen control, and modulates the immune-system (Guilloteau et al., 2010). Therefore, it is believed that butyrate may alleviate the negative effects of NE in chickens by modulating the intestinal microbiota, immune-system, and the intestinal barrier function. We recently showed that dietary supplementation of chickens with a protected source of sodium butyrate (SB) modulated the diversity, composition, and predictive function of the cecal microbiota (Bortoluzzi et al., 2017). Indeed, SB may directly modulate the intestinal microbiome, through its bactericidal effect, or

indirectly by stimulating the growth of beneficial lactic acid bacteria (Ahsan et al., 2016). Additionally, essential oils (EO) obtained from plant materials are known to possess antimicrobial, antioxidant, and anti-inflammatory properties (Brenes and Roura, 2010). Jerzsele et al. (2012) showed that a blend of ginger oils and carvacrol reduced the gross lesions caused by NE and had beneficial effects on the intestinal morphology.

Therefore, we hypothesized that supplementing SB alone or in combination with EO would beneficially modulate the cecal microbiota of broiler chickens challenged with *E. maxima* and *C. perfringens*, thereby alleviating the severity of challenge and improving bird's performance. The objective of this study was to evaluate the effects of SB alone or in combination with EO on the balance (in terms of diversity, composition, and predicted function) of the cecal microbiota of broiler chickens challenged with *E. maxima* and *C. perfringens*.

MATERIAL AND METHODS

Housing, birds and treatments

The animal care and use procedures followed the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) under supervision of a licensed poultry veterinarian. One thousand eight hundred fifty-six (1,856) one-day-old male Ross chicks were assigned to four (4) treatment groups with eight (8) replicate pens per treatment and 58 birds per pen raised on new litter. The treatments were: non-supplemented non-challenged group (unchallenged control); non-supplemented and challenged control; challenged and supplemented with SB (70% of sodium butyrate protected with sodium salts of palm fatty acids; ChaSB; 0.1% inclusion); and challenged and supplemented with SB plus essential oils (70% of sodium butyrate

protected with sodium salts of palm fatty acids, and carvacrol and ginger oil – 0.5% each in the commercial product; ChaSBEO; 0.1% inclusion). Each experimental diet was mixed separately, and the feed additives were supplemented to replace kaolin in the unsupplemented diet.

Feed consisted of a non-medicated commercial-type broiler starter, grower, and finisher diets compounded according to NRC guidelines (NRC, 1994) and contained feedstuffs commonly used in the United States (Table 4.1). The feeds were available *ad libitum* from date of chick arrival as follows: Starter, d 0 until d 13; grower, d 14 to d 34; and finisher, d 34 to d 41 (study termination). Diets were fed as crumbles (starter feed) or pellets (grower and finisher feed).

The challenge model consisted of coccidial vaccine (Coccivac®-B52) administered at d 0 by spray cabinet, and individual inoculation with ~5,000 sporulated oocysts of *Eimeria maxima* by oral gavage on d 13, kindly provided by Dr. Lorraine Fuller, from the Department of Poultry Science, University of Georgia. On d 18, and 19, the same birds were challenged with *C. perfringens*, as follow: feed was withdrawn for four hours and water was withdrawn for two to three hours prior to administration of *C. perfringens*. A measured amount of water (~200 mL with *C. perfringens*) that was consumed within 30 minutes was used for each pen. A *C. perfringens* culture (~$1x10^8$ cfu total) was added to this water and thoroughly mixed and given to birds in each challenge pen. Once the challenge water was consumed, treatment feed and water were returned to pen.

Sample collection and analysis performed on days 12, 18, 21, and 28

Two birds per pen were euthanized by cervical dislocation and the caeca were collected into a sterile Ziploc bag, immediately put in ice, and brought to the lab. Thus, the cecal content of the two birds was pooled in a new sterile Ziploc bag, and thoroughly homogenized by hand for further microbiota analysis. The content was then diluted in a 1:10 sterile PBS solution; 1mL of the solution was transferred to a 2 mL micro-tube, centrifuged for 3 m at 1,200 rcf, the supernatant discarded, and 200 µg of the content used for DNA isolation.

DNA extraction of the cecal microbiome

The DNA isolation was conducted following the manufacturer recommendations (PowerViral Environmental RNA/DNA Isolation Kit – Mo Bio; QIAGEN, Carlsbad, CA, USA), with a slight modification as described by Bortoluzzi et al. (2017). The quality and presence of DNA was verified by Nanodrop (NanoDrop 1000 Spectrophotometer; Thermo Fischer Scientific, Wilminton, DE, USA) and agarose gel electrophoresis (1.5%), respectively.

PCR amplification and sequencing

The V3-V4 hypervariable region of the 16S rRNA gene was amplified using the primer FwOvAd_341f and ReOvAd_785r as previously described (Klindworth et al., 2013). All the procedures followed a standardized protocol described previously (Bortoluzzi et al., 2017). The 16S rRNA gene was then sequenced using the Illumina Miseq platform.

Bioinformatics

All sequence data processing was performed using QIIME v. 1.9.1 software package (Caporaso et al., 2010). Sequences were paired-end and quality trimmed using Geneious (Newark, NJ). High-quality sequences were aligned against the SILVA database (Ribocon GmbH, Bremen, Germany) release 119 (Pruesse et al., 2007). UCHIME software (Tiburon, CA) was used to identify and remove chimeric sequences (Edgar et al., 2012). Number of sequences per sample was normalized based on the sample with the lowest number of reads for statistical comparison (Gihring et al., 2012). Operational taxonomic units (OTUs) were assigned at a 97% identity using SILVA database. Alpha (richness: Observed Species, Chao1; diversity: Shannon, and Phylogenetic Diversity (PD) of the Whole Tree), and beta diversity indices were calculated using QIIME v1.9.1. Nonparametric statistical tests PERMANOVA and ANOSIM were used to compare categories, and the similarity between the microbiome, respectively, using the weighted (quantitative) UniFrac metric measure. Principal Coordinates Analysis (PCoA) was used to visualize the data. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was carried out using the KEGG and Clusters of Orthologous Groups of proteins (COGs) databases. For this, a closed-reference OTU table was normalized by the 16S rDNA copy number, the metagenome was predicted, and categorized by function based on Kyoto Encyclopedia of Genes and Genomes (KEGG; Uji, Kyoto, Japan) pathway (Kanehisa and Soto, 2000). The obtained biome file was processed by STAMP (Halifax, Nova Scotia, Canada) version 2.1.3 (Parks et al., 2014).

Statistical analysis

The frequency of the main bacterial groups observed was submitted to a non-parametric one-way ANOVA (Kruskal-Wallis test) and, in case of significant difference ($P \leq 0.05$), means were separated by Dunn test, using SAS 9.4 (SAS, 2011). Welch's T-test was applied to compare the KEGG pathways ($P \leq 0.05$).

RESULTS

Alpha and Beta diversity

Chao index (minimal number of OTU present in a sample), number of observed species (number of species present in a community), phylogenetic diversity (PD) of the whole tree (minimum total length of all the phylogenetic branches required to span a given set of taxa on the phylogenetic tree), and Shannon index (abundance and evenness of the species present in a sample) were evaluated as measures of alpha diversity indices (Tables 4.2 and 4.3). Comparisons among timepoints were performed to evaluate the transition of the cecal microbiota within each experimental treatment. The different treatments differently changed the microbiota across ages, as shown by the diversity indexes.

When considering the unchallenged control group (Table 4.2), it was observed that richness and evenness were higher, mainly on d 28 of age when compared to earlier ages. However, considering the challenged control group, only few differences were observed in terms of richness and evenness across ages (Table 4.2). On the other hand, when the birds were challenged and supplemented with either SB or SBEO (Table 4.3) many differences were observed, showing that the supplementation of these feed additives had a strong effect on the cecal microbiota across ages.

The community structure and abundance of the cecal microbiome were significantly different across the experimental groups and ages. Both community membership (unweighted) and structure (weighted) contributed to differences in cecal microbiome across ages. PERMANOVA analysis was conducted to describe the strength and significance that treatment groups and age had in determining the variation among distance matrixes (weighted UniFrac). These differences, that are shown using Principal Coordinated Analysis (PCoA) plots, were evident across ages within each experimental treatment (Figure 4.1). ANOSIM was used to compare the similarity among the cecal microbiota. Differences were observed across ages within different treatment groups (Table 4.4). The largest difference in the microbiome was observed on d 12 vs. 28 when birds were challenged and supplemented with SB (R = 0.97 and P = 0.001; "R" is the index of ANOSIM that indicates the similarity of comparison between group pairs. "R" ranges from -1 to1: the pairs are more similar when the R index is closer to 0 and the pairs are different from each other when the R index is close to 1).

Composition of the cecal microbiome

The cecal microbiome in the four ages evaluated was dominated by bacteria belonging to the Phylum *Firmicutes*, followed by *Bacteroidetes* and *Proteobacteria* (Table 4.5). The effect of age on the relative abundance of the main phylum was accessed for the four experimental groups. First, it was observed that the frequency of *Firmicutes* significantly decreased (P = 0.0002), and *Bacteroidetes* increased (P = 0.034) in the cecal microbiome of unchallenged control birds on d 28 compared to d 12. The same effect was observed within the challenged control group; however, a higher frequency of *Proteobacteria* (P = 0.002) was observed after the birds were

challenged, which was not observed in the unchallenged control treatment. On the other hand, when the birds were challenged and supplemented with SB or SBEO, the differences in the frequency of *Firmicutes* and *Bacteroidetes* were observed at an early stage (d 21). The increase in the frequency of *Proteobacteria* due to the challenge when the birds were supplemented with SBEO was not as evident as in the other challenged treatments.

The most abundant genera observed in the cecal microbiome were *Ruminococcus*, *Lactobacillus*, and *Bacteroides*, followed by unclassified *Ruminococcaceae* and *Clostridiales* (Figure 4.2). Again, the effect across ages was evaluated within each experimental treatment. In the unchallenged control birds, it was observed that *Ruminococcus* decreased ($P = 0.006$), whereas *Bacteroides* and *Clostridiales* increased ($P \leq 0.02$) as the birds aged. In the challenged control group, however, besides similar effects on *Ruminococcus* ($P = 0.001$) and *Bacteroides* ($P = 0.001$), it was also observed that the frequency of *Coprococcus* and *Blautia* decreased as birds aged ($P \leq 0.01$). This was an effect caused by the challenge, as it was not observed in the unchallenged control group. *Clostridiales* did not increase as an effect of age in these challenged control birds (Figure 4.2).

In the challenged SB supplemented birds, the effect of age was also evident on the frequency of *Ruminococcus* ($P = 0.002$), *Lactobacillus* ($P = 0.01$), *Bacteroides* ($P = 0.001$), and *Coprococcus* ($P = 0.02$). The frequency of *Lachnospiraceae* also tended to be affected by age ($P = 0.07$) in this treatment. The frequency of *Lactobacillus* was lower at d 18, but not at d 21 and 28, compared to d 12; *Lachnospiraceae* tended to have higher abundance on d 18, compared to the other ages, and *Ruminococcus* and *Coprococcus* followed the same pattern as observed in the challenged control group. Supplementation of SB to challenged birds did not reduce the

abundance of *Blautia*, as observed in the challenged control group (Figure 4.2). Lastly, looking at the effects of age in the challenged and SBEO supplemented birds, similar results were observed as the challenged SB birds. *Ruminococcus* decreased (P = 0.001), *Bacteroides* increased (P = 0.001), and *Blautia* decreased (P = 0.02); additionally, *Lactobacillus* tended to be higher at d 21 (P = 0.06), and *Ruminococcaceae* had a higher abundance at d 18 compared to d 21 (P = 0.02; Figure 4.2).

Prediction of the cecal microbiota functions

To predict microbial community functions from the microbiome data, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis was carried out using the KEGG and Clusters of Orthologous Groups of proteins (COGs) databases. The focus of this analysis was to evaluate the changes in the predicted functions comparing d 12 (pre-challenge conditions) with d 21 (after coccidia and *C. perfringens* challenge) within each experimental treatment. A Venn diagram was constructed to visualize the shared and unique predicted functions among the treatments which were divided between upregulated (Figure 4.3A) and downregulated predictive functions (Figure 4.3B) on d 21 compared to d 12. Among the upregulated predicted functions, it was observed that 11, 11, and 9 were unique to the challenged, challenged supplemented with SB, and challenged supplemented with SBEO, respectively. Only few functions were downregulated on d 21 compared to d 12, wherein only 1, 4, and 2 were unique to the challenged, challenged supplemented with SB, and challenged supplemented with SBEO, respectively.

When evaluating the effect of the challenge, the predicted functions common to the unchallenged control treatment were removed. When evaluating the effect of the supplementation of SB and SBEO, the predicted functions shared with the unchallenged and challenged control were also filtered out, thereby the remaining functions were unique to SB or SBEO supplementation. In the unchallenged control treatment (Figure 4.4A) few changes were observed in the predictive functions of the cecal microbiota as the birds aged. A total of 14 pathways were changed ($P \leq 0.01$) comparing d 21 vs. d 12. A larger number of functional categories of the cecal microbiota in the challenged control treatment were changed on d 21 vs. d 12 (Figure 4.4B). These changes were due mainly to the challenge since the functions shared with the unchallenged control were filtered out. A total of 30 features were unique to the treatment supplemented with SB wherein most of them were related to metabolism, and one related to penicillin and cephalosporin biosynthesis (Figure 4.5A). Additionally, 23 functional categories were unique to the SBEO supplemented treatment which included, besides penicillin and cephalosporin biosynthesis, biosynthesis of vancomycin group of antibiotics, and streptomycin biosynthesis that were enriched on d 21 vs. d 12 (Figure 4.5B).

DISCUSSION

In the present study, SB improved FCR of the chickens before the challenge (1- 13 d), and both SB and SBEO completely recovered the performance of the birds in terms of FCR in the overall experimental period (1- 41 d) compared to challenged control birds (Appendices 1 and 2). The search for feed additives contributing to the health and formation of the gut has become even more important due to the recent restrictions to the use of antibiotics for growth

promotion by the poultry industry, which may increase the incidence of NE in broilers. The GIT acts as an interface between diet, host, and gut microbiota, and has vital role in the health status of an animal. In the present study, we evaluated the action of SB alone or in combination with EO in broiler chickens submitted to a NE infection model, and their effects on the establishment of the cecal microbiota. Nutrition affects the composition of the microbiota (Pan and Wu, 2014), and the functions that the microorganisms are going to perform on the host. Nutritional strategies targeting the modulation of the microbiota before and after a challenge have the potential to improve development and regeneration of the injured intestinal mucosa, digestive physiology, immune system and inflammation (Kogut, 2017).

Few studies have investigated the effect of *Eimeria* infection on the microbial community richness (Stanley et al., 2012; MacDonald et al., 2017; Zhou et al., 2017), and have observed that challenge itself does not have a strong effect on this parameter. Challenge with *Eimeria* plus *C. perfringens*, however, drastically changes the diversity and composition of the cecal microbiome (Stanley et al., 2014). Feed additives, such as SB and EO, may indirectly modulate the intestinal microbiome by their effects on the immune-system, or directly by their effects on the bacteria population. Indeed, supplementation of SB and SBEO, as well as the infection model used herein, modulated the diversity, composition, and predicted function of the cecal microbiome. The diversity of the cecal microbiome changed as the birds aged; however, SB and SBEO supplementation to challenged birds had a critical impact on the establishment of the microbiota over the rearing cycle of the birds. As shown in the PCoA plot (Fig. 1), the supplementation of SB reduced the variability between samples and promoted a more stable microbiota over time, which was not observed in the unsupplemented or SBEO supplemented challenged birds.

At the phylum level, *Firmicutes* decreased and *Bacteroidetes* increased over time, regardless the treatment group; the induction to NE, on the other hand, increased the frequency of *Proteobacteria*-related species, but the supplementation of SBEO prevented this effect (Table 5). Additionally, NE challenge impaired the increase of *Clostridiales*-related bacteria over time, and led to a reduction of *Blautia*, but SB supplementation prevented the later effect. Non-pathogenic *Clostridia* species, such as *Blautia*, can use carbohydrates as a fermentable substrate and produce SCFA as the major end products of glucose fermentation (Park et al., 2012; Bai et al., 2016). Therefore, dietary SB modulated the cecal microbiota of infected chickens, which can also explain its beneficial effects on the performance of the birds.

Bortoluzzi et al. (2017) observed that dietary inclusion of SB *per se* did not have a significant impact on the cecal microbiota of broiler chickens. However, when the birds were fed a nutritionally-reduced diet, the composition and predicted function of the cecal bacteria community drastically varied, and the supplementation of SB reduced these variations. Similar findings have been observed by Zhou et al. (2017) wherein coated SB had no significant effect on the cecal microbiota of healthy chickens but balanced the shifts of microbial composition caused by *E. tenella* infection. Additionally, the ANOSIM analysis revealed that the cecal microbiota showed more differences across ages when the birds were supplemented with SB or SBEO vs. the unsupplemented groups. Taken together, these results indicate that supplementation of SB mainly is beneficial in speeding the establishment of the cecal microbiota, which may be important in terms of resistance and recovery from enteric diseases.

In the absence of transcriptome data, PICRUSt was applied to predict the metagenome from 16S rRNA data and a reference genome database (Langille et al., 2013). Previously, SB

supplementation was shown to reduce the variations of the predicted functions caused by a nutritional challenge (Bortoluzzi et al., 2017). In the present study, we observed that SB and SBEO led to many variations in the predicted functions of the cecal microbiota over time, besides the variations related to the normal aging of the birds and/or challenge. The changes in the predicted functions of the cecal microbiota attributed to the supplementation of SB and SBEO may have contributed to the better performance observed in these group of birds, even under challenge conditions; however this cannot be directly ascertained without including these supplemented groups to unchallenged birds. Functions related to metabolism and synthesis of antibiotics were enriched when the birds were supplemented with SB (1 category of antibiotic synthesis) or SBEO (3 categories of antibiotic synthesis). Indeed, the cecal microbiota of birds presenting better feed efficiency was enriched with functions related to glucometabolism, ion transportation and amino acid metabolism (Yan et al., 2017). The enrichment of functional categories related to antibiotic synthesis may have reduced the impact of NE, promoted better feed efficiency in these group of birds, and help explain the mechanism of action of these feed additives.

CONCLUSION

Although the interactions between commensal bacteria and nutrition in health and challenge situations are not fully understood, dietary nutrients and feed additives are responsible for modulating the population of commensal and pathogenic microorganisms. As such, the understanding of these interactions, in both physiological and pathological situations, will allow the use of feed additives to promote a better growth during enteric pathogen challenges.

Overall, SB and SBEO supplementation to NE-challenged birds contributed to changes in the diversity, composition and predicted functions of the cecal microbiome. Older birds presented a cecal microbiome with lower and higher abundance of *Firmicutes* and *Bacteroidetes*, respectively, than younger birds. Additionally, *Proteobacteria* was observed in higher frequency after challenge, in challenged control vs. SBEO supplemented birds. NE challenge led to a decrease in the frequency of *Blautia* over time, but dietary SB prevented this effect. SB and SBEO modulated the predicted function of the many metabolic pathways of the cecal microbiota over time, which potentially explains the improvement in performance obtained with these feed additives.

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Table 4.1: Diet formulation and nutrient specifications

Ingredient, %	Starter	Grower	Finisher
Corn	51.8	56.5	60.3
Soybean meal, 47.5% CP	38.8	34.0	30.3
Soybean oil	5.08	5.70	5.82
Monocalcium phosphate	2.00	1.55	1.40
Limestone	0.89	1.07	1.05
Sodium chloride	0.45	0.40	0.40
L-lysine HCl	0.28	0.20	0.12
DL-methionine	0.40	0.33	0.28
L-threonine	0.11	0.08	0.04
Vitamin Premix- broilers[1]	0.05	0.05	0.05
Mineral Premix- broilers[2]	0.07	0.07	0.07
Kaolin	0.10	0.10	0.10
Formulated nutrient content			
ME, Kcal/Kg	3,050	3,150	3,200
CP, %	22.0	20.0	18.5
Lysine, %	1.43	1.24	1.09
Thr, %	0.94	0.83	0.74
Met+Cys, %	1.07	0.95	0.86
Available P, %	0.47	0.38	0.35
Ca, %	0.95	0.90	0.85
Na, %	0.20	0.18	0.18

[1]Supplied the following per kilogram of diet: vitamin A, 11,020 IU; vitamin D_3, 2,200 IU; vitamin E, 22 IU; thiamine, 441 µg; riboflavin, 882 µg; pantothenic acid, 2,205 µg; niacin, 8,818 µg; pyridoxine, 441 µg; folic acid, 110 µg; biotin, 22 µg; vitamin B_{12}, 2.5 µg; choline, 38,272 µg.

[2]Supplied the following per kilogram of diet: Ca, 15.7 mg; Mn, 65.7 mg; Zn, 52.4 mg; Mg, 13.1 mg; Fe, 12.9 mg; Cu, 4.9 mg; I, 4.9 mg; Se, 0.196 mg.

Table 4.2. Alpha diversity indexes of the cecal microbiome of broiler chickens at different timepoints considering the unsupplemented treatments and unchallenged or challenged with a necrotic enteritis infection model.

