Evaluation of potential alternatives to replace antibiotic growth promoters in broiler chicken diets

by

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ABSTRACT

The phasing out of antibiotic growth promoters (**AGP**) from livestock diets has increased the risk of enteric diseases in broilers; therefore, effective AGP replacements must be found. The objective of this Ph.D. thesis was to investigate three novel products as potential AGP replacements in broiler diets: chitosan oligosaccharides (**COS**), punicic acid (**PA**), and glucosamine-derived caramels. COS and PA were tested in broilers challenged with a natural, subclinical necrotic enteritis (**NE**) infection model. Birds were challenged with 15x the recommended dose of a commercial coccidiosis vaccine containing live *Eimeria* oocysts by gavage at 12 d, with a 24-hour feed withdrawal at 18 d. To investigate COS, a pilot study was conducted to evaluate the optimal molecular weight (14, 17, 25, 30, 95, 110, 180 or 220 kDa) and dietary inclusion level (0.2, 2 or 5 g/kg) compared with a Positive Control (**PC**; basal diet with antibiotic and coccidiostat) and Negative Control (**NC**; basal diet without medications). From this study, the most promising treatments to recover performance and mitigate NE were further investigated for their effects on gut health and immune response in broilers. Among the tested products, COS 95 kDa showed promise in enhancing broiler performance, increased the abundance of *Lactobacillus* species in the ceca compared to NC, enhanced the intestinal morphology integrity, and mitigated *Eimeria* infection at the same level as the PC. COS 95 kDa also contributed to a vigorous local immune response, increasing the serum levels of macrophage inflammatory protein-3α and interleukin-16 following the challenge. For the PA study, increasing levels of pomegranate seed oil (**PSO**), high in PA, were added to achieve a final PA concentration of 0.1, 0.25, 0.5, 1, 1.5, or 2% of the feed. Birds in these experimental treatments were compared to those fed PC and NC. Supplementation of PSO to achieve PA concentrations higher than 0.5% of the diet caused significant reductions in the feed intake, BW, and BW gain of broilers. In addition, PA at 2% predisposed the birds to more

severe NE intestinal lesions than PC birds following the challenge. For the glucosamine-derived caramels study, treatments included a Control (basal diet), Control plus glucosamine at 0.24% of the diet, and diets supplemented with glucosamine caramels: either Light Caramel produced at 50°C (**LC**), Brown Caramel produced at 90°C (**BC**), LC plus caramelized fructose (**LC+F**), or BC plus caramelized fructose (**BC+F**); each product was added at 0.08, 0.16 and 0.24% of the diet. Broilers were raised in wire-floored cages as a model to induce lameness. LC at 0.24% enhanced the BW of broilers at 10 d, and LC at 0.16% tended to enhance BW and BWG at 25 and 38 d ($P \leq 0.09$) compared to the Control. Regardless of dose, LC, BC, and BC+F reduced the lesions in the tibia of broilers at 40 d. None of the products tested were as effective as AGP for growth promotion and NE prevention. However, COS 95 kDa and LC should be further explored as a part of a strategy to replace in-feed AGP in broiler diets.

PREFACE

This thesis is an original work by Emanuele Goes. Emanuele was responsible for conducting the experiments, sample and data collection, laboratory work, statistical analyses, data interpretation, and thesis writing. Throughout this process, all experimental procedures and thesis writing were closely supervised by Dr. Douglas R. Korver. The research protocols of this thesis were approved by the University of Alberta Animal Care and Use Committee for Livestock and followed principles established by the Canadian Council on Animal Care guidelines and policies (CCAC, 2009). The animal care protocols for the research experiments used in this thesis were 1) AUP00003385: development of chitosan oligosaccharides as a nutritional bioactive ingredient in poultry feeds; 2) AUP00003160: development of functional yeast enriched in punicic acid for the reduced use of antibiotics in poultry, and 3) AUP00003587: bioactive glucosamine-derived caramels to improve locomotion problems and gut health in broiler chickens. Data from the chitosan oligosaccharides experiments are presented in Chapters 2 and 3, while punicic acid and glucosamine-caramel studies are presented in Chapters 4 and 5, respectively. Part of the data from these studies was presented and published as abstracts in the proceedings of the 2020, 2021, 2022, and 2023 Poultry Science Annual Meetings and in the 2021 Animal Nutrition Conference of Canada.