Unchallenged control					Challenged control				
Timepoint	Chao	OS	PD Whole tree	Shannon	Timepoint	Chao	OS	PD Whole tree	Shannon
12 days	296.5	239.0	16.3	5.08	12 days	296.5	239.0	16.3	5.08
18 days	323.2	258.9	18.5	5.19	18 days	322.0	261.5	18.6	5.32
21 days	297.7	234.5	17.3	5.04	21 days	311.4	250.7	17.9	5.09
28 days	355.4	283.3	21.3	5.26	28 days	323.9	253.5	19.4	4.88
		Probability					Probability		
12 vs. 18	0.19	0.26	0.13	0.55	12 vs. 18	0.15	0.19	**0.02**	0.33
12 vs. 21	0.72	0.93	0.37	0.93	12 vs. 21	0.28	0.62	0.18	0.98
12 vs. 28	**0.02**	**0.04**	**0.003**	0.44	12 vs. 28	0.13	0.56	**0.01**	0.43
18 vs. 21	0.36	0.34	0.52	0.69	18 vs. 21	0.88	0.63	0.56	0.52
18 vs. 28	0.11	0.28	**0.03**	0.77	18 vs. 28	0.88	0.58	0.45	0.14
21 vs. 28	**0.03**	**0.06**	**0.01**	0.55	21 vs. 28	0.73	0.94	0.30	0.54

OS: Observed species; PD whole tree: Phylogenetic diversity of the whole tree. Values are means of 8 replicates and a pool of 2 birds/replicate. Significant differences between time points for a given index are bolded.

Table 4.3. Alpha diversity indexes of the cecal microbiome of broiler chickens at different timepoints considering the necrotic enteritis challenged birds supplemented with SB or SBEO.

ChaSB					ChaSBEO				
Timepoint	Chao	OS	PD Whole tree	Shannon	Timepoint	Chao	OS	PD Whole tree	Shannon
12 days	268.1	218.4	14.6	4.8	12 days	247.8	202.0	14.0	4.7
18 days	333.3	275.2	19.3	5.5	18 days	335.6	272.9	19.4	5.4
21 days	284.8	220.0	16.7	4.6	21 days	285.5	219.9	17.0	4.7
28 days	349.6	281.4	21.5	5.3	28 days	330.9	261.4	20.3	5.0
		Probability					Probability		
12 vs. 18	**0.02**	**0.02**	**0.002**	**0.03**	12 vs. 18	**0.002**	**0.003**	**0.001**	**0.05**
12 vs. 21	0.63	0.99	0.12	0.44	12 vs. 21	0.09	0.37	**0.01**	0.85
12 vs. 28	**0.02**	**0.03**	**0.001**	0.15	12 vs. 28	**0.003**	**0.01**	**0.001**	0.36
18 vs. 21	**0.03**	**0.01**	**0.02**	**0.007**	18 vs. 21	**0.02**	**0.01**	**0.03**	**0.03**
18 vs. 28	0.51	0.82	0.13	0.35	18 vs. 28	0.99	0.56	0.45	0.32
21 vs. 28	**0.01**	**0.02**	**0.007**	**0.03**	21 vs. 28	**0.03**	**0.04**	**0.02**	0.30

OS: Observed species; PD whole tree: Phylogenetic diversity of the whole tree. Values are means of 8 replicates and a pool of 2 birds/replicate. Significant differences between time points for a given index are bolded.

Table 4.4. ANOSIM analysis results comparing the different timepoints within each of the treatment groups.

Treatment group	Age of Comparison	R[1]	Probability[2]
Control	12 d vs. 18 d	0.0007	0.39
	12 d vs. 21 d	0.08	0.13
	12 d vs. 28 d	0.53	**0.005**
	18 d vs. 21 d	-0.05	0.75
	18 d vs. 28 d	0.21	**0.048**
	21 d vs. 28 d	0.09	0.13
Challenged control	12 d vs. 18 d	0.10	0.08
	12 d vs. 21 d	0.42	**0.001**
	12 d vs. 28 d	0.81	**0.001**
	18 d vs. 21 d	0.09	0.12
	18 d vs. 28 d	0.55	**0.002**
	21 d vs. 28 d	0.21	**0.05**
ChaSB	12 d vs. 18 d	0.47	**0.001**
	12 d vs. 21 d	0.47	**0.001**
	12 d vs. 28 d	0.97	**0.001**
	18 d vs. 21 d	0.24	**0.01**
	18 d vs. 28 d	0.68	**0.002**
	21 d vs. 28 d	0.26	**0.004**
ChaSBEO	12 d vs. 18 d	0.22	**0.008**
	12 d vs. 21 d	0.37	**0.002**
	12 d vs. 28 d	0.85	**0.001**
	18 d vs. 21 d	0.12	0.11
	18 d vs. 28 d	0.35	**0.008**
	21 d vs. 28 d	0.15	**0.05**

[1]R is the index of ANOSIM that indicates the similarity of comparison between group pairs. “R” ranges from -1 to 1: the pairs are more similar when the R index is closer to 0 and the pairs are different from each other when the R index is close to 1. [2] Significant differences between time points for a given treatment groups are bolded.

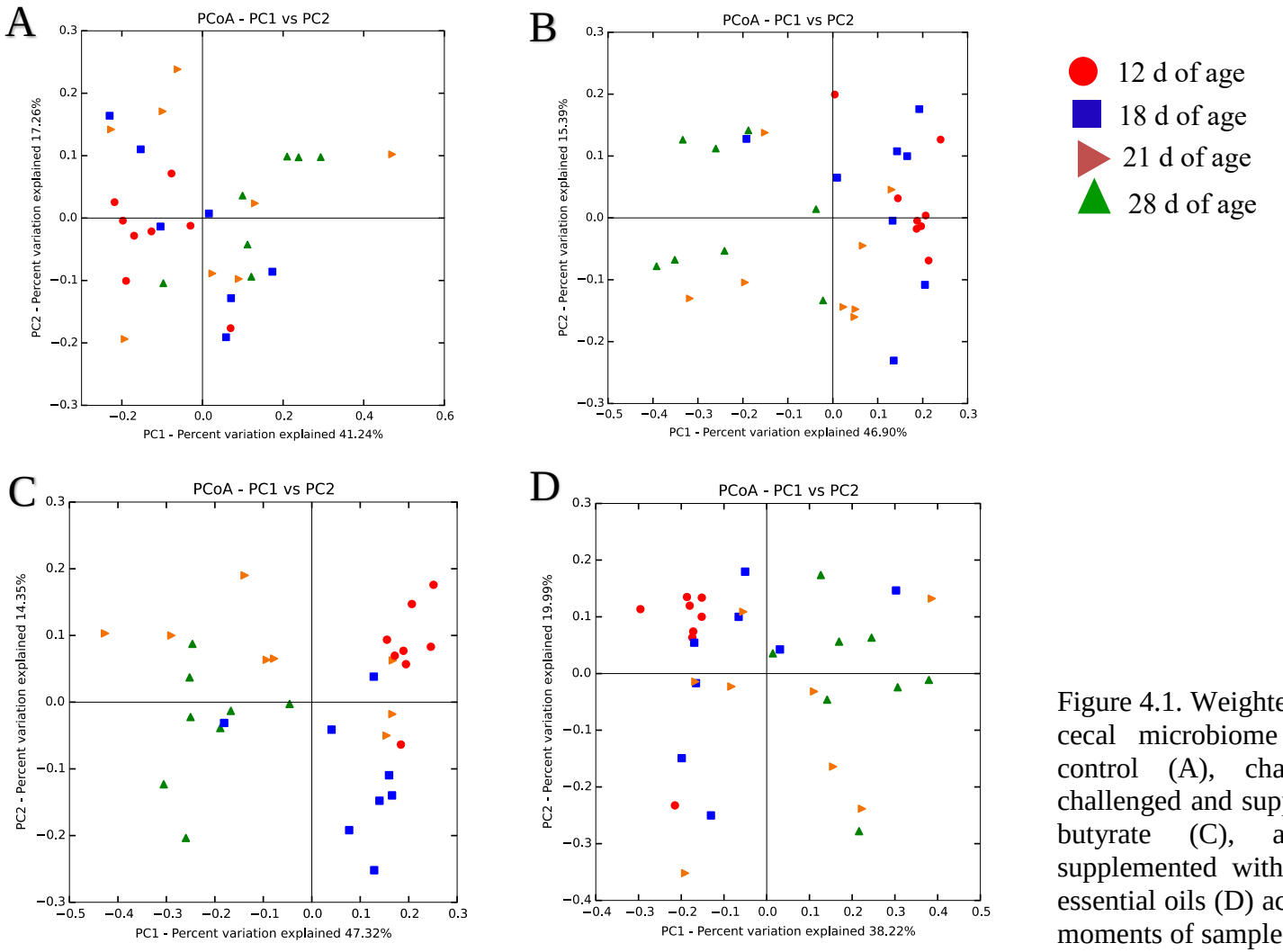

Figure 4.1. Weighted UniFrac PCoA of the cecal microbiome in the unchallenged control (A), challenged control (B), challenged and supplemented with sodium butyrate (C), and challenged and supplemented with sodium butyrate plus essential oils (D) according to the different moments of sample collection.

Table 4.5. Relative abundance (%) of the main phylum in the cecal microbiome of broiler chickens at different timepoints within each one of the experimental groups.

Unchallenged control				Challenged control			
Timepoint	*Firmicutes*	*Bacteroidetes*	*Proteobacteria*	Timepoint	*Firmicutes*	*Bacteroidetes*	*Proteobacteria*
12 days	87.1 [a]	9.9 [b]	0.8	12 d	97.3 [a]	0.8 [c]	0.1 [b]
18 days	78.8 [ab]	18.8 [ab]	1.2	18 d	89.8 [ab]	5.8 [bc]	1.7 [a]
21 days	77.9 [ab]	18.8 [ab]	0.7	21 d	78.4 [bc]	19.1 [ab]	1.1 [ab]
28 days	61.9 [b]	31.2 [a]	1.1	28 d	60.6 [c]	34.9 [a]	1.0 [ab]
SEM	2.3	2.3	0.1	SEM	3.1	3.1	0.2
Probability	0.002	0.01	0.64	Probability	<0.0001	<0.0001	0.004
Challenged and supplemented with SB				Challenged and supplemented with SBEO			
Timepoint	*Firmicutes*	*Bacteroidetes*	*Proteobacteria*	Timepoint	*Firmicutes*	*Bacteroidetes*	*Proteobacteria*
12 days	98.7 [a]	0.9 [b]	0.4 [b]	12 d	92.1 [a]	5.8 [b]	0.6
18 days	85.2 [ab]	12.8 [ab]	1.3 [ab]	18 d	76.1 [ab]	20.9 [ab]	1.4
21 days	76.5 [b]	22.3 [a]	0.9 [ab]	21 d	70.1 [b]	27.0 [a]	0.5
28 days	62.6 [b]	33.5 [a]	1.8 [a]	28 d	61.8 [b]	35.8 [a]	1.4
SEM	2.8	2.6	0.2	SEM	2.8	2.8	0.2
Probability	<.0001	<.0001	0.013	Probability	0.001	0.001	0.126

[a–b] Means with different superscripts in a column differ significantly ($P \leq 0.05$). Values are means of 8 replicates and a pool of 2 birds/replicate. SB: Sodium butyrate; SBEO: Sodium butyrate plus essential oils.

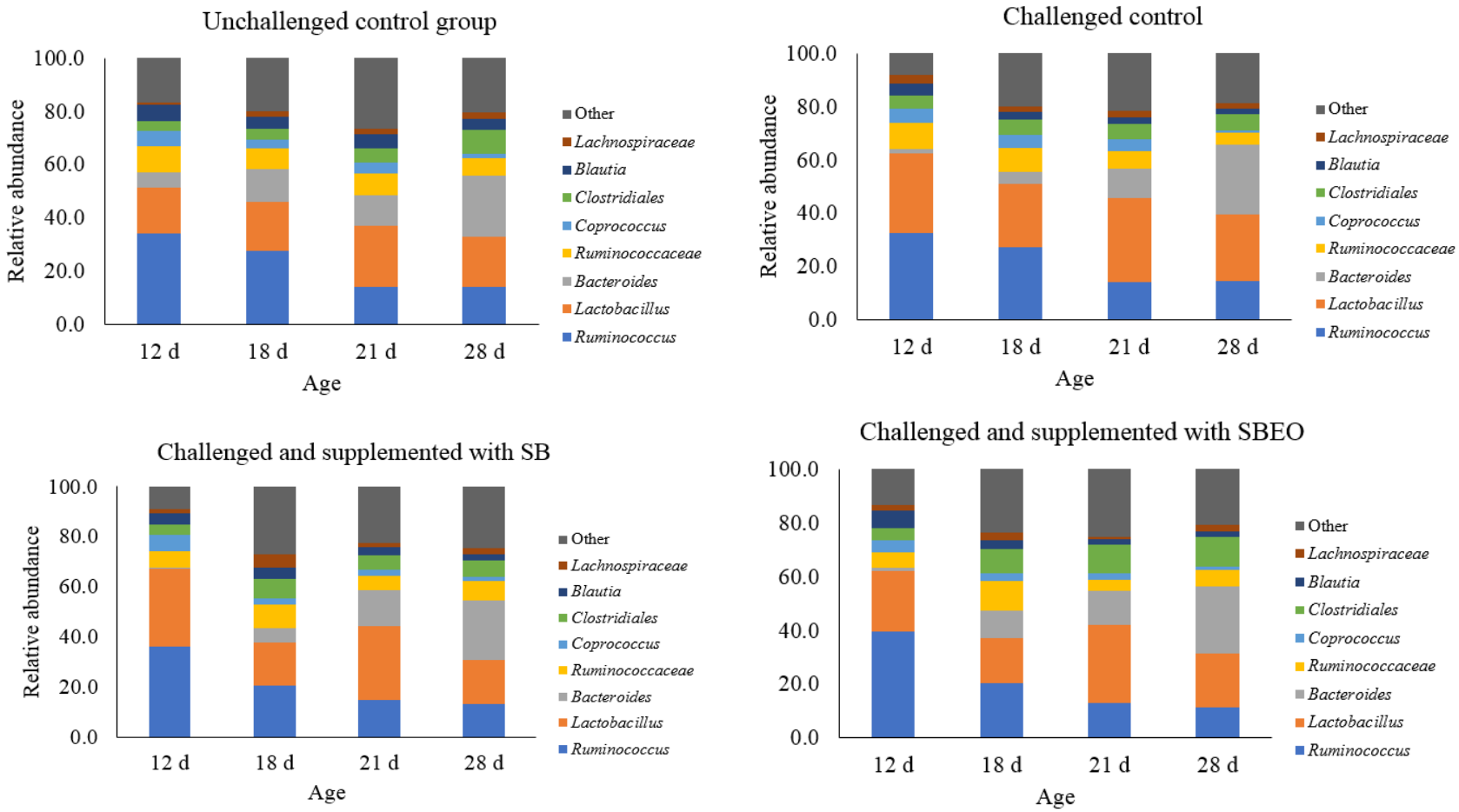

Figure 4.2. Relative abundance (%) of the main bacterial group present in the cecal microbiome of broiler chickens in different timepoints within each experimental treatment (Values are means of 8 replicates and a pool of 2 birds/replicate). SB: Sodium butyrate; SBEO: Sodium butyrate plus essential oils.

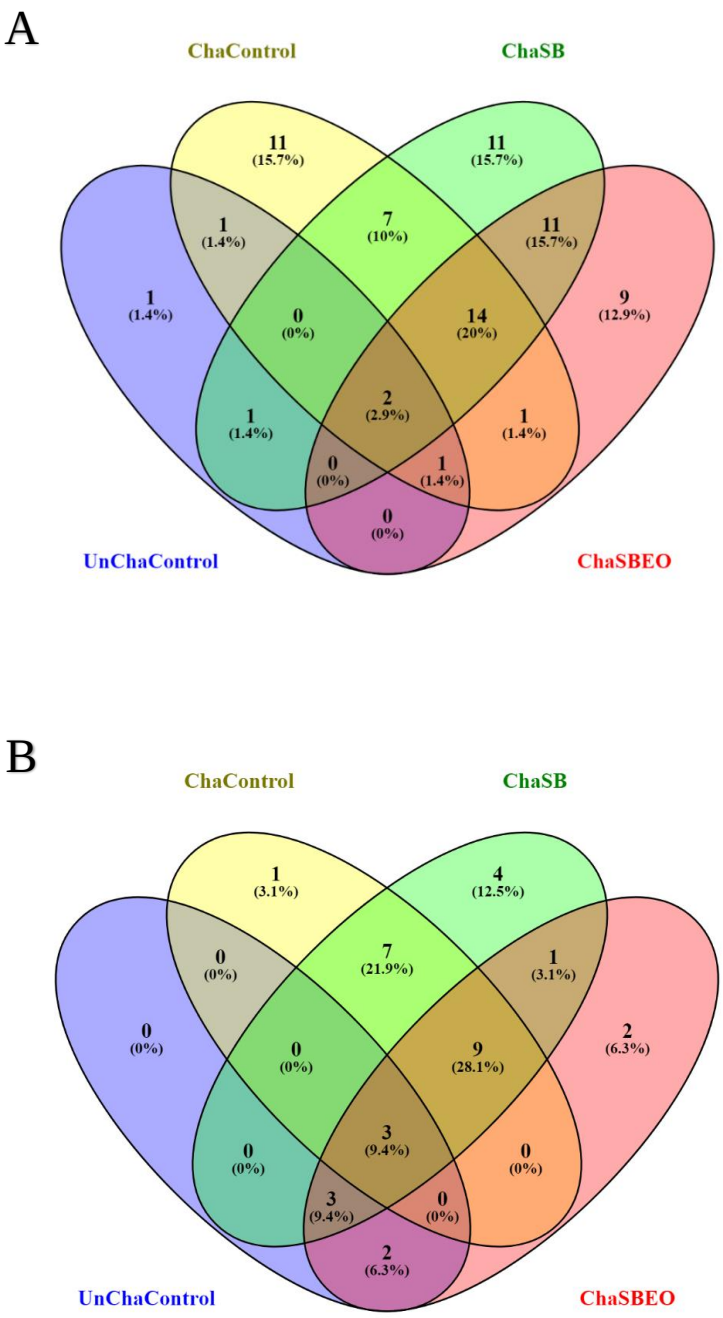

Figure 4.3. Venn diagram of the up (A) and downregulated (B) predicted functions on d 21 compared to d 12 according to each experimental group. Absolute number represent the number of different predicted functions among the groups, followed by its respective percentage. SB: Sodium butyrate; SBEO: Sodium butyrate plus essential oils.

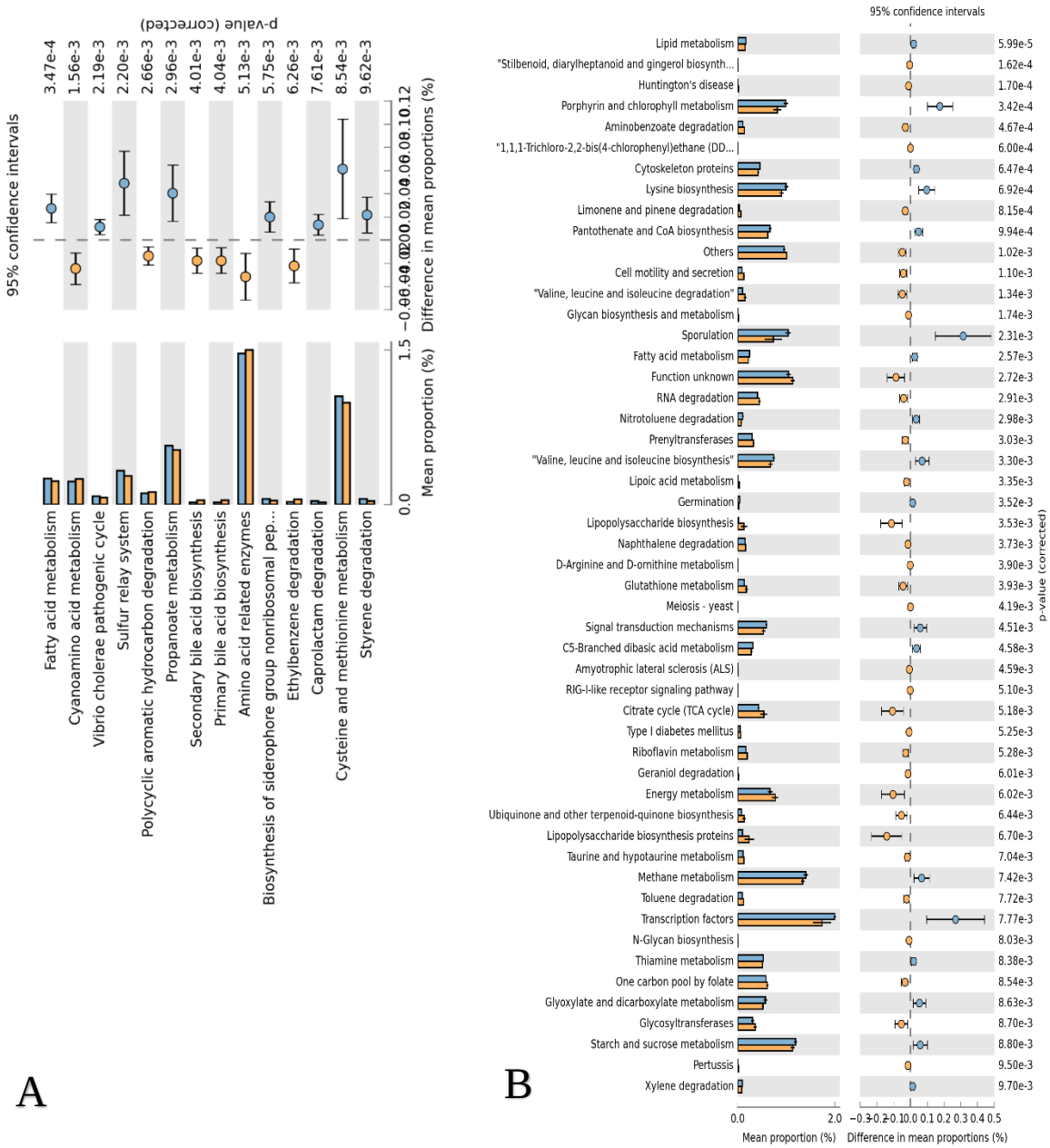

Figure 4.4. Predicted functions of the cecal microbiota unique to the unchallenged control birds (A) and unique to challenged control birds (B) comparing 12 d (blue bars) vs. 21 d (orange bars) with $P \leq 0.01$.

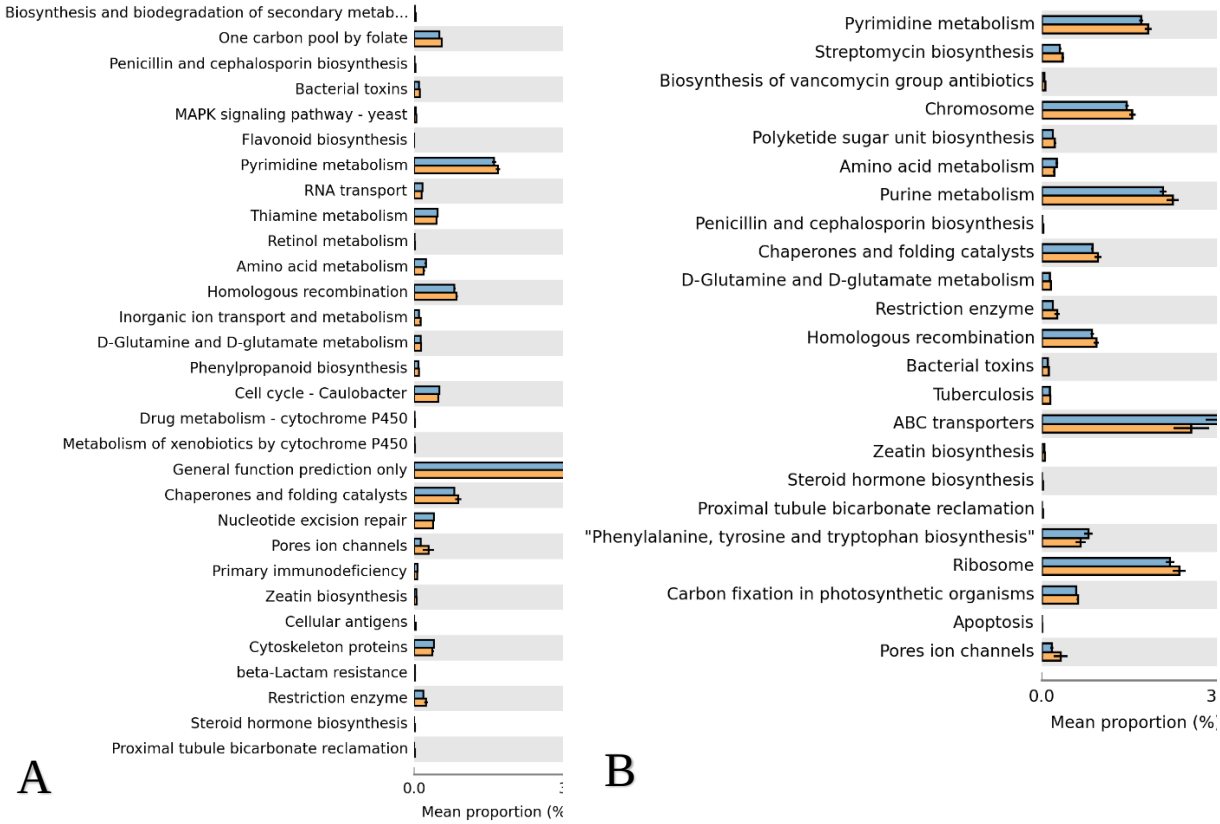

Figure 4.5. Predicted functions of the cecal microbiota unique to challenged and sodium butyrate supplemented birds (A) and unique to challenged and sodium butyrate plus essential oils supplemented birds (B) comparing 12 d (blue bars) vs. 21 d (orange bars) with $P \leq 0.01$.

CHAPTER 5

EXPRESSION OF TIGHT JUNCTIONS AND IMMUNE-RELATED GENES OF BROILER CHICKENS SUPPLEMENTED WITH SODIUM BUTYRATE ALONE OR IN COMBINATION WITH ESSENTIAL OILS UNDER COCCIDIA AND *CLOSTRIDIUM PERFRINGENS* CHALLENGE[1]

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[1]To be submitted to journal of *Avian Diseases*.

ABSTRACT

The objective of this study was to determine the effects of sodium butyrate (SB), and sodium butyrate plus essential oils (carvacrol and ginger; SBEO) on the expression of tight junction (TJ) and immune-related genes in the jejunum and cecal tonsils of coccidia and *Clostridium perfringens* challenged broiler chickens. Birds were assigned to 4 treatments (8 replicates of 58 birds each: basal diet and no challenge; basal diet and challenge; diet supplemented with SB and challenge; diet supplemented with SBEO and challenge). On d 13, the challenged birds were inoculated with ~5,000 oocysts of *Eimeria maxima* by oral gavage. On d 18-19, the same birds were inoculated with *C. perfringens* via drinking water. Jejunal and cecal tonsils samples were collected at d 12, 18, 21, and 28 to analyze the expression of TJ and immune-related genes. On d 12, previous to the challenge, SBEO upregulated the expression of IL-6 ($P = 0.01$) in the jejunum, and IgA ($P = 0.02$), IL-6 ($P = 0.02$), and TLR-2 ($P = 0.02$) in the cecal tonsils, when compared to the unsupplemented groups. Yet, SBEO upregulated the expression of occludin ($P = 0.02$). At d 18, five d after the *E. maxima* challenge, SB supplementation upregulated IL-1β ($P = 0.01$), and the *E. maxima* challenge downregulated the expression of IL-6 ($P = 0.03$) in the jejunum. SB supplementation upregulated claudin-4 expression ($P = 0.02$), tended to upregulate claudin-1 ($P = 0.08$), and partially attenuated the effect of the challenge on the expression of zonula occludens-1 ($P = 0.07$). At d 21, SB decreased IL-10 expression ($P = 0.001$), and SBEO increased the expression of IgA ($P = 0.001$) in the cecal tonsils. On d 28, the only effect was observed on the expression of claudin-1 wherein SB supplementation partially upregulated the its expression on compared to the challenged control group. In conclusion, SBEO modulated the expression of genes prior to the challenge, SB had beneficial effects on

the expression of TJ genes, and SB or SBEO supplementation showed immunomodulatory effects mainly on the jejunum after the *E. maxima* challenge.

Keywords: Broilers, butyrate, coccidiosis, essential oils, gene expression, necrotic enteritis

INTRODUCTION

The activation of the immune system stimulates the proliferation of defense cells, expression of receptors to recognize antigens, and production of cytokines and antibodies. Disease resistance and susceptibility are dependent upon the immune response to pathogens in the earliest stages of infection, in which the goal is to create antimicrobial immunity while limiting inflammation-induced tissue injury (Kogut, 2017). As stated by Kogut (2017) the benefits of modulating the innate response are paramount for the animal to fight infections because it is rapid, non-specific, and relies on different levels of therapeutic potential. Additionally, intestinal epithelial cells are strongly bound to each other by intercellular junctional complexes, known as tight junction (TJ) proteins, which are crucial for maintaining the integrity and function of the epithelial barrier as they also regulate the permeation between cells (Awad et al., 2017). Therefore, nutritional strategies aiming to strengthen the intestinal barrier and modulate of the innate immune-response may be beneficial for the animal to cope with enteric infections.

There has been an increasing number of studies looking for different alternatives to antibiotic growth promoters (AGPs) that could at least partially replace these substances in the diets of broiler chickens which has been driven by worldwide regulations and consumer pressure to reduce the use of AGP. Sodium butyrate (SB) and some essential oils (EO) are

potential candidates for promoting intestinal health mainly due to their effects on the epithelial barrier function and immune-modulating properties (Ahsan et al., 2016; Du et al., 2016; Bortoluzzi et al., 2017; Sikandar et al., 2017) prior or after an injury. As we have demonstrated previously, SB modulates the expression of immune-related genes in the jejunum of disease-free broiler chickens (Bortoluzzi et al., 2017) and inhibits the expression of cytokines in chicken macrophages stimulated by LPS (Zhou et al., 2014). Moreover, it has been reported that SB upregulates the expression of TJ protein encoding genes such as claudin-1 and 4, occludin and zonula occludens-1 after challenge to induce to necrotic enteritis (NE), with additional benefits to the intestinal morphology (Song et al., 2017). On the other hand, the essential oils thymol and carvacrol, have been shown to alleviate the severity of lesions caused by *Clostridium perfringens* challenge, but are not able to recover the expression of TJ protein encoding genes (Du et al., 2016).

Necrotic enteritis is caused primarily by *C. perfringens* infection, even though some predisposing factors are essential for the development of this disease, such as co-infection with parasites most commonly coccidiosis (Prescott et al., 2016). The damage caused by *Eimeria* infection increased the production of mucus by goblet cells (Collier et al., 2008), and causes leakage of plasma proteins into the intestinal lumen (Prescott et al., 2016) which facilitates the proliferation of *C. perfringens*. Additionally, NE infection has been shown to drastically alter the expression of genes, most of them related to the immune-response (Kim et al., 2014), which negatively impact the intestinal barrier function (Du et al., 2016; Song et al., 2017).

Therefore, understanding the mechanisms by which different feed additives act on the animal will help develop nutritional strategies to reduce the losses due to an enteric challenge.

We hypothesized that supplementing SB alone or in combination with EO would beneficially impact the innate immune response and the intestinal barrier of broiler chickens before and after being challenged to induce NE. The objective of this study was to evaluate the effects of SB alone or in combination with EO on the expression of immune-related and TJ proteins encoding genes in the jejunum and cecal tonsils of broiler chickens under coccidia and *C. perfringens* challenge.

MATERIAL METHODS

Housing, birds and treatments

The animal care and use procedures followed the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) under supervision of a licensed poultry veterinarian. One thousand eight hundred fifty-six (1,856) one-day-old Ross chicks were assigned to four (4) treatment groups with eight (8) replicate pens per treatment and 58 birds per pen raised on new litter. The treatments were: non-supplemented non-challenged group (unchallenged control); non-supplemented and challenged control; challenged and supplemented with a SB (70% of sodium butyrate protected with sodium salts of palm fatty acids; ChaSB; 0.1% inclusion); and challenged and supplemented with SB plus essential oils (70% of sodium butyrate protected with sodium salts of palm fatty acids, and carvacrol and ginger oil – 0.5% each in the commercial product; ChaSBEO; 0.1% inclusion). The feed additives were included in the diet to replace an inert substance (kaolin; Table 5.1).

Feed consisted of non-medicated commercial-type broiler starter, grower, and finisher diets compounded which met or exceeded NRC recommendations (NRC, 1994) and contained

feedstuffs commonly used in the United States (Table 5.1). The feeds were available *ad libitum* from date of chick arrival as follows: Starter - d 0 until d 13, grower d 14 to d 34, and finisher d 34 to d 41 (study termination). Diets were fed as crumbles (starter feed) or pellets (grower and finisher feed).

The challenge model consisted of coccidia vaccine (Coccivac®-B52) at d 0, and on d 13 each bird in the challenged groups was inoculated by oral gavage with ~5,000 sporulated oocysts of *Eimeria maxima*, donated by Dr. Lorraine Fuller from the Department of Poultry Science, University of Georgia. On d 18 and d 19, the same birds were challenged with *C. perfringens*, as follow: treatment feed was withdrawn for four (4) hours and water was withdrawn for two (2) to three (3) hours prior to administration of *C. perfringens*. A measured amount of water (~200 mL *C. perfringens*) that was consumed within 30 minutes was used for each pen. A *C. perfringens* culture (~$1x10^8$ cfu/mL) was added to this water and thoroughly mixed and given to birds in each challenge pen. Once the challenge water was consumed, treatment feed and water were returned to pen.

Sample collection and gene expression analysis

A section of the whole tissue (middle jejunum and cecal tonsils) was collected from one bird per experimental unit for gene expression analysis of the tight junction and immune-related genes on d 12, 18, 21, and 28. The preparation of the samples for the qPCR analyses was performed as described by Horn et al. (2014), and Bortoluzzi et al. (2017). Real-time PCR was conducted to determine the expression of tight junction and immune-related genes in the cecal tonsils. This reaction was performed in a 10 µL reaction mixture containing 1 µL of diluted

cDNA, 5 µL of 2x SYBR Green PCR Master Mix (Bio-Rad, Foster City, CA), 0.25 µL of each primer (Table 5.2), and 3.5 µL of PCR-grade water. The PCR procedure consisted of heating the reaction mixture to 95ºC for 10 min followed by 40 cycles of 95ºC for 15 s and 57ºC for 20 s, respectively, and 72ºC for 15 s. GAPDH was used as a reference gene. The mRNA relative abundance was calculated according to Livak and Schmittgen (2001). All samples were analyzed in duplicate.

Statistical analyses

The data were analyzed as a one-way ANOVA using the GLM procedure of the SAS 9.4 (SAS, 2011). The means showing significant ($P \leq 0.05$) treatment differences in the ANOVA were then compared using Tukey's test. All data were tested for normality and homogeneity of variances. The tests used herein were considered as significant when $P \leq 0.05$, and with trends when $0.05 < P \leq 0.10$.

RESULTS

Immune-related genes

The expression of immune-related genes was evaluated in the jejunum (Table 5.3) and cecal tonsils (Table 5.4) at different time points (12, 18, 21, and 28 d of age). In the jejunum, on d 12, the only effect observed was on the expression of IL-6 ($P = 0.01$) wherein the supplementation of SBEO upregulated its expression when compared to the unsupplemented birds. On d 18, after the *Eimeria maxima* challenge, SB supplementation upregulated the expression of IL-1β ($P = 0.01$); the challenged downregulated the expression of IL-6 ($P = 0.03$), but the supplementation of SB partially restored this effect. There was a trend towards increased

expression of A20, IgA, and TLR-2 (P = 0.07, 0.10, and 0.09, respectively) in challenged birds supplemented with SB when compared to challenged and unsupplemented, unchallenged and unsupplemented, and challenged supplemented with SBEO, respectively. On d 21 and 28, no effects of the treatments were observed on the expression of these immune-related genes (Table 5.3).

In the cecal tonsils before the challenge on d 12, the supplementation with SBEO upregulated the expression of IgA, IL-6, NFK-β, and TLR-2 (P = 0.02, 0.02, 0.05, and 0.02, respectively) when compared to control group (Table 5.4). On d 18, there was a trend (P = 0.07) toward increased expression of TLR-2 when challenged birds were supplemented with SBEO. On d 21, supplementation of SBEO upregulated the expression of IgA (P = 0.001) when compared to the unchallenged control birds, or when birds were supplemented with SB, and upregulated IL-10 (P = 0.001) when compared to challenged birds supplemented with SB. On d 28, however, there was no effect of treatments on the genes evaluated.

Tight junction proteins encoding genes

The expression of TJ genes was evaluated in the jejunum on d 12, 18, 21, and 28 (Table 5.3). On d 12, before the challenge, the supplementation of SBEO upregulated (P = 0.02) the expression of occludin. On d 18, after the *Eimeria maxima* challenge, supplementation of SB upregulated the expression of CLDN-4 (P = 0.02) and tended to upregulate the expression of CLDN-1 (P = 0.08) when compared to the challenged and unsupplemented birds; yet, the expression of zonula occludens-1 (ZO-1) tended to be downregulated in challenged birds supplemented with SBEO compared to the unchallenged and unsupplemented birds. No

differences on the expression of TJ genes was observed on d 21. On d 28, however, the induction to NE led to a reduction in the expression of claudin-1, but SB supplementation partially recovered this effect (P = 0.02).

DISCUSSION

In the present study, dietary supplementation of SB and SBEO completely recovered the performance of the birds in terms of feed conversion ratio compared to the challenged and unsupplemented birds; yet, SB increased villus height before challenge (d 12), but not after challenge, and SB and SBEO partially attenuated the severity of NE lesions (Appendices 1 and 2). The positive effects of SB and SBEO on the modulation of the immune-system prior to the challenge, as observed by higher gene expression of IgA may help explain the positive effects of these feed additives on the feed efficiency of the animals after the induction to NE (Bortoluzzi et al., 2017). Therefore, it is reasonable to argue that dietary SB and SBEO function by reducing the susceptibility of the animals to enteric challenges. Supplementation of SBEO upregulated the expression of IgA before and after the induction to NE. IgA is the primary antibody in mucosal secretions that prevents the entry of commensal or pathogenic bacteria into subepithelial areas (Brisbin et al., 2008), and may play an important role in keeping a healthy gut microbiota.

Butyrate has been shown to have a large array of effects on the host (Guilloteau et al., 2010; Bedford and Gong, 2017). Butyrate leads to epigenetic adaptations, changes in the gene function without changing the DNA sequence, due to its histone deacetylase inhibitory effects, which can result in hyperacetylation of histones, and can change the expression of a large

number of genes (Marks et al., 2000). This epigenetic effect caused by butyrate may be responsible for the changes in the expression of genes involved in the inflammatory process, reducing pro-inflammatory cytokines expression and upregulation of anti-inflammatory ones (Meijer et al., 2010; Vinolo et al., 2011; Fung et al., 2012). In the present experiment, SB combined with EO upregulated the expression of IL-6, NFK-b, and TLR-2 before the challenge, which can enhance the immune resistance against infections. In a previous report (Bortoluzzi et al., 2017), SB increased the expression of IL-6, in the absence of challenge. Therefore, one can assume that SB alone or in combination with EO regulate the expression of different immune-related genes to reduce the ability of pathogen to cause diseases, such as coccidiosis and/or NE.

Besides its effects on immune-modulation, butyrate serves as an energy source for enterocytes and colonocytes, stimulates mucus synthesis, promotes intestinal cell proliferation, differentiation and maturation, controls intestinal barrier function, decreases apoptosis of normal cells, and has antimicrobial effects against pathogenic bacteria (Guilloteau et al., 2010). In fact, the immune-modulation promoted by butyrate may be due to its indirect effect on the microbial population of the gut. Moreover, the combination of feed additives with different properties and mechanisms of action may be synergistic and contribute to the search for suitable alternatives to AGP. For instance, essential oils are secondary plant metabolites that may work by increasing the secretion of digestive enzymes, exert antioxidant and anti-inflammatory properties, decrease pathogenic bacteria load, and enhance the immune status (Brenes and Roura, 2010). Indeed, Timbermont et al. (2010) reported that the combination of butyric acid and EO decreased the number of birds with necrotic lesions, but not butyric acid alone. In

another study, a blend of carvacrol and ginger oil, the same used in the current study, was beneficial in attenuating the negative effects of NE in broilers (Jerzsele et al., 2012).

The intestinal barrier function is another aspect to be considered when developing products to maintain gut homeostasis. Coccidiosis and NE are diseases that are routinely found in flocks of broilers and may lead to degradation and/or reorganization of the TJ proteins (Fasano and Nataro, 2004), originating what is referred as a "leaky gut". A leaking intestine is thought to contribute to the severity of clinical signs, being a dominant characteristic of pathogenesis of many enteric pathogens (Awad et al., 2017). Therefore, dietary or immunotherapy interventions may have the ability to keep or restore, at least partially, the ability of the intestinal barrier to perform its function. The present study demonstrates that dietary supplementation of SBEO upregulated the expression of occludin before challenge, but after the coccidia challenge, SB alone restored the expression of claudin-1 and 4 in the jejunum. Wang et al. (2012) showed that butyrate upregulated the expression of claudin 1, and redistributed zonula occludens-1 and occludin in the cellular membrane. On the other hand, a blend of EO containing thymol and carvacrol tended to linearly upregulate the expression of occludin (Du et al., 2016). However, it should be noted that expression of tight junction genes alone does not relay paracellular barrier functionality. Thus, these measures should be in concert with other measures of plausible translocation.

CONCLUSION

The combination of different feed additives will be essential in the post-antibiotic era when looking for dietary interventions to mitigate the impact of enteric diseases and targeting

the strengthen of the intestinal immune-system and barrier. As it was presented herein, SBEO modulated the expression of genes prior to the challenge (d 12), and SB or SBEO supplementation showed immunomodulatory effects mainly in the jejunum after the *E. maxima* challenge (d 18). Sodium butyrate supplementation had beneficial effects on the expression of TJ genes. Future research is needed, however, to determine whether these changes would affect the leaking of plasma proteins into the intestinal lumen and decrease the translocation of undesirable molecules and/or bacteria.