The challenge model used to induce necrotic enteritis in broilers in the studies reported in Chapters 2, 3, and 4 of this thesis was validated and published as follows: He, W., E. C. Goes, J. Wakaruk, D. R. Barreda, and D. R. Korver, “A poultry subclinical necrotic enteritis disease model based on natural *Clostridium perfringens* uptake.” *Frontiers in Physiology*, vol.13, article 788592. Emanuele was responsible for conducting one of the trials described in the paper and for laboratory analysis.

The chitosan oligosaccharide studies reported in Chapters 2 and 3 of this thesis are being submitted for publication in Poultry Science. Punicic acid and glucosamine-derived caramels have received funding for follow-up studies, and their effects on broilers will be further explored before publication.

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LIST OF ABBREVIATIONS

AGP	antibiotic growth promoters
AMP	antimicrobial peptides
AMR	antimicrobial resistance
ASV	amplicon sequence variant
BC	brown caramel
BC+F	brown caramel plus caramelized fructose
BCO	bacterial chondronecrosis with osteomyelitis
BMD	bacitracin methylene disalicylate
BW	body weight
BWG	body weight gain
COS	chitosan oligosaccharide
DD	deacetylation degree
DOFR	deoxyfructosazine
FAME	fatty acid methyl esters
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
GlcN	glucosamine
FI	feed intake
FCR	feed conversion ratio
FOS	fructo-oligosaccharides
FPD	footpad dermatitis
FR	fructosazine

GIT	gastrointestinal tract
GPR	G protein-coupled
IgY	egg yolk antibodies
IL	interleukin
IFN	interferon
kDa	kilodaltons
LC	light caramel
LC+F	light caramel plus caramelized fructose
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
M-CSF	macrophage colony-stimulating factor
MIC	minimum inhibitory concentration
MIP	macrophage inflammatory protein
MOS	mannan-oligosaccharides
Mw	molecular weight
NC	negative control
NE	necrotic enteritis
NF-κB	nuclear factor kappa
NSP	non-starch polysaccharides
PA	punicic acid
PC	positive control
PGN	peptidoglycans
PPAR	peroxisome proliferator-activated receptor

PRR	pattern recognition receptors
PSO	pomegranate seed oil
PUFA	polyunsaturated fatty acids
RANTES	regulated on activation, normal T cell expressed and secreted
ROS	reactive oxygen species
SCFA	short-chain fatty acid
VEGF	vascular endothelial growth factor
WHO	World Health Organization

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1. LITERATURE REVIEW

1.1 INTRODUCTION

Antibiotic growth promoters (**AGP**) have been used to increase the growth rates of farm animals since the 1940s. They played an important role in enhancing animal health status, uniformity, and production efficiency (Bedford, 2000). However, due to the emergence of bacterial resistance to antibiotics used to treat animal and human infections, the European Union officially banned the use of AGP for livestock animals in 2006 (Dibner and Richards, 2005). This decision, coupled with increased antimicrobial resistance concerns, stimulated other countries to create prohibitive legislation or enhance veterinary oversight of the use of AGP in animal diets. It also changed consumer perspectives, increasing demand for poultry products from antibiotic-free production (Dibner and Richards, 2005; Castanon, 2007).

Although the mechanisms of action of AGP are not fully understood, their growth-promoting effects appear linked to controlling intestinal and systemic inflammation, as well as inhibiting bacterial growth characteristics and virulence factors (Niewold, 2007; Broom, 2017a; Oh et al., 2019). Therefore, the removal of AGP from poultry production eliminated this preventive support, increasing the exposure of birds to potentially harmful pathogens and resulting in a recurring activation of their innate immune system. This increases the risks of clinical and subclinical enteric diseases in broilers (Broom and Kogut, 2018). One of the major enteric diseases in poultry that has become more prevalent since the withdrawal of AGP is necrotic enteritis (**NE**) (Williams, 2005; Timbermont et al., 2011), which causes losses of $6 billion annually worldwide (Wade and Keyburn, 2015). NE in poultry develops due to infection with pathogenic strains of *Clostridium perfringens* facilitated by predisposing factors (Annett et al., 2002; Keyburn et al., 2008; Wu et al., 2014; Bortoluzzi et al., 2019). This enteric disease causes a reduction in the performance and

welfare of broilers, increasing the mortality of the flocks and also increasing the risk of bacterial contamination of poultry products for human consumption (Timbermont et al., 2011; Grass et al., 2013).