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Table 5.1: Diet formulation and nutrient specifications

Ingredient, %	Starter	Grower	Finisher
Corn, yellow dent	51.8	56.5	60.3
Soybean meal, 47.5% CP	38.8	34.0	30.3
Soybean oil	5.08	5.70	5.82
Monocalcium phosphate	2.00	1.55	1.40
Limestone	0.89	1.07	1.05
Sodium chloride	0.45	0.40	0.40
L-lysine HCl	0.28	0.20	0.12
DL-methionine	0.40	0.33	0.28
L-threonine	0.11	0.08	0.04
Vitamin Premix- broilers[1]	0.05	0.05	0.05
Mineral Premix- broilers[2]	0.07	0.07	0.07
Kaolin	0.10	0.10	0.10
Formulated nutrient content			
ME, Kcal/Kg	3,050	3,150	3,200
CP, %	22.0	20.0	18.5
Lysine, %	1.43	1.24	1.09
Thr, %	0.94	0.83	0.74
Met+Cys, %	1.07	0.95	0.86
Available P, %	0.47	0.38	0.35
Ca, %	0.95	0.90	0.85
Na, %	0.20	0.18	0.18

[1]Supplied the following per kilogram of diet: vitamin A, 11,020 IU; vitamin D_3, 2,200 IU; vitamin E, 22 IU; thiamine, 441 µg; riboflavin, 882 µg; pantothenic acid, 2,205 µg; niacin, 8,818 µg; pyridoxine, 441 µg; folic acid, 110 µg; biotin, 22 µg; vitamin B_{12}, 2.5 µg; choline, 38,272 µg.

[2]Supplied the following per kilogram of diet: Ca, 15.7 mg; Mn, 65.7 mg; Zn, 52.4 mg; Mg, 13.1 mg; Fe, 12.9 mg; Cu, 4.9 mg; I, 4.9 mg; Se, 0.196 mg.

Table 5.2. Primer sequences for RT-PCR

Objective	RNA Target	Primer sequence (5′–3′)	
RT - PCR	*IL-6*	F: ATAAATCCCGATGAAGTGG R: GCAGGTGAAGAAGCGGTGA	Gao et al., (2012)
	IL-10	F: CAATCCAGGGACGATGAAC R:TCCAAGCACACCTCTCTTCCATCC	Gao et al., (2012)
	INF- γ	F: TGAGCCAGATTGTTTCGA R: ACGCCATCAGGAAGGTTG	Gao et al., (2012)
	IL-1β	F: AGAAGAAGCCTCGCCTGGAT R: CCGCAGCAGTTTGGTCAT	Gao et al., (2012)
	NF-κB p65	F:GTGTGAAGAAACGGGAACTG R:GGCACGGTTGTCATAGATGG	Li et al., (2015)
	A20	F:GACATCGTGCTAACAGCTTGGA R:AGAAAAGAGGGTATCAGGCACAAC	Li et al., (2015)
	IL-8	F: GCGGCCCCCACTGCAAGAAT R:TCACAGTGGTGCATCAGAATTGAGC	Tan et al., (2014)
	iNOS	F: CCTGTACTGAAGGTGGCTATTGG R: AGGCCTGTGAGAGTGTGCAA	Tan et al., (2014)
	MUC2	F: CAGCACCAACTTCTCAGTTC R: TCTGCAGCCACACATTCTTT	Tan et al., (2014)
	TLR2	F: AGGCACTTGAGATGGAGCAC R: CCTGTTATGGGCCAGGTTTA	Wu et al., 2014
	IgA	F: ACCACGGCTCTGACTGTACC R: CGATGGTCTCCTTCACATCA	Tan et al., (2014)
	Claudin-1	F: CATACTCCTGGGTCTGGTTGGT R: GACAGCCATCCGCATCTTCT	Du et al., (2016)
	Claudin-2	F: CCTGCTCACCCTCATTGGAG R: GCTGAACTCACTCTTGGGCT	Chen et al., (2016)
	Claudin-4	F: GAAGCGCTGAACCGATACCA R: TGCTTCTGTGCCTCAGTTTCC	Shao et al., (2013)
	Occludin	F: CCGTAACCCCGAGTTGGAT R: ATTGAGGCGGTCGTTGATG	Chen et al., (2016)
	Zonula occludens-1	F: TGTAGCCACAGCAAGAGGTG R: CTGGAATGGCTCCTTGTGGT	Chen et al., (2016)
	GAPDH	F: ACTGTCAAGGCTGAGAACGG R: CATTTGATGTTGCTGGGGTC	Gao et al., (2012)

Table 5.3: Expression of tight junction and immune-related genes in the jejunum of broiler chickens at different time points, supplemented with SB and SBEO and challenged with *Eimeria maxima* at d 14 and *Clostridium perfringens* at 18 and 19 d.

	CLDN1	CLDN2	CLDN4	ZO1	OCCLU	A20	IgA	IL-1b	IL-6	IL-10	MUC2	TLR-2
Treatment						12 days						
Control	1.10	1.06	1.05	1.06	1.04^{b}	1.10	3.30	0.98	0.94b	1.13	1.07	1.19
SB	0.94	1.02	1.35	1.07	0.98^{b}	0.96	2.76	1.00	1.10ab	1.10	0.99	1.18
SBEO	1.12	1.06	1.35	1.12	1.27^{a}	1.22	4.62	0.96	1.34a	0.90	1.09	1.30
SEM	0.08	0.08	0.15	0.06	0.08	0.10	0.84	0.10	0.11	0.18	0.06	0.16
Probability	0.21	0.94	0.16	0.77	0.02	0.21	0.36	0.95	0.01	0.59	0.49	0.85
Treatment						18 days						
NC	0.89	1.02	0.91^{ab}	1.01	1.01	1.01	1.64^{b}	0.86^{b}	1.04^{a}	1.01	1.01	1.07
CC	0.82	0.79	0.62^{b}	0.84	0.90	0.84	3.30^{ab}	0.94^{b}	0.76^{b}	0.97	0.93	0.93
CC+SB	1.18	0.85	1.14^{a}	0.90	0.92	1.15	5.79^{a}	1.58^{a}	0.87^{ab}	1.53	1.03	1.29
CC+SBEO	0.87	0.80	0.72^{b}	0.77	0.83	0.84	3.28^{ab}	0.98^{b}	0.71^{b}	0.97	0.94	0.84
SEM	0.11	0.10	0.13	0.07	0.07	0.10	0.99	0.18	0.09	0.23	0.06	0.13
Probability	0.08	0.35	0.02	0.07	0.39	0.07	0.10	0.01	0.03	0.31	0.65	0.09
Treatment						21 days						
NC	0.94	0.90	1.12	1.04	1.03	1.01	1.58	1.06	0.93	0.98	1.08	0.92
CC	0.83	0.73	1.05	0.91	0.88	0.86	5.55	1.07	0.86	1.09	0.82	0.79
CC+SB	0.71	0.91	0.79	0.95	1.09	0.92	4.85	0.74	0.72	0.65	0.98	0.73
CC+SBEO	0.66	0.84	0.99	0.93	1.01	0.80	10.74	1.02	0.65	1.28	0.88	0.90
SEM	0.11	0.11	0.16	0.09	0.10	0.07	2.45	0.14	0.15	0.27	0.13	0.12
Probability	0.33	0.69	0.67	0.78	0.49	0.19	0.19	0.34	0.60	0.51	0.54	0.81
Treatment						28 days						
NC	1.04^{a}	1.04	0.93	1.01	1.01	1.06	1.89	0.94	1.01	0.93	1.01	1.08
CC	0.73^{b}	0.95	0.80	1.06	0.99	1.08	1.60	0.92	0.83	1.21	0.94	1.05
CC+SB	0.78^{ab}	0.83	0.77	0.97	0.95	0.99	1.82	1.00	0.88	0.99	0.88	1.35
CC+SBEO	0.70^{b}	0.93	0.81	0.97	0.89	1.03	1.54	0.99	0.87	0.99	0.90	1.11
SEM	0.08	0.09	0.08	0.06	0.05	0.09	0.29	0.08	0.07	0.20	0.06	0.15
Probability	0.02	0.53	0.71	0.74	0.46	0.94	0.90	0.92	0.42	0.85	0.48	0.54

$^{a–b}$ Means with no common superscripts in a column are significantly different ($P \leq 0.05$). Values are means ± SEM (1 bird/pen; 8 birds/treatment). NC: non-challenged control; CC: challenged control; SB: sodium butyrate; SBEO: sodium butyrate plus essential oils.; SEM: standard error of mean.

Table 5.4: Expression of immune-related genes in the cecal tonsils of broiler chickens at different time points, supplemented with SB and SBEO and challenged with *Eimeria maxima* at d 14 and *Clostridium perfringens* at 18 and 19 d.

	A20	IgA	IL-1b	IL-6	IL-8	IL-10	INF-y	iNOS	LITAF	NFK-β	TLR-2
Treatment						12 days					
Control	1.17	1.69^{b}	1.12	0.98^{b}	0.92	1.16	1.13	0.92	1.12	1.06^{b}	1.15^{ab}
SB	1.22	1.50^{b}	1.13	1.12^{ab}	1.25	1.19	1.21	0.77	1.20	1.11^{ab}	0.96^{b}
SBEO	1.29	3.15^{a}	1.27	1.28^{a}	0.96	1.16	1.31	0.94	1.02	1.18^{a}	1.37^{a}
SEM	0.10	0.49	0.10	0.09	0.17	0.16	0.15	0.12	0.08	0.04	0.11
Probability	0.59	0.02	0.46	0.02	0.30	0.99	0.63	0.51	0.34	0.05	0.02
						18 days					
NC	1.02	0.92	1.06	1.03	1.12	1.23	1.07	1.06	1.02	1.02	1.02
CC	1.07	1.87	1.28	1.14	1.06	1.40	1.27	0.91	0.91	1.17	1.32
CC+SB	1.15	1.88	1.10	1.12	0.98	1.53	1.38	1.06	1.04	1.11	1.14
CC+SBEO	1.07	2.26	1.18	1.30	0.70	1.59	1.47	1.23	0.81	1.22	1.51
SEM	0.08	0.39	0.14	0.12	0.15	0.27	0.13	0.14	0.08	0.07	0.14
Probability	0.75	0.12	0.75	0.44	0.25	0.82	0.14	0.48	0.14	0.21	0.07
						21 days					
NC	1.04	1.42^{c}	1.03	1.04	0.84	0.93^{a}	1.03	1.08	0.95	1.01	0.93
CC	0.84	3.53^{ab}	1.15	1.21	1.23	0.72^{a}	0.99	0.92	0.92	0.96	1.07
CC+SB	0.88	2.00^{bc}	1.04	1.06	0.99	0.50^{b}	0.87	0.76	1.02	1.09	1.04
CC+SBEO	0.91	4.14^{a}	1.40	1.16	1.00	0.76^{a}	0.92	0.98	0.97	1.07	1.06
SEM	0.08	0.58	0.15	0.12	0.14	0.08	0.10	0.12	0.05	0.05	0.10
Probability	0.33	0.001	0.28	0.73	0.33	0.001	0.71	0.29	0.62	0.31	0.75
						28 days					
NC	1.03	1.33	1.04	1.04	1.12	1.15	1.03^{a}	1.03	1.03	1.01	1.09
CC	1.06	1.09	0.83	0.83	0.68	0.82	0.75^{ab}	0.67	0.99	0.98	0.97
CC+SB	0.96	1.26	1.04	1.04	0.85	0.89	0.66^{b}	0.78	0.87	0.99	1.01
CC+SBEO	1.07	1.08	0.88	0.88	0.82	0.97	0.82^{ab}	0.76	0.95	1.04	1.07
SEM	0.11	0.25	0.10	0.10	0.15	0.14	0.11	0.11	0.09	0.05	0.17
Probability	0.92	0.90	0.33	0.33	0.23	0.41	0.10	0.14	0.62	0.86	0.97

[a–b] Means with no common superscripts in a column are significantly different ($P \leq 0.05$). Values are means ± SEM (1 bird/pen; 8 birds/treatment). NC: non-challenged control; CC: challenged control; SB: sodium butyrate; SBEO: sodium butyrate plus essential oils.; SEM: standard error of mean.

CHAPTER 6

ZINC SOURCE MODULATES INTESTINAL INFLAMMATION AND INTESTINAL INTEGRITY OF BROILER CHICKENS CHALLENGED WITH COCCIDIA AND *CLOSTRIDIUM PERFRINGENS*[1]

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[1]Submitted to *Poultry Science*.

ABSTRACT

Two dietary sources of zinc ($ZnSO_4$ or organic Zn) were tested in chickens challenged with coccidiosis (Co) or coccidiosis plus *Clostridium perfringens* (CoCPF). On d 14, the chickens were orally gavaged with ~5,000 *Eimeria maxima* sporulated oocysts. On d 19, 20 and 21 chickens challenged with *C. perfringens* were given a broth culture containing 10^8 cfu of this bacterium. Productive performance parameters were determined at d 14, 21, and 28. On d 21, necrotic enteritis (NE) lesions were scored, and intestinal permeability was evaluated. Jejunum and cecal tonsils were collected for morphology and gene expression analysis. On d 21, organic Zn improved BW gain by 18.6% ($P = 0.07$), and FCR by 12% ($P = 0.09$) in CoCPF challenged chickens vs. birds fed $ZnSO_4$. From 1 to 28, organic Zn increased BW gain ($P = 0.02$), and improved FCR ($P = 0.03$) vs. birds fed $ZnSO_4$. At 21 d, NE lesions were only observed in CoCPF birds ($P < 0.001$), and mortality due to NE was only observed when CoCPF birds were fed $ZnSO_4$ ($P = 0.001$). Organic Zn fed birds had increased villus height in the jejunum ($P = 0.005$) and decreased intestinal permeability ($P=0.01$) vs. $ZnSO_4$. In the jejunum, organic Zn fed birds showed a downregulation of expression of IL-8 ($P = 0.02$), and upregulation of IL-10 ($P = 0.05$) in CoCPF birds vs. $ZnSO_4$- CoCPF birds. As main effect, birds supplemented with organic Zn had higher mRNA expression of TLR-2 ($P = 0.02$) and IgA ($P = 0.01$). In the cecal tonsils, organic Zn fed birds showed upregulation of iNOS ($P = 0.008$) in CoCPF birds vs. $ZnSO_4$- CoCPF birds. Organic Zn supplementation reduced intestinal permeability and attenuated intestinal inflammation of broilers co-challenged with coccidia and *C. perfringens*.

Keywords: Broiler, coccidiosis, necrotic enteritis, zinc supplementation

INTRODUCTION

Necrotic enteritis (NE), caused by *Clostridium perfringens*, has its pathogenesis commonly divided into phases starting with colonization followed by multiplication, acquisition of nutrients, and evasion of immune-response. These often lead to intestinal damage, and finally transmission (Prescott et al., 2016). Although a division exists for didactical purposes, these processes typically occur simultaneously (Prescott et al., 2016). It is known that coccidiosis, caused by *Eimeria* spp. (Blake and Tomley, 2014), is a well-characterized predisposing factor for the development of NE. This is because coccidiosis causes leakage of plasma proteins into the intestinal lumen, and stimulates mucogenesis, which favors the proliferation of *C. perfringens* (Collier et al., 2008; Prescott et al., 2016). Consequently, there is intestinal inflammation (Collier et al., 2008), disruption of the intestinal epithelia through necrosis, and changes in the structure and function of the tight junction (TJ) proteins (Awad et al., 2017).

Zinc (Zn) is an essential micromineral required for growth, and influences intestinal development and/or regeneration during and after enteric diseases (MacDonald, 2000). Studies have shown the impact of Zn on growth performance and antioxidant system (Mwangi et al., 2017); immune defense and inflammation (Kidd, 1996; Prasad et al., 2011; Li et al., 2015); intestinal microbial community (Zhang et al., 2012; Shao et al., 2014; Starke et al., 2014), and intestinal permeability (Zhang and Guo, 2009). It has been demonstrated that dietary Zn concentrations higher than the 40 mg/kg recommended by NRC (1994) lowers the impact of coccidiosis in broilers (Bafundo et al., 1984; Troche, 2012). Inorganic sources of Zn have been used for many years but are known to be less available for absorption than organic sources

(Mwangi et al., 2017; Yu et al., 2017). Thus, it is reasonable to argue that when the absorptive capacity of the intestine is impaired, a more available source of Zn may be needed.

The effects of Zn on intestinal immunology and permeability have been observed previously (Prasad et al., 2011; Troche, 2012; Li et al., 2015). Organic Zn induced higher expression of A20, an anti-inflammatory regulator, downregulated the expression of inflammatory inducers, including NF-kB p65 (Prasad et al., 2011; Li et al., 2015), and promoted MUC2 and IgA production, when compared to its inorganic counterpart (Prasad et al., 2011). The higher expression of A20 promoted by organic Zn is most likely due to an epigenetic effect by lowering DNA methylation (Li et al., 2015). On the other hand, inorganic Zn enhanced intestinal permeability and upregulated the expression of occludin and zonula occludens- 1 in the ileum of weanling piglets (Zhang and Guo, 2009).

We hypothesized that organic Zn would improve the performance of broiler chickens challenged with coccidia (Co) or coccidia plus *Clostridium perfringens* (CoCPF) by enhancing intestinal integrity (jejunum morphology, intestinal permeability, and expression of TJ encoding genes) and by modulating intestinal inflammation (expression of innate immune-related genes). The purpose of this study was to build on prior work by determining the effects of Zn source on the performance and intestinal physiology and immunology of broiler chickens challenged with Co or CoCPF.

MATERIAL AND METHODS

Housing, birds and treatments

The animal care and use procedures followed the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) under supervision of a licensed poultry veterinarian. One-day-old male Cobb 500 chickens (256) were used in the experiment. Chicks were weighed individually and by pen such that body weight (BW) did not differ between pens. Birds were placed into 32 cages (8 birds/cage and 8 cages/treatment), using a completely randomized design, in an environmentally controlled room. Chickens had a*d libitum* access to water and feed in mash form during the entire experimental period. An unmedicated basal starter diet was formulated with corn and soybean meal with a Zn free premix to meet or exceed NRC broiler starter requirements (NRC, 1994; except for Zn; Table 6.1). Experimental diets were remixed from this basal diet, and the Zn source was added to obtain the experimental diets.

The experiment was arranged in a 2 x 2 factorial design (4 treatments) with Zn source and challenge as factors. Zinc source included 90 mg/kg of supplemental zinc from $ZnSO_4$ or organic Zn (a partially hydrolyzed soy protein, Bioplex® Zn 10%, Alltech Inc.; Mwangi et al., 2017) fed from d 1 to 28. Birds were challenged with coccidiosis (Co) or coccidiosis plus *C. perfringens* (CoCPF). Zinc concentration in the feed was determined by Inductive Coupled Plasma (ICP) at University of Arkansas – Central Analytical Lab (Fayetteville, AR, Table 6.1). Briefly, 250 mg of feed was diluted into 10 mL of HNO_3, heated to 100^{o}C for 5 h, followed by the addition of 20 mL of nanopure water. The samples were then analyzed by Inductive Coupled Plasma (ICP). Crude fiber, crude protein, and crude fat (methods 962.09, 990.03, and 920.39 respectively; AOAC International, 2006) were determined by Dairy One (Ithaca, NY). As authors, we recognize that there was an analytical difference in the Zn concentration

between diets; therefore, the differences in results between Zn sources may not be completely attributable to the source.

On d 14, all birds were given ~5,000 oocysts of *E. maxima* via oral gavage. Starting on d 19, two groups of birds fed $ZnSO_4$ or organic Zn were given a fresh broth culture of *C. perfringens* with ~10^8 CFU/mL once daily for 3 d (on d 19, 20, and 21). On d 21, three birds from each cage were randomly selected, euthanized, weighed, and examined for the presence and degree of necrotic enteritis lesions. Lesion scoring was based on a 0 to 3 score (Hofacre et al., 1998), as follow: 0: normal; 1: slight mucus covering the small intestine; 2: necrotic small intestine mucosa; and 3: sloughed and blood small intestinal mucosa and contents. The sample collection was performed at 21 d of age.

Sample collection and analyses performed

Birds and feed were weighed weekly by cage and mortality was recorded daily. Average feed intake (FI) and body weight gain (BW gain) were corrected for mortality when calculating feed:gain ratio (FCR) for each cage. On d 21, one bird per cage (8 birds/treatment) was randomly selected, euthanized by cervical dislocation, and the jejunum and cecal tonsils were collected, placed in liquid nitrogen, and frozen at -80^{o}C for subsequent gene expression analysis. Additionally, a section of the jejunum was collected for morphology and microscopic lesion score analysis.

Jejunum morphology, microscopic lesion score, and intestinal permeability

A section of the jejunum was collected at the midpoint between the bile duct papilla and Meckel's diverticulum. Tissue was fixed in 10% neutral-buffered formalin, routinely processed and embedded in Tissue Path (Fisher Scientific). Two sections were cut per sample and stained with hematoxylin/eosin to determine villus height, crypt depth, and villus:crypt ratio. Measurements of villi and crypts were performed in 10 intact villi, and 10 crypts per bird. Intestinal sections were also evaluated by histopathology and scored based on the degree of intestinal necrosis and number of coccidia (*E. maxima*) as described: necrotic enteritis score: 0: no necrotic lesions (absence of enterocyte degeneration/necrosis, intestinal epithelium attached to lamina propria); 1: mild single cell enterocyte degeneration/necrosis detachment from lamina propria; 2: mild necrosis at villi tips; 3: moderate necrosis affecting half of the villi; 4: severe necrosis affecting entire villi and/or submucosa. Coccidia scores were: 1: 0-2 coccidia/section; 2: 3-30 coccidia/section; 3: 31-100 coccidia/section; 4: >100 coccidia/section. The necrotic enteritis microlesion score used herein has not been described previously and was developed by Dr. Monique França (Poultry Diagnostic and Research Center of the University of Georgia). The coccidia score, however, was developed by Dr. Frederic Hoerr and Dr. Joan Schrader (Veterinary Diagnostic Pathology, LLC).

To evaluate gut permeability, oral administration of fluorescein isothiocyanate dextran (FITC-d, 100mg, MW 4,000; Sigma-Aldrich, Canada) and its passage into the blood was used (Zhang et al., 2016). Briefly, at 21 d FITC-d was diluted in distilled water and given by oral gavage to 1 bird/cage (2.2 mg/bird). Two hours after inoculation, the birds were euthanized. Blood collected from the heart was used to determine the FITC-d concentration per mL of serum using a microplate reader at a wavelength of 485 nm and emission wavelength of 528

nm. The FITC-d concentration per mL of serum was calculated based on a standard curve. The higher the gut permeability, the higher the blood level of FITC-d.

C. perfringens *enumeration by qPCR*

Quantification of *C. perfringens* in the cecal content was performed by real time PCR (Wise and Siragusa, 2005). First, the DNA isolation was conducted following manufacturer recommendations (PowerViral Environmental RNA/DNA Isolation Kit – Mo Bio; QIAGEN, Carlsbad, CA, USA), and a slight modification established by our lab (Bortoluzzi et al., 2017). The presence and quality of DNA was verified by agarose gel electrophoresis (1.5%). Quantitative real time PCR was performed using a pair of primers (Wise and Siragusa, 2005; CPerf165F [5-CGCATAACGTTGAAAGATGG-3] and CPerf269R [5-CCTTGGTAGGCCGTTACCC- 3]. The DNA isolated from the cecal digesta was normalized to 100 ng of DNA in each sample, and then 8 µl of sample was used in PCR amplification in a total volume of 20 µl. A pure *C. perfringens* DNA (the same which was used for challenging the birds) with known concentration was used to construct a standard curve. The cycling conditions were as follows: an initial 10 min step at 94°C, followed by 45 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 20 s, and extension at 70°C for 10 s. Fluorescence was acquired following the annealing step. The corresponding number of cells was calculated by taking the genome size of *C. perfringens* into consideration (Dumonceaux et al., 2006). Bacterial numbers were expressed as log10 genomic DNA copy number per gram of digesta (wet weight).

Intestinal gene expression - Real-Time PCR (qPCR) analysis

The mRNA production of several immune-related genes was evaluated, including cytokines, TLR-2, IgA, MUC2, TJ proteins, Zn binding proteins, and matrix metalloproteinases 7 and 9 (MMP7 and MMP9). Preparation of the samples for qPCR analysis was performed as described by Horn et al. (2014) and the real-time PCR amplification was performed according to Bortoluzzi et al. (2017). Synthesized cDNA was diluted (2X) with sterile water and stored at -20^{o}C before use. Primers used are described in Table 6.2. The PCR procedure consisted of heating the reaction mixture to 95^{o}C for 10 min followed by 40 cycles of 95^{o}C for 15 s, the corresponding annealing temperature for 20 s, and 72^{o}C for 15 s. The relative standard curve method was used to quantify mRNA concentrations of each gene in relation to the reference gene (GAPDH) which did not show variations according to the treatments. The mRNA relative abundance was calculated according to the method of Livak and Schmittgen (Livak and Schmittgen, 2001), using the treatment challenged only with Co and supplemented with $ZnSO_4$ as control. All samples were analyzed in duplicate.

Statistical analysis

The data were analyzed as a 2-way ANOVA using the GLM procedures of SAS (SAS, 2011). Factors were Zn source and challenge method. Cage was the experimental unit. Means were considered significantly different at $P \leq 0.05$ with trends with P between 0.05 and 0.10. Treatment differences were then compared using the least square mean procedure of SAS. All data were tested for normality and homogeneity of variance, using the UNIVARIATE procedure and Bartlett test of SAS (9.4), respectively.

RESULTS

Performance and mortality

The objective of this study was to evaluate the mechanism of action of different sources of Zn in chickens under coccidia plus *C. perfringens* challenge which is why the birds were raised in battery cage for up to d 28. The next step will be to conduct a similar study on floor pens up to d 42 to evaluate the growth performance. Growth performance was calculated from 1 to 14 d (before the challenge), i.e., only the effect of Zn source was evaluated (Table 6.3). During this time, $ZnSO_4$ supplemented birds had better FCR than organic Zn supplemented birds ($P = 0.01$); BW gain, however, tended to be higher in birds fed organic Zn ($P = 0.08$). From 1 to 21 d (data not shown), there was an interaction between Zn source and method of challenge for BW gain and FCR. Organic Zn showed a trend towards improved BW gain by 18.6% ($P = 0.07$), and FCR by 12% ($P = 0.09$) in CoCPF challenged chickens when compared to the same group of birds supplemented with $ZnSO_4$. From 1 to 28 d (data not shown), no interaction was observed for any of the variables ($P > 0.05$). However, regardless of the challenge method, organic Zn fed birds had increased BW gain ($P = 0.02$), and improved FCR ($P = 0.03$) on d 28. The addition of CPF to Co impaired FCR by 8% ($P = 0.04$). An interaction was observed for mortality, wherein CoCPF challenge caused mortality only in birds fed $ZnSO_4$ ($P = 0.001$; Table 6.4). No interaction was observed for NE gross lesions ($P > 0.05$), however, gross lesions, characteristic of NE, were only detected when birds were submitted to the CoCPF challenge ($P < 0.001$; Table 6.4).

Growth performance of birds during the experimental challenge (14 to 21 d) and from d 14 to end of the trial (14 to 28 d) is presented in Table 6.4. From 14 to 21 d of age, an

interaction was observed for BW gain and FCR wherein the CoCPF challenged birds supplemented with organic Zn tended to have higher BW gain (P = 0.09), and better FCR (P = 0.04) when compared to the CoCPF challenged birds fed $ZnSO_4$. Taking the recovery phase into consideration, 14 to 28 d, an interaction between Zn source and method of challenge was observed for FCR; there was a trend toward significance (P = 0.09), wherein CoCPF challenged birds fed organic Zn had better FCR than the same group of birds fed $ZnSO_4$. As a main effect, organic Zn increased BW gain of the birds by 17% when compared to $ZnSO_4$ supplemented birds (P = 0.02).

Jejunum morphology and histopathology, intestinal permeability, and* C. perfringens *enumeration

No interaction between Zn source and method of challenge was observed for these variables. However, organic Zn by itself increased villus height by 16% (Table 6.5; P = 0.005). No effect was observed for crypt depth, and villus:crypt ratio (P > 0.05). No characteristic microscopic lesions of necrotic enteritis were observed in the jejunum (data not shown); however, coccidiosis lesions were observed, with no differences among the different experimental groups (Table 6.5).

There was no interaction between Zn source and method of challenge for gut permeability. Organic Zn, as main effect, decreased intestinal permeability (P = 0.01; Table 6.5), as shown by the lower FITC-d concentration in the serum. The inoculation of *C. perfringens* on top of *E. maxima* challenge did not alter the translocation of FITC-d (4 kd) from

intestinal lumen to blood. There was no effect of Zn source or method of challenge ($P > 0.05$) on the *C. perfringens* enumeration in the caeca content.

Gene expression

Gene expression data of the jejunum and cecal tonsils are presented in Tables 6.6 and 6.7, respectively. In the jejunum, there was an interaction between Zn source and CoCPF challenge for IL-8 ($P = 0.02$), IL-10 ($P = 0.05$), INF-γ ($P = 0.07$), and LITAF ($P = 0.06$). Expression of IL-8 was downregulated while IL-10, INF-γ, and LITAF were upregulated in CoCPF challenged birds fed organic Zn vs. those fed $ZnSO_4$. CoCPF challenge downregulated the expression of iNOS ($P = 0.07$). There was an effect of Zn source on TLR-2 expression, wherein organic Zn upregulated its expression. Regarding the gut mucosal immunity, organic Zn upregulated the expression of IgA ($P = 0.01$). CoCPF challenge also upregulated the expression of this gene ($P = 0.03$) vs. birds challenged only with Co. Organic Zn upregulated ($P = 0.07$) the expression of zonula occludens-1 in CoCPF challenged birds. Ultimately, the CoCPF challenge downregulated the expression of MMP9 ($P = 0.03$), regardless of the Zn source.

In the cecal tonsils, an interaction was observed for the expression of iNOS ($P = 0.008$) and MMP9 ($P = 0.08$) wherein supplementation with organic Zn upregulated mRNA of iNOS and tended to upregulate MMP9 when birds were challenged with CoCPF vs. birds supplemented with $ZnSO_4$. Organic Zn upregulated the mRNA expression of NFK-β ($P = 0.047$) and tended to upregulate A20 ($P = 0.08$) and TLR-2 ($P = 0.10$).

DISCUSSION

Zinc is an essential trace mineral possessing unique chemical properties, which allows it to serve structural and catalytic roles for proteins and enzymatic reactions, acting mainly as an important cofactor (Troche et al., 2015). Zinc is a cofactor in antioxidant enzymes as well as a mediator of T cell development, and modulator of cytokine production (Prasad et al., 2011). Higher concentrations of Zn are rationalized based on literature wherein Zn metabolism changes with coccidial and bacterial challenges. Specifically, plasma Zn greatly decreases (Turk and Stephens, 1966, 1967; Southern and Baker, 1983; Turk, 1986; Richards and Augustine, 1988) while hepatic Zn greatly increases as it is bound through up-regulation of metallothionein (MT) during an acute phase response to these challenges (Richards and Augustine, 1988). On the other hand, Zn source may also influence the cellular trafficking of Zn (Troche et al., 2015); in a study presented from our lab, dietary organic Zn supplementation partially prevented the decrease in the serum Zn concentration due to the challenge (He et al., 2017), which help explain the better performance observed in organic Zn supplemented birds after the challenge.

Epigenetic mechanisms alter gene expression without changes in DNA sequence and can explain the effects of Zn on the cell. Li et al. (2015) observed that Zn attenuated intestinal inflammation by epigenetic mechanisms by modulating the expression of cytokines involved in the inflammatory process. In the present study, organic Zn upregulated the expression of IL-10 and downregulated the expression of IL-8 in CoCPF infected birds, which suggests that organic Zn was beneficial in attenuating jejunal inflammation in CoCPF infected broilers vs. birds fed $ZnSO_4$. This lower level of inflammatory signaling helps explain the higher villus height and

lower intestinal permeability observed in birds supplemented with organic Zn, and consequently lower mortality and better performance. Even though our results show that CoCPF impaired growth performance and promoted a certain degree of inflammation, the absence of microscopic lesions characteristic of NE suggests that the birds developed a mild and subclinical form of NE.

Enteric challenges may alter the mechanisms by which the development of the intestinal immune response occurs, and consequently the nutrient requirement of the animal. In the present study, organic Zn led to an average 3.16- fold increase in the expression of the IgA gene transcript in the jejunum. IgA is the main immunoglobulin present in mucosal secretions that prevents the entry of harmful bacteria (Brisbin et al., 2008). While there was no interaction between Zn source and challenge, organic Zn caused a much higher fold increase in the expression of IgA when the birds were challenged with CoCPF compared to birds given inorganic Zn (4.22 vs. 1.87- fold). This finding agrees with previous observations where higher secretory IgA was observed in birds receiving organic Zn supplementation (Bun et al., 2011). On the other hand, Zn source did not change the expression of MUC2, the main gene responsible for the synthesis of mucin. However, CoCPF infection tended to upregulate its expression.

In a previous study, subtle differences were observed when the basolateral surface of intestinal tissues from birds challenged with a mild coccidial vaccine were exposed *ex vivo* to a secretory stimulant (carbachol; Troche, 2012). Birds fed a moderately deficient Zn diet dramatically increased mucosal secretion of chloride. Feeding $ZnSO_4$ alone (90 mg of total Zn/kg diet) was unable to mitigate this hyper-chloride secretion/anaphylactic response, whereas

a blend of $ZnSO_4$ and a Zn amino acid complex restored chloride secretion back to levels of unchallenged birds (Troche, 2012). Those findings are in line with the results herein, where a proteinate organic Zn decreased intestinal permeability, as measured by FITC-d translocation from the intestinal lumen to the blood. Thus, form and delivery of Zn to tissues along the digestive tract may play a functional role in immunological and intra- cellular responses to pathogens such as *Eimeria* and *C. perfringens*.

Changes in the intestinal permeability may be influenced by modulation (down or up-regulation) and/or functionality of TJ proteins, in which bacterial derived proteases may cause its degradation (Awad et al., 2017) by a broad range of mechanisms. However, it has been demonstrated that *C. perfringens* enterotoxins are able to attach to the cell surface by binding to the TJ proteins, especially to the claudin family proteins (Eichner et al., 2017). In the present study, CoCPF challenge tended to upregulate the expression of claudin 2, a pore-forming TJ protein, probably as a mechanism to increase the efflux of water and ions into the intestinal lumen, and consequently promote pathogen clearance (Tsai et al., 2017). On the other hand, organic Zn tended to upregulate the expression of zonula occludens-1 only in CoCPF challenged birds, which may help explain the lower intestinal permeability effects of organic Zn.

Additional transcriptional profiling in a necrotic enteritis model suggests that MMP 7 and 9, both of which contain Zn, are critical for differential inflammatory responses by intestinal intra-epithelial lymphocytes (Kim et al., 2014). We observed that CoCPF challenge significantly decreased the expression of MMP9 in the jejunum but was unaffected by Zn source. In the cecal tonsils, however, organic Zn tended to upregulate its expression in CoCPF

challenged birds. Influence of Zn source on translocation to MMP 7 and 9 proteins, however, remains uncertain. Recent work has demonstrated the impact of coccidiosis on the movement of Zn inside the cell (Troche et al., 2015), and further work is being done by our lab to elucidate the effect of Zn source and bacterial challenge on the Zn status of the animal and cellular Zn trafficking.

CONCLUSION

Overall, organic Zn improved performance from 1 to 28 d of age and prevented the mortality up to 28 d of age in CoCPF challenged chickens. Additionally, organic Zn increased jejunum villus height, decreased intestinal permeability, and modulated the expression of genes related to the immune response, including IL-8, IL-10, IgA, TLR-2, A20, and iNOS. In conclusion, organic Zn vs. $ZnSO_4$ had beneficial effects on the performance of chickens challenged with coccidiosis plus *C. perfringens* inoculation by enhancing the intestinal integrity and partially attenuating the inflammation in the jejunum and cecal tonsil under the conditions of this study.

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Table 6.1: Starter (1-28 d) basal diet formulation, and formulated energy and nutrient composition.

Ingredient, %	Basal diet + $ZnSO_4$	Basal diet + Organic Zn
Corn, yellow dent	58.50	58.50
Soybean meal, 47.5% CP	35.60	35.60
Soybean oil	2.100	2.100
Dicalcium phosphate	1.734	1.734
Calcium carbonate	1.150	1.150
NaCl	0.390	0.390
L-lysine HCl	0.113	0.113
Methionine hydroxy analog, MHA	0.386	0.386
L-threonine	0.012	0.012
Vitamin premix[1]	0.065	0.065
Trace mineral premix[2]	0.075	0.075
$MnSO_4$	0.029	0.029
$ZnSO_4$	0.025	-
Organic Zn[3]	-	0.090
Formulated energy and nutrient composition		
ME Kcal/Kg	3,050	3,050
Crude Protein, %	21.70	21.70
Fat, %	4.480	4.480
Lysine, %	1.300	1.300
Thr, %	0.890	0.890
Met+Cys, %	0.910	0.910
Non phytate phosphorus, %	0.400	0.400
Total Ca, %	0.900	0.900
Na, %	0.180	0.180
Supplemental Zn, mg/kg	90	90
Determined nutrient content		
Crude Fiber, %	2.20	1.80
CP, %	21.1	20.9
Crude Fat, %	4.90	4.90
Zn, mg/kg	116	133

[1]Supplied per kg of diet: vitamin A, 13,233 IU; vitamin D_3, 6,636 IU; vitamin E, 44.1 IU; vitamin K, 4.5 mg; thiamine, 2.21 mg; riboflavin, 6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2 mg; pyridoxine, 3.31 mg; folic acid, 1.10 mg; biotin, 0.33 mg; vitamin B_{12}, 24.8 µg; choline, 669.8 mg; [2]Supplied per kg of diet: Fe, 1.99 mg; Cu, 3 mg; I, 2.25 mg; Se, 0.3 mg, Mg, 20.9 mg.
[3]Bioplex® Zn 10%: Zinc proteinate derived from partially hydrolyzed soy protein.

Table 6.2. Primer sequences used for quantitative real time-PCR.

Primer name	Primer sequence (5′–3′)	Reference
IL-10	F: CAATCCAGGGACGATGAAC R:TCCAAGCACACCTCTCTTCCATCC	(Gao et al., 2012)
INF- γ	F: TGAGCCAGATTGTTTCGA R: ACGCCATCAGGAAGGTTG	(Gao et al., 2012)
NF-κB p65	F:GTGTGAAGAAACGGGAACTG R:GGCACGGTTGTCATAGATGG	(Li et al., 2015)
A20	F:GACATCGTGCTAACAGCTTGGA R:AGAAAAGAGGGTATCAGGCACAAC	(Li et al., 2015)
IL-8	F: GCGGCCCCCACTGCAAGAAT R:TCACAGTGGTGCATCAGAATTGAGC	(Tan et al., 2014)
LITAF	F: TGTGTATGTGCAGCAACCCGTAGT R: GGCATTGCAATTTGGACAGAAGT	(Tan et al., 2014)
iNOS	F: CCTGTACTGAAGGTGGCTATTGG R: AGGCCTGTGAGAGTGTGCAA	(Tan et al., 2014)
MUC2	F: CAGCACCAACTTCTCAGTTC R: TCTGCAGCCACACATTCTTT	(Tan et al., 2014)
TLR2	F: AGGCACTTGAGATGGAGCAC R: CCTGTTATGGGCCAGGTTTA	(Wu et al., 2014)
IgA	F: ACCACGGCTCTGACTGTACC R: CGATGGTCTCCTTCACATCA	(Tan et al., 2014)
Claudin 2	F: CCTGCTCACCCTCATTGGAG R: GCTGAACTCACTCTTGGGCT	(Chen et al., 2017)
Zonula occludens 1	F: TGTAGCCACAGCAAGAGGTG R: CTGGAATGGCTCCTTGTGGT	(Chen et al., 2017)
MMP7	F: CGCTGCGCTTCAAAAGAGTT R: GCCACCTCTTCCATCAAAAGG	(Velada et al., 2011)
MMP9	F: GCCATCACTGAGATCAATGGAG R: GATAGAGAAGGCGCCCTGAGT	(Velada et al., 2011)
GAPDH	F: ACTGTCAAGGCTGAGAACGG R: CATTTGATGTTGCTGGGGTC	(Gao et al., 2012)

Table 6.3: Performance of broiler chickens from 1 to 14 d of age (before the challenge), fed diets supplemented with different sources of Zn.

	1 to 14 d		
Zn source	BW gain, g	FI, g	FCR
$ZnSO_4$	152	245	1.508 [b]
Organic Zn	162	264	1.644 [a]
SEM	12	7.5	0.05
Probability	0.08	0.54	0.01

[a–b]Means with different superscripts in a column differ ($P \leq 0.05$). Values are means ± SEM of 8 cages (8 birds/cage), except for lesion score (3 birds/cage). BW gain: body weight gain; FI: feed intake; FCR: feed conversion ratio.

Table 6.4: Performance of broiler chickens from 14 to 21 and 14 to 28 d of age, fed diets supplemented with different sources of Zn challenged with *E. maxima* (14d; Co) alone or with *C. perfringens* (19, 20, and 21 d; CoCPF).

		14 to 21 d			14 to 28 d			Lesion score	Mortality,
		BW gain, g	FI, g	FCR	BW gain, g	FI, g	FCR	21 days	%
$ZnSO_4$	Co	164	320	1.983[b]	434	859[b]	2.007	0.00	0.00[b]
	CoCPF	128	349	2.967[a]	412	982[ab]	2.508	0.96	12.50[a]
Organic Zn	Co	170	347	2.061[b]	563	1064[a]	1.904	0.00	0.00[b]
	CoCPF	171	340	2.011[b]	456	879[b]	1.949	0.75	0.00[b]
SEM		11.70	17.20	0.27	0.04	64.10	0.15	0.17	2.51
Main effect means									
Zn source	ZnSO4	146 [b]	334	2.475	423 [b]	920	2.257 [a]	0.481	6.25 [a]
	Organic Zn	170 [a]	343	2.036	509 [a]	972	1.926 [b]	0.375	0.00 [b]
Method of challenge	Co	167	333	2.022	498	962	1.955 [b]	0.000 [b]	0.00 [b]
	CoCPF	150	344	2.489	434	930	2.228 [a]	0.856 [a]	6.25 [a]
Source of variation		Probability							
Zn source		0.03	0.60	0.08	0.02	0.40	0.02	0.11	0.001
Method of challenge		0.11	0.51	0.07	0.07	0.60	0.04	<0.001	0.001
Zn source*method of challenge		0.09	0.29	0.04	0.22	0.02	0.09	0.11	0.001

[a–b]Means with different superscripts in a column differ ($P \leq 0.05$). Values are means ± SEM of 8 cages (8 birds/cage). BW gain: body weight gain; FI: feed intake; FCR: feed conversion ratio.

Table 6.5: Jejunum morphology, microscopic lesion score of coccidiosis in the jejunum, gut permeability, and *C. perfringens* enumeration in the cecal content of broiler chickens fed diets supplemented with different sources of Zn and challenged with *E. maxima* (14d; Co) alone or with *Clostridium perfringens* (19, 20 and 21 d; CoCPF).