Given the global shift towards antibiotic-free production and the significant impact of enteric diseases on the poultry industry, there is an urgent need to develop effective AGP alternatives in broiler production. Based on what is known about the mechanism of action of AGP, alternatives that can modulate gut health and the immune system of broilers may have the potential to maintain or enhance performance (M'Sadeq et al., 2015; Pham et al., 2020; Emami et al., 2021). Over the past two decades, extensive research has focused on the development of AGP alternatives. Several categories of products, such as prebiotics, probiotics, exogenous enzymes, organic acids, and phytogenics, are being explored as potential AGP replacements (Emami et al., 2012; Flores et al., 2016; Froebel et al., 2019; Zhang et al., 2021; Rivera-Pérez et al., 2021; Zhen et al., 2023). Although some of these products show potential, the results are still inconsistent, and their mechanisms of action remain not fully elucidated.

It is unlikely that a single alternative product will be able to compensate fully for the loss of AGP benefits in broiler production. Consistent success in AGP-free broiler production will likely depend on a combination of products with different mechanisms of action, complemented by effective management and biosafety practices (Gadde et al., 2017). Therefore, understanding the mechanisms of action of candidate products is essential to use them effectively. Moreover, exploring novel products and technologies in broilers under conditions similar to those in the field can help us to develop effective strategies to control or mitigate field issues, such as the occurrence of NE in broilers. This review explores the importance of maintaining broiler gut health to sustain

productivity in the absence of AGP. It also summarizes current knowledge about NE and potential non-antibiotic alternatives to prevent or mitigate enteric diseases in broilers.

1.2 A BRIEF HISTORY OF THE USE OF ANTIBIOTICS GROWTH PROMOTERS IN POULTRY DIETS

AGP have been used to increase the growth rates of farm animals since the 1940s. The first indications of the potential benefits of AGP on production efficiency in poultry were reported by Moore et al. (1946) when they observed that birds fed streptomycin had increased growth rates. After that, many other experiments with chickens were conducted in the early 1950s to test the effects of in-feed, subtherapeutic doses of antibiotics on growth performance (Stokstad and Jukes, 1950; Whitehill et al., 1950; Groschke and Evans, 1950). In 1951, the Food and Drug Administration (**FDA**) in the United States approved antibiotics in feed without a veterinary prescription, setting the stage for the wide use of antibiotics in poultry feed for the next 50 years (Jones and Ricke, 2003). The remarkable success of using AGP was established due to its potential to enhance animal growth performance regardless of the type of diet used, which was allied to the intensification of animal farming in the 1950s. Although the mechanisms of action of AGP were not fully understood at that time, the use of in-feed AGP played a significant role in enhancing animal health, uniformity, and production efficiency (Bedford, 2000). A meta-analysis conducted by Cromwell (2002) revealed that in-feed AGP increased growth rates by up to 16% and feed efficiency by up to 7% in young pigs. In broilers, a similar meta-analysis revealed that in-feed AGP increased body weight gain by 3.85% and decreased the feed conversion ratio by 3.6% in a 42-day period compared to birds raised without AGP. This reduced the production cost by $0.03 per bird (Cardinal et al., 2019). The AGP help birds to get closer to their maximum genetic potential for growth and, simultaneously, helping the industry meet consumer demand. However,