		21 days					
		Villus, µm	Crypt, µm	Villus: crypt	Coccidiosis lesions*	Gut permeability FITC ng/mL	*C. perfringens*, log_{10} DNA copy number/g
$ZnSO_4$	Co	602	104	6.30	3.5	1,393	5.21
	CoCPF	627	118	5.69	3.1	1,381	5.55
Organic Zn	Co	732	125	5.79	3.3	1,366	5.38
	CoCPF	731	99	7.52	3.4	1,347	5.55
SEM		40.2	10.0	0.70	0.17	10.9	0.16
Main effect means							
Zn source	$ZnSO_4$	614 [b]	111	5.99	3.31	1,387 [a]	5.35
	Organic Zn	732 [a]	112	6.59	3.33	1,357 [b]	5.46
Method of challenge	Co	656	114	6.06	3.38	1,380	5.30
	CoCPF	676	104	6.47	3.27	1,364	5.55
Source of variation		Probability					
Zn source		0.005	0.96	0.37	0.93	0.01	0.64
Method of challenge		0.75	0.57	0.45	0.75	0.16	0.16
Zn source*method of challenge		0.72	0.07	0.12	0.38	0.74	0.65

[a–b]Means with different superscripts in a column differ ($P \leq 0.05$). Values are means ± SEM of 8 cages (1 bird/cage). BW gain: body weight gain; FI: feed intake; FCR: feed conversion ratio. *Coccidiosis lesion score: 1: 0-2 coccidia/section; 2: 3-30 coccidia/section; 3: 31-100 coccidia/section; 4: >100 coccidia/section.

Table 6.6: Expression of immune-related genes, tight junction, and Zn binding proteins-encoding genes in the jejunum of broiler chickens fed diets supplemented with different sources of Zn, and challenged with *E. maxima* (14d; Co) alone or with *C. perfringens* (19, 20, and 21 d; CoCPF).

Gene/Treatment	$ZnSO_4$ + Co	$ZnSO_4$ + CoCPF	Organic Zn + Co	Organic Zn + CoCPF	SEM	Zn	Method of challenge	Zn*method of challenge
Inflammatory response related genes						Probability		
INF-γ	1.30	0.99	1.09	1.44	0.17	0.49	0.90	0.07
LITAF	1.27	0.97	1.24	1.40	0.12	0.10	0.57	0.06
IL-8	0.95^{ab}	1.20^{a}	1.22^{a}	0.88^{b}	0.12	0.82	0.73	0.02
IL-10	1.35^{ab}	0.85^{b}	1.15^{ab}	1.54^{a}	0.22	0.25	0.80	0.05
iNOS	1.07	0.73	0.78	0.50	0.16	0.13	0.07	0.85
TLR-2	0.89	1.14	1.23	1.43	0.13	0.02	0.10	0.89
Gut immunity								
IgA	0.92	1.87	2.10	4.22	0.70	0.01	0.03	0.39
MUC2	1.09	1.23	1.01	1.41	0.13	0.72	0.08	0.39
Tight junction encoding genes								
Claudin 2	1.09	1.32	1.11	1.37	0.12	0.77	0.06	0.91
Zonula occludens 1	1.25	1.22	1.12	1.41	0.09	0.70	0.15	0.07
Zinc binding protein								
MMP 9	1.54	1.09	1.67	0.50	0.35	0.54	0.03	0.32

[a–b] Means with different superscripts in a row differ ($P \leq 0.05$). Values are means ± SEM of 8 cages (1 bird/cage).

Table 6.7: Expression of immune-related genes, and Zn binding protein-encoding gene in the cecal tonsils of broiler chickens fed diets supplemented with different sources of Zn and challenged with *E. maxima* (14d; Co) alone or with *C. perfringens* (19, 20, and 21 d; CoCPF).

Gene/Treatment	$ZnSO_4$ + Co	$ZnSO_4$ + CoCPF	Organic Zn + Co	Organic Zn + CoCPF	SEM	Zn	Method of challenge	Zn*method of challenge
Inflammatory response related genes						Probability		
A20	0.91	0.88	1.16	1.21	0.09	0.08	0.91	0.79
NFK- β	0.95	0.97	1.11	1.10	0.05	0.047	0.93	0.75
IL-10	0.96	0.94	1.15	1.33	0.10	0.14	0.66	0.58
iNOS	1.28[a]	0.85[ab]	0.72[b]	1.24[a]	0.10	0.54	0.73	0.008
TLR-2	0.88	0.86	1.16	1.19	0.12	0.10	0.97	0.90
Zinc binding protein								
MMP 9	1.10	0.78	0.87	1.11	0.13	0.72	0.79	0.08

[a–b] Means with different superscripts in a row differ ($P \leq 0.05$). Values are means ± SEM of 8 cages (1 bird/cage).

CHAPTER 7

CAN DIETARY ZINC DIMINISH THE IMPACT OF NECROTIC ENTERTIS ON GROWTH PERFORMANCE OF BROILER CHICKENS BY MODULATING THE INTESTINAL IMMUNE-SYSTEM AND MICROBIOTA?[1]

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[1]Submitted to *Poultry Science*.

ABSTRACT

The objective of this study was to compare the effects of inorganic and proteinate Zn in chickens challenged with coccidia and *Clostridium perfringens.* A 3x2 factorial design was used, with 3 dietary formulations (0 or 90 mg/kg supplemental Zn from $ZnSO_4$ or Zn proteinate), with or without challenge. On d 14, challenged birds were orally gavaged with ~5,000 *Eimeria maxima* sporulated oocysts, and on d 19- 21 with *Clostridium perfringens* (10^8 cfu/mL/d). Productive performance was assessed at d 21 and 28. At 21 d, necrotic enteritis (NE) lesion severity, intestinal permeability, gene expression, and ileal and cecal microbiota were evaluated. An interaction of Zn source by challenge was observed for lesion score and mortality, wherein Zn supplementation decreased the degree of NE lesions (P=0.02) and mortality due to NE (P=0.008). In the jejunum, an interaction of Zn source by challenge was observed for the expression of IL-8 (P=0.001) and INF-γ (P=0.03), wherein the NE challenge upregulated their expression, but not in the Zn proteinate supplemented birds. Zn proteinate supplementation downregulated iNOS vs. ZnSO4 supplemented birds (P=0.0003), and supplemental Zn downregulated TLR-2 (P=0.05) and ZnT5 (P=0.04), regardless of the source. In the ileal microbiota, Zn proteinate supplementation decreased the frequency of *Lactobacillus* (P=0.01), and the challenge increased *Enterobacteriaceae* (P=0.01). Dietary Zn decreased NE lesion severity and mortality due to NE; Zn proteinate led to lower expression of IL-8 and INF-γ in challenged birds which may be an indicative of a lessened inflammatory response.

Keywords: Broilers, immune system, microbiota, necrotic enteritis, zinc.

INTRODUCTION

Over the years inorganic sources of zinc (Zn) such as oxides and sulfates have been used to supplement the diets of broiler chickens above the NCR recommended levels (Lesson, 2005). When inorganic trace minerals are fed and reach the upper parts of the gastrointestinal tract (GIT) they may dissociate due to the low pH and interact with other minerals or dietary compounds (Mwangi et al., 2017), decreasing their bioavailability. Thus, the use of organic Zn to supplement broiler diets is becoming a common practice when looking to enhance mineral uptake, improve growth performance, and reduce mineral excretion (Burrell et al., 2004; Yan and Waldroup, 2006; Nollet et al., 2007; Mwangi et al., 2017). Adequate Zn intake and absorption is essential for many metabolic and biological functions, including growth, reproduction, meat quality, and immune response against pathogens challenge (Salim et al., 2008).

Zinc is paramount for adequate functioning of heterophils, mononuclear phagocytes and T lymphocytes (Kidd et al., 1996). In poultry, serum and plasma concentrations of Zn decrease during infection with *Salmonella* (Hill, 1989), or LPS (Butler and Curtis, 1973), mainly because there is upregulation of metallothionein in the liver (Richards and Augustine, 1988). Thus, it is reasonable to argue that when the absorptive capacity of the intestine is impaired due to an enteric infection, such as coccidiosis and necrotic enteritis (NE), a more available source of Zn may be needed. Yet, because organic sources of Zn are generally bound to a specific amino-acid complex, or proteinates, it may be more available to exert effects on the immune-system upon an infection. In our previous study (Bortoluzzi et al., 2018), Zn proteinate improved growth

performance, intestinal barrier function, and modulated the expression of cytokines of chickens under coccidia plus *Clostridium perfringens* challenge.

The indispensability of Zn in the diets of animals has been recognized for years (Salim et al., 2008). Zinc is a hydrophilic ion and cannot cross cellular membranes by simply diffusion, which requires specialized mechanisms for its cellular uptake. Integral membrane transport proteins are used to move Zn across the lipid bilayer of the plasma membrane (Tako et al., 2005). Zinc transporters are responsible for the movement of Zn out of the cytoplasm (ZnT) and into the cytoplasm (Zip). There is a limited number of studies evaluating the expression of these transporters in birds, but it is known that it can be changed according to the Zn source of the diet and presence or absence of challenge (Troche et al., 2015; Bin et al., 2018). For instance, Troche et al. (2015) observed a reduction of intracellular free Zn in the cecal tonsils and upregulation of Zip transporters following a coccidial challenge. Yet, Bin et al. (2018) found out that organic Zn downregulated the expression of Zip 5 and 11 and upregulated the expression of ZnT1 in the jejunum.

Despite the fact that Zn is widely used in animal nutrition in high concentrations with the objective of promoting growth, there is a lack of studies available on the effects of Zn on the intestinal bacteria community (Starke et al., 2014). Starke et al. (2014) observed that high dietary Zn (2,425 mg/kg) for weaned piglets decreased *Lactobacillus amylovorus* which also coincided with lower intestinal lactic acid, propionate and n-butyrate concentrations. Lower concentration of Zn (120 mg/kg) restored the cecal microbial community balance after *Salmonella* Typhimurium challenge by increasing *Lactobacillus* and reducing *Salmonella* colonization (Shao et al., 2014). Therefore, we hypothesized that Zn proteinate would improve the performance of

broiler chickens challenged with coccidia plus *C. perfringens* by enhancing intestinal integrity, attenuating intestinal inflammation, and restoring the balance of the ileal and cecal microbiota. The aim of this study was to build upon previous work in our lab (Troche et al., 2015; Bin et al., 2018; Bortoluzzi et al., 2018) in determining the effects of supplemental Zn sources on the performance, expression of genes (immune, tight junction related, and Zn transporter genes), and diversity and composition of the ileal and cecal microbiota of broiler chickens under coccidia and *C. perfringens* challenge.

MATERIAL AND METHODS

Housing, birds and treatments

The animal care and use procedures followed the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) under supervision of a licensed poultry veterinarian. One-day-old male Cobb 500 (384) were used in the experiment. Chicks were weighed individually and by pen for equal weight distribution and placed into 48 cages (8 birds/cage and 8 replicates/treatment), in a completely randomized design. Chickens had a*d libitum* access to water and feed in mash form during the entire experimental period. An unmedicated chicken starter diet based on corn and soybean meal was formulated. The diet was representative of a local commercial formulation and calculated analyses met or exceeded NRC broiler starter requirements. Experimental treatment feeds were prepared from this basal starter feed (Table 7.1).

The experiment was a 3 x 2 factorial design: basal diet (no added Zn); 90 mg/kg of supplemental Zn from $ZnSO_4$ (97.4 mg/kg of analyzed Zn) or 90 mg/kg of supplemental Zn from

Zn proteinate (Bioplex® Zn, Alltech Inc.; 104 mg/kg of analyzed Zn) and challenged or not with coccidia and *C. perfringens*. On d 14, all birds in the challenged treatments were given ~5,000 oocysts of *E. maxima,* kindly donated by Dr. Lorraine Fuller from the Department of Poultry Science, University of Georgia. Starting on day 19, the challenged birds were given a broth culture of *C. perfringens* ~10^8 CFU/mL. The birds were administered a fresh broth culture once daily for 3 days (on days 19, 20, and 21). On day 21, three birds from each cage were selected, sacrificed, weighed, and examined for the degree of presence of necrotic enteritis lesions. The sampling was performed at 21 days of age, to be representative of peak lesion severity.

Sample collection and analysis performed

Birds and feed were weighed weekly by cage and the mortality recorded daily. Average feed intake (FI) and body weight gain (BW gain) were corrected for mortality when calculating feed conversion ratio (FCR) for each cage. On day 21, one bird per cage (eight birds/treatment) was selected, euthanized by cervical dislocation, and had the jejunum and cecal tonsils collected for gene expression analysis. In order to evaluate gut permeability, oral administration of fluorescein isothiocyanate dextran (FITC-d, 100mg, MW 4,000; Sigma-Aldrich, Canada) and its passage into blood was used. At 21 days, FITC-d was orally gavaged to 1 bird/cage (2.2 mg/bird). Two hours after the oral gavage, the birds were euthanized, and blood collected from the heart to determine the FITC-d concentration per mL of serum, according to Bortoluzzi et al. (2018). The higher the gut permeability, the higher the blood level of FITC-d. The same serum was used for determination of Zn concentration.

Serum and liver samples were also sent to Central Analytical Lab at University of Arkansas for determination of zinc concentration. Briefly, 1 mL and 250 mg of serum and freeze-dried liver, respectively, were diluted in 9 mL of HNO_3, and placed in water bath for 2 h at 75ºC, and then at room temperature to allow it to cool, followed by the addition of 20 mL of nanopure water. The samples were then analyzed by Inductive Coupled Plasma (ICP).

A section of the jejunum from one bird per cage was collected at the midpoint between the bile duct entry and Meckel's diverticulum. Tissue was fixed in 10% buffered formalin routinely processed and embedded in Tissue Path (Fisher Scientific). Two sections were cut per sample, and HE stained to determine villus height, crypt depth, and villus-to-crypt ratio. Measurements of villus and crypts were performed in 5 intact villi, and 5 crypts per bird. Additionally, the whole GIT of two birds per cage was aseptically collected into a Ziploc bag, put in ice, and brought to the lab to separate the ileal and cecal digesta for microbiota analysis according to Bortoluzzi et al., (2018).

Intestinal gene expression - Real-Time PCR (qPCR) analysis

The preparation of samples for qPCR analysis was performed as described by Horn et al. (2014). Briefly, total RNA was isolated from ileal samples using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The precipitated RNA was suspended in 20 µL of RNAse-free water, and then stored at -80 ºC. RNA quantity was assessed by UV spectrophotometer, and then treated with DNAse (Invitrogen, China). First-strand cDNA was synthetized from 5 µL of total RNA using oligodT primers and Superscript II reverse

transcriptase, according to the manufacturer's instructions (Invitrogen, China). Synthetized cDNA was diluted (5X) with sterile water and stored at -20 ^{o}C before use.

Primers used are described below (Table 7.2). The real-time PCR amplification was performed in 10 µL of reaction mixture containing 1 µL of diluted cDNA, 5 µL of 2x SYBR Green PCR Master Mix (Bio-Rad, USA), 0.25 µL of each primer, and 3.5 µL of PCR-grade water. The PCR procedure consisted of heating the reaction mixture to 95^{o}C for 10 min followed by 40 cycles of 95^{o}C for 15 s, 57^{o}C for 20 s, and 72^{o}C for 15 s.

The relative standard-curve method was used to quantify the mRNA concentrations of each gene in relation to the reference gene (GAPDH). The mRNA relative abundance was calculated according to the method of Livak and Schmittgen (2001). All samples were analyzed in duplicate.

Ileal and cecal microbiota analysis

DNA isolation of the ileal and cecal digesta, and PCR amplification and sequencing were done in accordance with Bortoluzzi et al. (2017). The 16S rRNA was sequenced using the Illumina Miseq platform. The bioinformatic analysis was done according to Bortoluzzi et al. (2018). Alpha (Chao 1, Observed species, Phylogenetic diversity (PD) of the Whole Tree, and Shannon indexes) and beta diversity indexes were calculated using QIIME v1.9.1. PERMANOVA and ANOSIM tests were used to compare categories and similarity between the microbiota, respectively.

Statistical analysis.

The data were analyzed as a 2-way ANOVA using the GLM procedure of the SAS system (9.4, SAS Institute Inc., Cary, NC). The model included the main effect of diet, challenge, and their interaction. Cage was considered as an experimental unit. The means showing significant ($P \leq 0.05$) and trending (P between 0.05 and 0.10) treatment differences in the ANOVA were then compared using least square mean procedure of SAS. All data were tested for normality and homogeneity of variances, using the UNIVARIATE procedure and Bartlett test of SAS (9.4), respectively. The frequency of bacterial groups was submitted to a non-parametric one-way ANOVA (Kruskal-Wallis test) considering the main effects of Zn supplementation and challenge, and the means separated by Dunn test, using SAS 9.4 (SAS, 2011).

RESULTS

Growth performance and jejunum morphology

No interaction between Zn source by challenge nor main effect of Zn source was observed for BW gain, FI, and FCR in any of the periods evaluated (Tables 7.3 and 7.4). The challenge, however, significantly impaired ($P \leq 0.05$) the performance traits evaluated in all the experimental phases, except for FI from 14 to 28 and 21 to 28 d of age (Tables 7.3 and 7.4). An interaction was observed for lesion score and mortality due to NE wherein, regardless of the source, Zn supplementation reduced the lesion score characteristic of NE and decreased the mortality caused by this disease (Table 7.3). The number of oocysts the gram of excreta (OPG)

was also only affected by the challenge with higher counts in the challenge treatments ($P \leq 0.05$; Table 7.3).

The results regarding jejunum morphology are shown in Table 7.5. An interaction was observed for Zn source by challenge wherein the supplementation of $ZnSO_4$ decreased crypt depth ($P = 0.008$) and increased crypt:villus ratio ($P = 0.004$) when the birds were challenged with coccidia plus *C. perfringens*.

Serum and liver Zn concentration and gut permeability

There was no interaction between Zn source and challenge for serum and liver Zn concentrations, nor for FITC-d concentration (Table 7.6). However, it was observed that the challenge significantly decreased the concentration of Zn in the serum ($P = 0.001$) and increased its concentration in the liver ($P = 0.001$) on d 21, but not on d 27 (Table 7.6). Additionally, the dietary Zn source affected serum Zn concentration on d 27 wherein it was increased by $ZnSO_4$ when compared to the birds fed the basal diet or proteinate Zn ($P = 0.02$; Table 7.6). Gastro-intestinal tract permeability, measured by the passage of FITC-d from the intestinal lumen to the blood, increased ($P < 0.0001$) in challenged birds compared to the non-challenged group, without effect of the Zn source (Table 7.6).

Expression of immune, Zn transporters, and tight junction-related genes

In the jejunum, an interaction between Zn source and challenge was observed on the expression of IL-8 ($P = 0.001$) and INF-γ ($P = 0.03$) wherein the supplementation of Zn proteinate downregulated the expression of these genes in challenged birds fed the basal diet or

fed either the basal diet or diet supplemented with $ZnSO_4$, respectively (Table 7.7). On the other hand, an effect of the Zn source was observed on the expression of iNOS ($P = 0.0003$) wherein Zn proteinate supplementation downregulated its expression when compared to the birds supplemented with $ZnSO_4$; additionally, regardless of the source, Zn supplementation decreased the expression of TLR2 ($P = 0.05$). Moreover, the challenge with coccidia plus *C. perfringens* led to an upregulation of IL-1β ($P = 0.04$), IL-10 ($P = 0.001$), and iNOS ($P = 0.0003$; Table 7.7). In the cecal tonsils, no interaction was observed between Zn source by challenge ($P \geq 0.05$; Table 7.9). As a main effect, Zn proteinate tended to downregulate the expression of IL-8 ($P = 0.08$). The challenge with coccidia plus *C. perfringens*, however, upregulated the expression of IL-8 ($P = 0.02$), INF-γ ($P = 0.02$), iNOS ($P = 0.0007$), and NFk-β ($P = 0.0003$; Table 7.9)

No interaction was observed regarding the expression of TJ and Zn transporter genes in the jejunum ($P \geq 0.05$; Table 7.8); however, it was observed that, regardless of the source, Zn supplementation downregulated the expression of ZnT5 ($P = 0.04$; Table 7.8) and tended to downregulate the expression of ZIP9 ($P = 0.09$) and ZIP13 ($P = 0.08$). The challenge with coccidia plus *C. perfringens* upregulated the expression of CLDN-1 ($P = 0.002$) and ZIP13 ($P = 0.04$) and downregulated the expression of occludin ($P = 0.02$), ZO-1 ($P = 0.05$), and ZnT7 ($P = 0.03$; Table 8) and tended to downregulate the expression of ZIP9 ($P = 0.08$; Table 7.8). In the cecal tonsils, the challenge with coccidia plus *C. perfringens* downregulated the expression of all Zn transporters evaluated (ZIP9, ZIP13, ZnT5, ZnT7; $P \leq 0.05$; Table 7.9).

Alpha and Beta diversity of the ileal and cecal microbiota

The following comparisons were done when analyzing alpha (variability within sample) and beta (variability between samples) diversity indices: basal diet vs. basal diet plus challenge, $ZnSO_4$ supplementation vs. $ZnSO_4$ supplementation plus challenge, and Zn proteinate supplementation vs. Zn proteinate plus challenge. The ileal microbiota showed small changes in terms of diversity. It was observed that the Chao index (minimal number of OTU present in a sample) was higher (P = 0.05), and the number of observed species tended to be higher (P = 0.10) in challenged chickens and supplemented with $ZnSO_4$ compared to the same group of birds without challenge (Table 7.10). The beta diversity was not different among treatments in the ileal microbiota.

The cecal microbiota showed differences in alpha diversity in terms of Chao index and number of observed species. It was observed that challenged chickens had a cecal microbiota with higher Chao index and number of observed species when fed basal diet or diet supplemented with $ZnSO_4$. However, when chickens were fed a diet supplemented with Zn proteinate there was no difference in alpha diversity indexes related to the challenge (Table 7.10). The beta diversity, as measured by the PERMANOVA analysis, showed that the cecal microbiota quantitatively (Weighted UniFrac) changed according to the experimental treatments. These differences, that are shown using Principal Coordinated Analysis (PCoA), were evident comparing the presence or absence of challenge in chickens supplemented with ZnSO4 (Figure 7.1A) or Zn proteinate (Figure 7.1B), which was also noted in the ANOSIM analysis (Table 7.11).

Composition of the ileal and cecal microbiota

The microbial composition of the ileal and cecal microbiota was assessed by comparing the main effects of diet and challenge (Table 7.12). The ileal microbiota was found to have a predominance of bacteria belonging to the phylum *Firmicutes*, *Proteobacteria*, and *Tenericutes*. There was an effect of the challenge wherein chickens induced to NE had an increase in the frequency of *Proteobacteria* related bacteria (P = 0.02) when compared to non-challenged chickens. Downstream, the challenge increased the frequency (P = 0.01) of members of the family *Enterobacteriaceae* vs. non-challenged birds, and the supplementation with Zn proteinate decreased the frequency (P = 0.01) of *Lactobacillus*.

The cecal microbiota was dominated by members of the phylum *Firmicutes*, *Proteobacteria, Tenericutes*, and *Bacteroidetes* (Table 7.12). The phylum *Tenericutes* had its frequency decreased (P = 0.04) in birds supplemented with Zn proteinate compared to birds fed the basal diet or supplemented with $ZnSO_4$. Additionally, *Lactobacillus* (P = 0.003) and members of the family *Enterobacteriaceae* increased (P = 0.02) in challenged birds, and *Coprobacillus* decreased (P = 0.02) in chickens supplemented with $ZnSO_4$ compared to birds fed the basal diet.

DISCUSSION

Necrotic enteritis is a reemerging disease due to the limited use of sub-therapeutic antibiotics (Van Immerseel et al., 2016). This disease is estimated to cause losses of over $6 billion annually, mainly due to the impaired growth and veterinary costs (Wade and Keyburn, 2015). Since *Clostridium perfringens*, the causative agent of NE, is part of the normal intestinal

microbiota of birds, some predisposing factors, such as coccidiosis, are necessary to optimize the growth of *C. perfringens* (Prescott et al., 2016) and the occurrence of the disease. Therefore, such diseases will most likely change the nutritional demands of the intestine and higher amounts as well as more available nutrients may be necessary to maintain essential immunologic and metabolic functions of the intestine. For instance, Zn concentrations higher than those recommended by NRC (1994) have been shown to maximize growth performance of chickens (Lesson, 2005). Yet, studies have demonstrated that coccidiosis increased Zn requirement when targeting the optimization of BW gain (Bafundo et al., 1984; Troche, 2012). In the present study, growth performance of the birds was drastically impacted by the challenge, and dietary Zn supplementation did not ameliorate this effect.

Although the field of nutritional immunology is advancing, there is still a lack of knowledge about the relationships between immune system, nutritional processes, and intestinal microbiota (Kogut, 2017). In the present study, the effect of different sources of dietary Zn supplementation was evaluated on the intestinal physiology and immunology of chickens induced to NE. It was observed that regardless of the source, Zn supplementation reduced the severity of NE and mortality due to NE. Nevertheless, Zn proteinate showed to be beneficial in attenuating the intestinal inflammation by reducing the expression of IL-8 and INF-γ in challenged birds. These results agree with our previous publication (Bortoluzzi et al., 2018) in which Zn proteinate supplementation improved the intestinal barrier function and ameliorated the intestinal inflammation after coccidia plus *C. perfringens* challenge.

Zinc deficiency triggers the NFk-β mediated inflammatory response and consequent production of other inflammatory cytokines, such as IL-1β, TNF-α, and IL-8 (Li et al., 2015).

This response was not demonstrated in our study, as the expression of NFk-β did not change according to the diet. This may be explained by the fact that the basal diet used herein had a Zn concentration of 36.7 mg/kg which was most likely not enough to cause deficiency. However, Zn proteinate only, downregulated the expression of IL-8 and INF-γ in challenged birds, and regardless of the source Zn supplementation downregulated TLR-2 expression in the jejunum. Similarly, Tan et al. (2014), showed that arginine suppressed inflammation originated by LPS by reducing the expression of TLR-4. Therefore, it is plausible to argue that Zn is exerting its anti-inflammatory effect by downregulating TLR-2, besides IL-8 and INF-γ which help explain the lower severity of lesions and mortality due to NE.

Furthermore, Zn supplementation did not affect the intestinal barrier function, as measured by the passage of FITC-d from the intestinal lumen to the blood, nor the expression of TJ protein encoding genes. On the other hand, $ZnSO_4$ decreased crypt depth and increased villus:crypt ratio without affecting villus height in challenged birds, showing that Zn in its inorganic presentation ($ZnSO_4$), but not its proteinate counterpart, lowered the epithelial cell proliferation at the crypt region in challenged birds. Li et al. (2015) reported that organic Zn supplemented to broiler breeders increased intestinal cell proliferation of the progeny as measured by the proliferating cell nuclear antigen (PCNA) methodology, and decreased apoptosis-related proteins expression; yet, Zn deficient diets inhibited enterocyte proliferation activity in the jejunum of rats (Southon et al., 1985). More recently, Wen et al. (2018) observed that Zn supplementation increased villus height in the jejunum of pekin ducks and upregulated the expression of most of the TJ proteins evaluated.

It is known that the intestine has unique mechanisms for Zn transport during inflammation (Troche et al., 2015). In the present study, it was observed in the cecal tonsils that all the transporters were downregulated by the challenge. In the jejunum, on the other hand, Zn supplementation downregulated the expression of ZnT5, and the challenge upregulated ZIP13 and downregulated ZnT7, suggesting that the challenge increased movement of Zn from the Golgi to the cytoplasm (Troche et al., 2015). These authors proposed that upregulation of ZIP transporters is due to the fact that Zn is being incorporated into intracellular proteins and, during a challenge, the cell may be Zn deficient. This observation would make sense as it was observed in the present study that there was an increase in the concentrations of Zn in the liver, which is most likely due to an upregulation of metallothionein in the liver (Richards and Augustine, 1988).

Despite the well-recognized use of dietary Zn in animal production, only few studies have looked at its effects on the intestinal bacterial community. There seems to be an agreement throughout different experiments towards an increase of enterobacteria and decrease of *Lactobacillus* species in the intestine of weaning piglets fed high dietary Zn (Mores et al., 1998; Hojberg et al., 2005; Broom et al., 2006). Using DNA sequencing techniques, Vahjen et al. (2011) demonstrated that high dietary Zn increased the diversity of enterobacteria in the intestinal microbiota of piglets. To the best of our knowledge, this is first study to look at the effect of different sources of Zn supplementation and induction to NE on the intestinal microbiota of chickens using 16S rRNA sequencing, but previous studies with swine partially agree with the findings reported herein. In the present study, Zn proteinate, but not $ZnSO_4$, decreased the frequency of *Lactobacillus*; however, this effect of Zn against *Lactobacillus* is

probably not specific to the whole genus, but rather species specific (Starke et al., 2014). These authors observed that high dietary Zn decrease the amount of *L. amylovorus*, but other species of *Lactobacillus* were less affected. Nevertheless, Torok et al. (2011) reported that the presence of three species of *Lactobacillus* (*L. salivarius*, *L. aviarius* and *L. crispatus*) in the ileum of broiler chickens is correlated with worse performance. Therefore, the reduction of *Lactobacillus* by Zn proteinate cannot be linked with worse performance, since Zn supplementation did not change the growth performance of the chickens.

CONCLUSION

Induction to NE impaired the performance of the chickens, and dietary Zn supplementation (regardless the source) did not counteract this effect, but decreased NE lesions and mortality due to NE. Zn proteinate increased the crypt depth, indicating higher cellular proliferation in the intestine. Yet, Zn proteinate led to lower expression of IL-8 and INF- γ which indicates a lessened inflammatory response. Additionally, the challenge itself changed the expression of several genes in both jejunum and cecal tonsils, but Zn supplementation did not have much impact on the expression of these genes, regardless of the source. Zn proteinate supplementation reduced the frequency of *Lactobacillus* in the ileal microbiota, and the induction to NE increased *Lactobacillus* and enterobacteria in the cecal microbiota.

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Table 7.1: Starter (1-28 d) basal diet formulation.

Ingredient, %	Basal diet	Basal diet + $ZnSO_4$	Basal diet + Proteinate Zn
Corn	58.51	58.51	58.51
Soybean meal, 47.5% CP	35.55	35.55	35.55
Soybean oil	2.100	2.100	2.100
Dicalcium phosphate	1.734	1.734	1.734
Calcium carbonate	1.150	1.150	1.150
NaCl	0.305	0.305	0.305
L-lysine HCl	0.113	0.113	0.113
Methionine Hydroxy Analogue, MHA	0.386	0.386	0.386
L-threonine	0.012	0.012	0.012
Vitamin Premix- broilers[1]	0.065	0.065	0.065
Trace mineral[2]	0.075	0.075	0.075
$ZnSO_4$	-	0.026	-
Bioplex, Zn 15%	-	-	0.060
Formulated energy and nutrient composition			
ME Kcal/Kg	3,050	3,050	3,050
CP, %	21.7	21.7	21.7
Fat, %	4.48	4.48	4.48
Lysine, %	1.30	1.30	1.30
Thr, %	0.890	0.890	0.890
Met+Cys, %	0.910	0.910	0.910
nPP, %	0.400	0.400	0.400
Ca, %	0.900	0.900	0.900
Na, %	0.180	0.180	0.180
Determined Zn, mg/kg	36.7	97.4	104

[1]Supplied per kg of diet: vitamin A, 13,233 IU; vitamin D_3, 6,636 IU; vitamin E, 44.1 IU; vitamin K, 4.5 mg; thiamine, 2.21 mg; riboflavin, 6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2 mg; pyridoxine, 3.31 mg; folic acid, 1.10 mg; biotin, 0.33 mg; vitamin B_{12}, 24.8 µg; choline, 669.8 mg; [2]Supplied per kg of diet: Fe, 1.99 mg; Cu, 3 mg; I, 2.25 mg; Se, 0.3 mg, Mg, 20.9 mg.

[3]Bioplex® Zn 15%: Zinc proteinate derived from partially hydrolyzed soy protein.

Table 7.2. Primer sequences for RT-PCR

Objective	RNA Target	Primer sequence (5′–3′)	
RT - PCR	*IL-6*	F: ATAAATCCCGATGAAGTGG R: GCAGGTGAAGAAGCGGTGA	Gao et al., (2012)
	IL-10	F: CAATCCAGGGACGATGAAC R:TCCAAGCACACCTCTCTTCCATCC	Gao et al., (2012)
	INF- γ	F: TGAGCCAGATTGTTTCGA R: ACGCCATCAGGAAGGTTG	Gao et al., (2012)
	IL-1β	F: AGAAGAAGCCTCGCCTGGAT R: CCGCAGCAGTTTGGTCAT	Gao et al., (2012)
	NF-κB p65	F:GTGTGAAGAAACGGGAACTG R:GGCACGGTTGTCATAGATGG	Li et al., (2015)
	IL-8	F: GCGGCCCCCACTGCAAGAAT R:TCACAGTGGTGCATCAGAATTGAGC	Tan et al., 2014
	iNOS	F: CCTGTACTGAAGGTGGCTATTGG R: AGGCCTGTGAGAGTGTGCAA	Tan et al., 2014
	MUC2	F: CAGCACCAACTTCTCAGTTC R: TCTGCAGCCACACATTCTTT	Tan et al., 2014
	TLR2	F: AGGCACTTGAGATGGAGCAC R: CCTGTTATGGGCCAGGTTTA	Wu et al., 2014
	Claudin 1	F: CATACTCCTGGGTCTGGTTGGT R: GACAGCCATCCGCATCTTCT	Du et al., 2016
	Claudin 2	F: CCTGCTCACCCTCATTGGAG R: GCTGAACTCACTCTTGGGCT	Chen et al., 2016
	Claudin 4	F: GAAGCGCTGAACCGATACCA R: TGCTTCTGTGCCTCAGTTTCC	Shao et al., 2013
	Occludin	F: CCGTAACCCCGAGTTGGAT R: ATTGAGGCGGTCGTTGATG	Chen et al., 2016
	Zonula occludens 1	F: TGTAGCCACAGCAAGAGGTG R: CTGGAATGGCTCCTTGTGGT	Chen et al., 2016
	Zip9	F: CATTGGCAGCACCTGTTATG R: CGCGATTCCTCCTACTTCTG	Troche et al., 2015
	Zip13	F: AGCTTTGCAATTGGTGGACT R: GGGCACTCCTCCTCTTCTTT	Troche et al., 2015
	ZnT5	F: GGACATTCCCATGTGTCCTC R: AGAGCAGAGGGGATCAGCTA	Troche et al., 2015
	ZnT7	F: ATTTTGATGCAGCGAACTCC R: ATCCACCTTCCATCAGCATC	Troche et al., 2015

GAPDH	F: ACTGTCAAGGCTGAGAACGG R: CATTTGATGTTGCTGGGGTC	Gao et al., (2012)

Table 7.3: Performance of broiler chickens from 1 to 21 and 1 to 28 days of age, fed diets supplemented with different sources of Zn under coccidia and *Clostridium perfringens* challenge.

		BW gain, g 0-21d	FI, g 0-21d	FCR 0-21d	BW gain, g 0-28d	FI, g 0-28d	FCR 0-28d	OPG	Lesion score[1]	NE mort, %
No Zn		459	729	1.609	777	1194	1.532	0	0 c	0 c
ZnSO4		493	726	1.468	855	1261	1.493	0	0 c	0 c
Pro Zn		476	711	1.507	820	1206	1.471	0	0 c	0 c
No Zn + Challenge		299	596	2.014	484	992	2.072	22,704	1.46 a	28.1 a
$ZnSO_4$ + Challenge		337	660	1.998	536	1113	2.137	20,592	1.29 b	14.1 b
Pro Zn + Challenge		353	648	1.845	506	1038	2.071	23,334	0.92 b	15.6 b
Main effects										
Zn	No Zn	379	662	1.811	630	1,093	1.802	11,352	0.73	14.1
	$ZnSO_4$	415	693	1.733	695	1,187	1.815	10,296	0.65	7.03
	Proteinate Zn	415	680	1.676	663	1,122	1.771	11,667	0.46	7.81
Cha	No Cha	476 [a]	722 [a]	1.531 [b]	817 [a]	1,221 [a]	1.497 [b]	0 [b]	0	0
	With Cha	330 [b]	635 [b]	1.952 [a]	507 [b]	1,045 [b]	2.093 [a]	24,615 [a]	1.22	19.3
SEM		36.1	39.3	0.112	77.9	94.0	0.132	1,984	0.241	4.35
P Value	Zn	0.26	0.70	0.19	0.51	0.58	0.84	0.56	0.02	0.008
	Cha	<0.0001	0.005	<0.0001	<0.0001	0.03	<0.0001	<0.0001	<0.0001	<0.0001
	Zn*Cha	0.71	0.55	0.46	0.95	0.96	0.79	0.58	0.02	0.008

[a–b] Means with no common superscripts in a column are significantly different ($P \leq 0.05$). Values are means ± SEM of 8 cages (8 birds/cage). BW gain: body weight gain; FI: feed intake; FCR: feed conversion ratio; OPG: oocysts per gram; NE: necrotic enteritis; Pro: proteinate; Cha: challenge. [1] The scoring was based on a 0 to 3 score, with 0 being normal and 3 being the most severe.

Table 7.4: Performance of broiler chickens from 14 to 21 and 14 to 28 days of age, fed diets supplemented with different sources of Zn under coccidia and *Clostridium perfringens* challenge.

		BW gain, g 14-21d	FI, g 14-21d	FCR 14-21d	BW gain, g 14-28d	FI, g 14-28d	FCR 14-28d	BW gain, g 21-28d	FI, g 21-28d	FCR 21-28d
No Zn		267	463	1.768	585	936	1.599	318	465	1.460
$ZnSO_4$		290	472	1.637	664	1035	1.585	401	570	1.445
Pro Zn		280	447	1.612	624	947	1.517	343	495	1.431
No Zn + Challenge		131	374	2.751	311	725	2.546	213	454	2.152
$ZnSO_4$ + Challenge		139	363	2.696	331	901	2.628	268	433	2.152
Pro Zn + Challenge		150	380	2.547	307	823	2.857	187	416	2.161
Main effects										
Zn	no Zn	199	419	2.259	448	831	2.072	265	460	1.806
	$ZnSO_4$	214	418	2.167	498	968	2.106	334	502	1.799
	Proteinate Zn	215	414	2.080	465	885	2.187	265	455	1.796
Cha	No Cha	279 [a]	460 [a]	1.674 [b]	624 [a]	974	1.565 [b]	352 [a]	509	1.445 [b]
	With Cha	140 [b]	372 [b]	2.661 [a]	316 [b]	812	2.677 [a]	222 [b]	435	2.155 [a]
SEM		27.5	21.3	0.193	70.7	94.0	0.23	40.1	67.6	0.164
P Value	Zn	0.43	0.95	0.13	0.39	0.40	0.73	0.17	0.82	0.99
	Cha	<0.0001	<0.0001	<0.0001	0.03	0.07	<0.0001	<0.0001	0.25	<0.0001
	Zn*Cha	0.71	0.37	0.8	0.88	0.89	0.29	0.66	0.76	0.99

[a–b] Means with no common superscripts in a column are significantly different ($P \leq 0.05$). Values are means ± SEM of 8 cages (8 birds/cage). BW gain: body weight gain; FI: feed intake; FCR: feed conversion ratio; Pro: proteinate; Cha: challenge.

Table 7.5: Jejunum morphology of broiler chickens fed diets supplemented with different sources of Zn under coccidia and *Clostridium perfringens* challenge.

		Villus	Crypt	villus:crypt
No Zn		938	195 [b]	5.06 [a]
$ZnSO_4$		801	209 [b]	4.00 [a]
Pro Zn		769	160 [b]	5.05 [a]
No Zn + Challenge		659	315 [a]	2.22 [b]
$ZnSO_4$ + Challenge		645	170 [b]	3.94 [a]
Pro Zn + Challenge		596	311 [a]	2.20 [b]
Main effects				
Zn	No Zn	798	255	3.64
	$ZnSO_4$	723	190	3.97
	Proteinate Zn	682	235	3.63
Cha	No Cha	836 [a]	188	4.71
	With Cha	633 [b]	265	2.79
SEM		60.0	31.91	0.53
P Value	Zn	0.14	0.10	0.68
	Cha	<0.0001	0.005	<0.0001
	Zn*Cha	0.49	0.008	0.004

[a–b] Means with no common superscripts in a column are significantly different ($P \leq 0.05$). Values are means ± SEM of 8 cages (1 birds/cage). Pro: proteinate; Cha: challenge.

Table 7.6: Serum and liver zinc concentration, and gut permeability of broiler chickens fed diets supplemented with different sources of Zn under coccidia and *Clostridium perfringens* challenge.

		Zn serum, 21 d	Zn serum, 27 d	Zn liver, 21 d	Zn liver, 27 d	FITC-d ng/mL
No Zn		1.29	1.28	68.2	71.0	485.1
$ZnSO_4$		1.46	1.58	84.3	71.2	479.9
Pro Zn		1.49	1.59	78.1	78.6	409.3
No Zn + Challenge		1.09	1.33	92.9	73.5	909.9
$ZnSO_4$ + Challenge		1.27	1.54	90.1	74.1	950.5
Pro Zn + Challenge		1.07	1.48	87.2	72.3	948.5
Main effects						
Zn	No Zn	1.19	1.30 [b]	80.6	72.3	697
	$ZnSO_4$	1.36	1.56 [a]	87.6	73.0	715
	Proteinate Zn	1.28	1.53 [ab]	82.7	75.4	679
Cha	No Cha	1.41 [a]	1.48	76.9 [b]	73.8	463 [b]
	With Cha	1.14 [b]	1.45	90.3 [a]	73.3	935 [a]
SEM		0.10	0.10	5.15	2.41	66.2
P Value	Zn	0.20	0.02	0.33	0.43	0.89
	Cha	0.001	0.68	0.001	0.81	<0.0001
	Zn*Cha	0.41	0.67	0.14	0.16	0.74

[a–b] Means with no common superscripts in a column are significantly different ($P \leq 0.05$). Values are means ± SEM of 8 cages (1 birds/cage). FITC-d: fluorescein isothiocyanate-dextran; Pro: proteinate; Cha: challenge.

Table 7.7: Expression of immune-related genes in the jejunum of broiler chickens fed diets supplemented with different sources of Zn under coccidia and *Clostridium perfringens* challenge.

		IL1-β	IL-6	IL-8	IL-10	INF-γ	iNOS	MUC2	NFk β	TLR2
No Zn		0.89	1.04	0.71 ^{c}	1.25	1.04 ^{b}	1.20	1.01	1.01	1.10
$ZnSO_4$		1.19	0.87	1.72 ^{b}	1.83	1.54 ^{b}	3.09	0.71	0.89	0.71
Pro Zn		0.95	1.19	1.48 ^{bc}	1.17	1.16 ^{b}	1.80	0.84	1.02	0.88
No Zn + Challenge		1.33	1.07	2.98 ^{a}	3.09	3.03 ^{a}	3.63	0.82	0.98	1.44
$ZnSO_4$ + Challenge		1.56	0.98	2.42 ^{ab}	2.69	2.84 ^{a}	4.88	0.74	0.92	1.00
Pro Zn + Challenge		1.28	1.02	1.69 ^{b}	2.17	1.61 ^{b}	3.03	0.69	0.81	0.83
Main effects										
Zn	no Zn	1.11	1.05	1.85	2.17	2.04	2.42 ^{b}	0.92	1.00	1.27 ^{a}
	$ZnSO_4$	1.38	0.92	2.07	2.26	2.19	3.98 ^{a}	0.72	0.91	0.86 ^{b}
	Proteinate Zn	1.12	1.11	1.59	1.67	1.39	2.41 ^{b}	0.77	0.92	0.85 ^{b}
Cha	No Cha	1.01	1.03	1.30	1.42	1.25	2.03	0.85	0.97	0.89
	With Cha	1.39	1.02	2.36	2.65	2.49	3.85	0.75	0.91	1.09
SEM		0.20	0.11	0.37	0.44	0.36	0.55	0.10	0.07	0.19
P Value	Zn	0.37	0.34	0.36	0.37	0.01	0.0003	0.15	0.36	0.05
	Cha	0.04	0.92	0.0004	0.001	<0.0001	<0.0001	0.23	0.25	0.23
	Zn*Cha	0.97	0.49	0.001	0.45	0.03	0.33	0.53	0.23	0.55

$^{a–b}$ Means with no common superscripts in a column are significantly different ($P \leq 0.05$). Values are means ± SEM of 8 cages (1 bird/cage). Pro: proteinate; Cha: challenge.

Table 7.8: Expression of tight junction and zinc transporter genes in the jejunum of broiler chickens fed diets supplemented with different sources of Zn under coccidia and *Clostridium perfringens* challenge.

		CLDN-1	CLDN-2	CLDN-4	Occludin	ZO-1	ZIP9	ZIP13	ZnT5	Znt7
No Zn		0.84	1.04	0.94	1.01	1.02	1.02	1.01	1.01	1.01
$ZnSO_4$		0.87	0.98	1.00	0.81	0.89	0.77	0.89	0.77	0.95
Pro Zn		0.93	0.95	1.36	0.96	1.16	0.97	1.00	0.93	1.04
No Zn + Challenge		1.90	1.04	1.09	0.71	0.87	0.92	1.25	1.08	0.92
$ZnSO_4$ + Challenge		3.64	1.15	1.44	0.77	0.87	0.73	1.06	0.88	0.83
Pro Zn + Challenge		1.72	0.65	1.63	0.86	0.87	0.66	0.98	0.76	0.73
Main effects										
Zn	no Zn	1.37	1.04	1.01	0.86	0.95	0.97	1.13	1.04 [a]	0.96
	$ZnSO_4$	2.26	1.07	1.22	0.79	0.88	0.75	0.97	0.83 [b]	0.89
	Proteinate Zn	1.32	0.80	1.49	0.91	1.01	0.82	0.99	0.84 [b]	0.89
Cha	No Cha	0.88	0.99	1.10	0.93	1.02	0.92	0.97	0.90	1.00
	With Cha	2.42	0.95	1.38	0.78	0.87	0.77	1.09	0.90	0.83
SEM		0.59	0.13	0.24	0.07	0.09	0.10	0.08	0.09	0.09
P Value	Zn	0.20	0.14	0.18	0.25	0.38	0.09	0.07	0.04	0.60
	Cha	0.002	0.71	0.18	0.02	0.05	0.08	0.04	0.99	0.03
	Zn*Cha	0.19	0.25	0.84	0.18	0.36	0.38	0.19	0.30	0.41

[a–b] Means with no common superscripts in a column are significantly different ($P \leq 0.05$). Values are means ± SEM of 8 cages (1 bird/cage). Pro: proteinate; Cha: challenge.

Table 7.9: Expression of immune-related and zinc transporter genes in the cecal tonsils of broiler chickens fed diets supplemented with different sources of Zn under coccidia and *Clostridium perfringens* challenge.

		IL1-β	IL-6	IL-8	IL-10	INF-γ	iNOS	NFk β	TLR2	ZIP9	ZIP13	ZnT5	ZnT7
No Zn		1.10	1.08	1.15	1.11	1.10	1.10	1.03	1.09	1.02	1.03	1.02	1.03
$ZnSO_4$		1.20	1.06	1.80	0.76	1.33	1.40	1.05	0.85	0.93	0.95	0.90	0.98
Pro Zn		1.07	1.11	1.38	0.80	0.99	1.04	1.09	0.90	1.07	1.02	1.04	1.09
No Zn + Challenge		1.84	1.25	1.84	0.81	1.51	2.09	0.86	0.90	0.80	0.84	0.83	0.79
$ZnSO_4$ + Challenge		1.12	1.15	2.45	0.80	1.58	1.91	0.88	0.88	0.85	0.87	0.86	0.88
Pro Zn + Challenge		1.29	1.03	1.83	0.73	1.46	2.02	0.78	0.79	0.68	0.72	0.67	0.71
Main effects													
Zn	no Zn	1.47	1.17	1.49	0.96	1.30	1.60	0.94	0.99	0.91	0.93	0.93	0.91
	$ZnSO_4$	1.16	1.11	2.13	0.78	1.45	1.66	0.97	0.86	0.89	0.91	0.88	0.93
	Proteinate Zn	1.57	1.14	1.84	0.77	1.49	2.05	0.82	0.85	0.74	0.78	0.75	0.75
Cha	No Cha	1.13	1.09	1.44	0.89	1.14	1.18	1.06	0.95	1.01	1.00	0.99	1.03
	With Cha	1.42	1.14	2.04	0.78	1.52	2.01	0.84	0.86	0.78	0.81	0.79	0.79
SEM		0.22	0.11	0.30	0.12	0.19	0.29	0.07	0.11	0.09	0.07	0.08	0.08
P Value	Zn	0.30	0.74	0.08	0.21	0.48	0.90	0.91	0.40	0.93	0.62	0.64	0.90
	Cha	0.13	0.56	0.02	0.29	0.02	0.0007	0.0003	0.36	0.003	0.0009	0.003	0.0002
	Zn*Cha	0.20	0.59	0.90	0.38	0.82	0.63	0.47	0.64	0.25	0.25	0.13	0.17

[a–b] Means with no common superscripts in a column are significantly different ($P \leq 0.05$). Values are means ± SEM of 8 cages (1 bird/cage). Pro: proteinate; Cha: challenge.

Table 7.10. Alpha diversity indexes of the ileal and cecal microbiota of broiler chickens fed diets supplemented with different sources of Zn under coccidia and *Clostridium perfringens* challenge.

	Chao	OS	PD	Shannon	Chao	OS	PD	Shannon
	Ileal microbiota				Cecal microbiota			
No Zn (A)	170	140	4.59	2.86	112	81	6.56	3.45
$ZnSO_4$ (B)	152	122	4.36	2.59	114	82	6.86	3.29
Pro Zn (C)	161	127	3.97	2.8	132	91	7.3	3.61
No Zn + Challenge (D)	193	145	5.06	3.08	154	111	7.11	3.64
$ZnSO_4$ + Challenge (E)	179	141	4.93	2.88	150	99	6.38	3.51
Pro Zn + Challenge (F)	137	108	4.27	2.87	124	94	6.39	3.21
			Probabilities					
A vs D	0.18	0.55	0.41	0.29	0.02	0.01	0.14	0.34
B vs E	0.05	0.10	0.22	0.31	0.03	0.08	0.40	0.34
C vs F	0.32	0.34	0.43	0.82	0.73	0.84	0.11	0.22

OS: Observed species; PD whole tree: Phylogenetic diversity of the whole tree; Pro: proteinate. Values are means of 8 replicates and a pool of 2 birds/replicate.

Table 7.11. ANOSIM analysis of the ileal and cecal microbiota of broiler chickens fed diets supplemented with different sources of Zn under coccidia and *Clostridium perfringens* challenge.

	Ileal microbiota		Cecal microbiota	
	Probability	R	Probability	R
No Zn vs. No Zn + challenge	0.46	-0.007	0.19	0.08
ZnSO4 vs. ZnSO4 + challenge	0.93	-0.08	0.02	0.22
Pro Zn vs. Pro Zn + challenge	0.41	-0.002	0.003	0.34

R is the index of ANOSIM that indicates the similarity of comparison between group pairs. “R” ranges from -1 to 1: the pairs are more similar when the R index is closer to 0 and the pairs are different from each other when the R index is close to 1. Pro: proteinate.

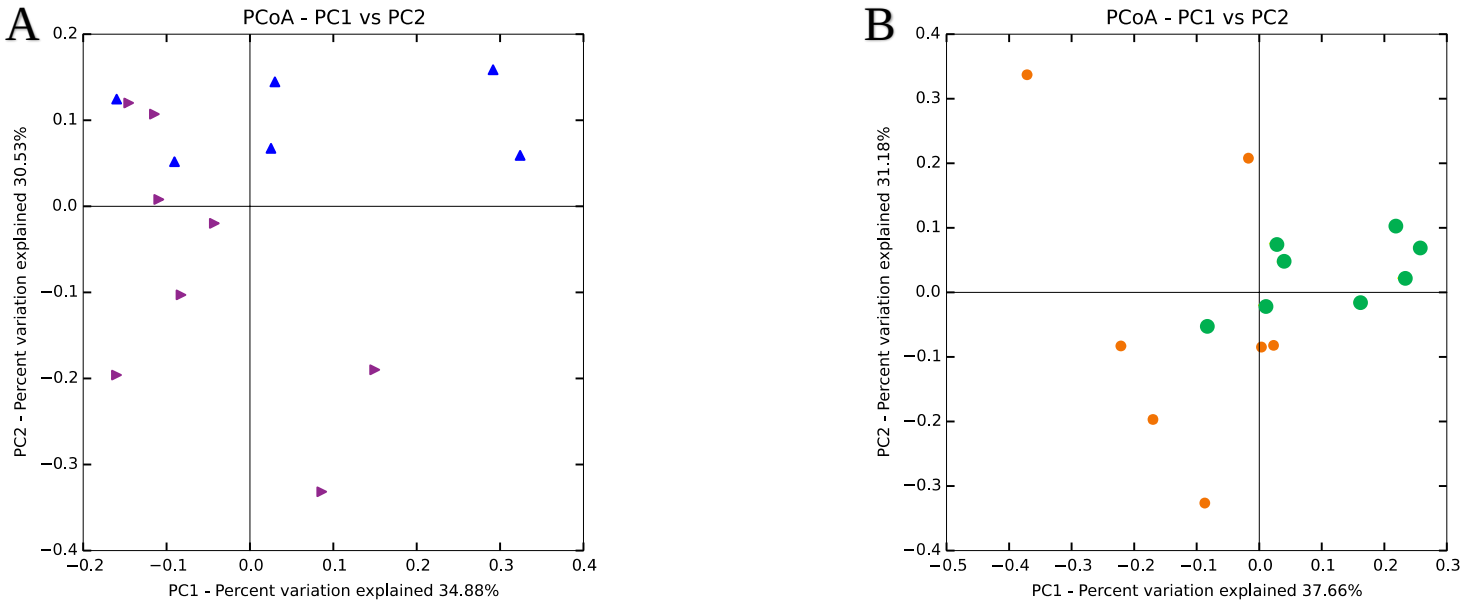

Figure 7.1: Weighted UniFrac PCoA of the cecal microbiota of broiler chickens fed diets supplemented with $ZnSO_4$ (A) or Zn proteinate (B) under coccidia and *Clostridium perfringens* challenge. A: supplemented with $ZnSO_4$ and P = 0.01. Blue is not challenged, and purple is challenged; B: Supplemented with Zn proteinate and P = 0.01. Orange is not challenged, and green is challenged.

Table 7.12. Relative abundance (%) of the main bacterial group present in the ileal and cecal microbiome of broiler chickens fed diets supplemented with different sources of Zn under coccidia and *Clostridium perfringens* challenge.

Ileal microbiota							
	Firmicutes	*Proteobacteria*	*Tenericutes*	*Lactobacillus*	*Streptococcus*	*Enterococcus*	*Enterobacteriaceae*
No Zn	98.26	1.17	0.04	95.71 [a]	1.09	0.75	0.95
ZnSO4	99.05	0.58	0.04	95.12 [a]	2.10	0.64	0.41
Pro Zn	90.33	8.97	0.01	72.10 [b]	2.24	0.98	4.03
No Cha	97.14	2.32 [b]	0.03	90.57	2.67	0.93	0.33 [b]
With Cha	95.02	4.44 [a]	0.03	85.21	0.90	0.61	3.03 [a]
Probabilities							
Zn	0.16	0.15	0.16	0.01	0.89	0.74	0.12
Challenge	0.20	0.02	0.81	0.34	0.55	0.12	0.01
SEM	1.35	1.34	0.01	2.90	0.67	0.26	0.69
Cecal microbiota							
	Firmicutes	*Proteobacteria*	*Tenericutes*	*Lactobacillus*	*Clostridiales*	*Coprobacillus*	*Enterobacteriaceae*
No Zn	76.69	17.75	4.18 [a]	30.82	12.55	2.0 [a]	12.21
$ZnSO_4$	69.33	24.21	5.61 [a]	31.33	9.98	0.7 [b]	15.19
Pro Zn	63.57	32.57	2.57 [b]	25.86	6.34	1.4 [ab]	26.78
No Cha	75.75	17.79	5.30	16.65 [b]	14.47	1.67	10.31 [b]
With Cha	64.94	30.75	3.12	39.32 [a]	5.65	1.17	24.64 [a]
Probabilities							
Zn	0.30	0.22	0.04	0.71	0.28	0.02	0.24
Challenge	0.17	0.09	0.77	0.003	0.25	0.12	0.02
SEM	3.00	2.99	0.97	3.94	2.12	0.26	2.88

[a–b] Means with no common superscripts in a column are significantly different ($P \leq 0.05$). Values are means of 8 replicates and a pool of 2 birds/replicate. Pro: proteinate; Cha: Challenge.

CHAPTER 8

CONCLUSIONS

CONCLUSION

Although the interactions between commensal bacteria and nutrition in health and challenge situations are not fully understood, dietary nutrients and feed additives are responsible for modulating the population of commensal and pathogenic microorganisms. As such, the understanding of these interactions, in both physiological and pathological situations, will allow the use of feed additives to promote a better growth during enteric pathogen challenges. In the studies presented in this dissertation, we evaluated the effect of different challenge models and their effects on the intestinal microbiota, as well as the effects of supplementing SB, EO, and different sources of Zn to mitigate the negative effects of NE on the intestinal microbiota and immune-system of broiler chickens.

Regardless of the challenge model, the induction of NE negatively impacted the growth performance of the birds. Birds that were raised on floor pens with recycled litter, vaccinated on d 1, and challenged with *C. perfringens* on d 18-20 had the largest variations in terms of diversity and composition of the ileal and cecal microbiota. Yet, the α-diversity indices decreased due to the challenge. Therefore, the use of vaccination on d 1 and challenge with *C. perfringens* on d 18-20 is the method of choice to reproduce necrotic enteritis when evaluating its effect on the intestinal microbiota of broilers raised on floor pens with recycled litter.

In Chapters 3, 4 and 5 the supplementation of sodium butyrate alone or combination with essential oils was evaluated in the presence or absence of a challenge. Overall, it was observed that the nutritional reduction of energy and amino acids impaired the performance of broiler chickens in terms of BW gain and FCR, but the supplementation of SB partially counteracted this effect. The cecal microbiota of chickens showed a large amount of fiber degraders and SCFA producers, especially in the groups fed a nutritionally reduced diet supplemented with SB. The nutritional reduction changed the predicted function performed by the microbiota, and the SB supplementation reduced this variation. The frequency of bacterial species presenting the butyryl-CoA:acetate CoA-transferase gene related to butyrate production was increased in the microbiota of chickens fed a nutritionally reduced diet and reduced with SB supplementation.

In challenge studies (Chapter 4 and 5) SBEO modulated the expression of genes prior to the challenge (d 12), and SB or SBEO supplementation showed immunomodulatory effects mainly in the jejunum after the *E. maxima* challenge (d 18). Sodium butyrate supplementation had beneficial effects on the expression of TJ genes. Additionally, SB and SBEO supplementation to NE-challenged birds contributed to changes in the diversity, composition and predicted functions of the cecal microbiome. Older birds presented a cecal microbiome with lower and higher abundance of *Firmicutes* and *Bacteroidetes*, respectively, than younger birds. Additionally, *Proteobacteria* was observed in higher frequency after challenge, in challenged control vs. SBEO supplemented birds. NE challenge led to a decrease in the frequency of *Blautia* over time, but dietary SB prevented this effect. SB and SBEO modulated

the predicted function of the many metabolic pathways of the cecal microbiota over time, which potentially explains the improvement in performance obtained with these feed additives.

In Chapter 6 and 7, the effect of organic Zn supplementation was evaluated in broilers under coccidia plus *C. perfringens* challenge. In the first study, organic Zn improved the performance from 1 to 28 d of age and reduced the mortality up to 28 d of age in birds challenged with coccidia plus *C. perfringens*. Additionally, organic Zn increased jejunal villus height, decreased intestinal permeability, and modulated the expression of genes related to the immune response, including IL-8, IL-10, IgA, TLR-2, A20, and iNOS. In the second study, induction to NE impaired the performance of the chickens, and dietary Zn supplementation (regardless the source) did not counteract this effect, but decreased NE lesions and mortality due to NE. Organic Zn increased crypt depth, indicating higher cellular proliferation in the intestine. Yet, organic Zn led to lower expression of IL-8 and INF- γ which indicated a lowered inflammatory response. Additionally, the challenge itself changed the expression of several genes in both jejunum and cecal tonsils, but Zn supplementation did not have much impact on the expression of these genes, regardless of the source. Organic Zn supplementation reduced the frequency of *Lactobacillus* in the ileal microbiota, and the induction to NE increased *Lactobacillus* and enterobacteria in the cecal microbiota.

Even though we now have a clearer picture of how the gut microbiota and immune-system changes according to the challenge and feed supplements, we still do not know beyond these changes, i.e., information is missing with regards to microbial-cross talk, modulation of pathogenicity factors by nutrients and feed additives, and endogenous losses associated with microbial dysbiosis and immune response. Future research must be conducted in order to study

the mechanisms of action of these feed additives and other nutrients using different approaches. For example, other-omic tools such as transcriptome, proteome, and metabolome will give further insights into the mechanism of action of feed additives such as butyrate and phytogenics. Yet, in future studies, when conducting studies to investigate the effects of butyrate on the development of the intestine, earlier stages of the life of the chicks should be targeted.

Additionally, as it has been shown by this dissertation, different conditions in which the birds are raised may affect the intestinal microbiota. Therefore, further study must be done with Zn when raising chickens on floor pens with recycled litter. Nevertheless, higher doses of organic Zn should be evaluated when targeting the replacement of inorganic sources, such as Zn oxide.

APPENDICES

APPENDIX 1

A1. Performance of broiler chickens from 1 to 13 and 1 to 21 days of age, fed diets with sodium butyrate alone or in combination with essential oils, and submitted to a necrotic enteritis challenge model.

Treatment	1 to 13 d			1 to 21 d		
	WG, g	FI, g	FCR	WG, g	FI, g	FCR
Unchallenged control	334	353	1.065 [a]	667 [a]	896 [a]	1.347
Challenged control	---	---	---	633 [ab]	854 [ab]	1.381
ChaSB	346	353	1.019 [b]	645 [ab]	847 [ab]	1.291
ChaSBEO	318	340	1.073 [a]	616 [b]	825 [b]	1.320
SEM	0.008	0.008	0.021	0.01	0.02	0.027
Probability	0.18	0.28	0.03	0.05	0.05	0.19

[a–b] Means with different superscripts in a column differ significantly ($P \leq 0.05$). Values are means of 8 pens (58 birds/pen). WG: weight gain; FI: feed intake; FCR: feed conversion ratio; ChaSB: challenged supplemented with sodium butyrate; ChaSBEO: challenged supplemented with sodium butyrate plus essential oils; SEM: standard error of mean.

APPENDIX 2

A2. Performance of broiler chickens from 1 to 34 and 1 to 41 days of age, fed diets with sodium butyrate alone or in combination with essential oils, and submitted to a necrotic enteritis challenge model.

Treatment	1 to 34 d			1 to 41 d			
	WG, g	FI, g	FCR	WG, g	FI, g	FCR	Mortality, %
Unchallenged control	1,797	2,507	1.409	2,331	3,576 [ab]	1.535 [b]	3.02 [b]
Challenged control	1,745	2,562	1.465	2,291	3,764 [a]	1.620 [a]	12.71 [a]
ChaSB	1,758	2,390	1.383	2,361	3,459 [b]	1.536 [b]	15.73 [a]
ChaSBEO	1,778	2,438	1.371	2,361	3,558 [b]	1.507 [b]	15.83 [a]
SEM	0.02	0.05	0.025	0.02	0.06	0.023	3.22
Probability	0.13	0.15	0.12	0.13	0.01	0.01	0.001

[a–b] Means with different superscripts in a column differ significantly ($P \leq 0.05$). Values are means of 8 pens (58 birds/pen). WG: weight gain; FI: feed intake; FCR: feed conversion ratio; ChaSB: challenged supplemented with sodium butyrate; ChaSBEO: challenged supplemented with sodium butyrate plus essential oils; SEM: standard error of mean.