soon after the discovering that AGP could enhance growth and feed efficiency, concerns were expressed about a potential relationship between such use of in-feed antibiotics and the development of resistance by bacteria (Dibner and Richards, 2005; Castanon, 2007). Sub-therapeutic doses of antibiotics associated with long-term use in the feed could result in bacterial resistance to specific antibiotics or entire classes of antimicrobial agents. Thus, the main concern was related to the potential horizontal transfer of resistance-conferring genes from animal-associated bacteria to human pathogens, thereby posing a significant threat to public health (Dibner and Richards, 2005). This scenario could limit the choice of treatments for human diseases caused by these bacteria, increasing the potential for treatment failures and adverse clinical outcomes (van den Bogaard and Stobberingh, 2000). Although contrary opinions were raised, the World Health Organization (**WHO**) recognized the use of antibiotics in food animals as a public health issue and suggested that the use of AGP in classes also used in human medicine be terminated or rapidly phased out, by legislation, if necessary, unless and until risk assessments were conducted (WHO, 1997, 2000). After the initial speculations, several studies showed that there might be a connection between the use of AGP and the development of antimicrobial resistance among the microflora (Witte, 1998; van den Bogaard, 2001; Medeiros et al., 2011; Roth et al., 2019). These concerns lead to prohibitive legislation or guidance by individual countries and regions. Sweden was the first country to officially eliminate the use of antimicrobials for the growth promotion of livestock animals in 1986. Following this, Denmark banned the use of avoparcin in 1995 (Dibner and Richards, 2005). Later, the European Union imposed a ban on the use of AGP in 2006, thereby putting pressure on other countries to move toward antibiotic-free production in order to trade their products and meet consumer demand (Castanon, 2007). In 2017, the FDA officially implemented changes in the regulation of antibiotics use in animal feed and water in the United States, removing

production uses of medically important antibiotics for human health. In 2021, the FDA implemented greater veterinary oversight to ensure the proper use of antibiotics in animal production (Gens et al., 2022).

In Canada, antibiotics are categorized by their importance relative to human medicine. Four main categories are classified according to their ability to effectively treat a human infection (Table 1.1). In 2014, sub-therapeutic doses of antibiotics of category I (very high importance to treat human diseases) were eliminated from animal production. In 2018, those from category II (high importance to treat human diseases) were also eliminated. Category III antibiotics (medium importance to treat human diseases) are still allowed to be used to prevent poultry diseases in the field; however, only under a veterinarian's prescription and oversight (Chicken Farmers of Canada, 2018). So far, there is no restriction in the use of antibiotics of category IV (low importance or not used in human medicine) such as ionophores or in the use of chemical coccidiostats.

The ongoing process of removing AGP from animal diets remains a significant challenge for the poultry industry. This scenario has opened the door for a massive amount of research on the development of alternatives to antibiotics that can maintain or improve poultry health and performance (Huyghebaert et al., 2011; Gadde et al., 2017). Over the past two decades, several products and strategies have been evaluated in poultry for their potential to replace antibiotics. Although the benefits of many alternatives have been demonstrated, results vary significantly from farm to farm, leading to a lack of consistency (Gadde et al., 2017). Furthermore, products often show efficacy under research conditions but not in commercial settings. So far, no single product has yet demonstrated to be as widely effective as AGP.

1.3 POTENTIAL MODE OF ACTION OF ANTIBIOTIC GROWTH PROMOTERS

Understanding the biological mechanisms of action of AGP is essential in order to develop effective non-antibiotic alternatives for animal production. Even though numerous hypotheses have been proposed to explain how AGP allow animals to grow faster and more efficiently, the precise mechanisms of action are still not fully understood (Broom, 2017a). The challenge in understanding the actual mechanism of action of AGP is related to antibiotics having varying effects on animals of different species (e.g., poultry and pigs) raised in different environments, facing different challenges, consuming different diets, and consequently, with different immune status and intestinal microbiota. Preliminary theories have linked AGP efficacy to their antimicrobial action (Dibner and Richards, 2005). It was proposed that the direct effects of antibiotics on gut microbiota are related to 1) reduction of the total microbial density in the gastrointestinal tract (**GIT**), decreasing the competition for nutrients with the host, and reducing the levels of microbial metabolites that depress growth (such as ammonia and bile degradation products); 2) reduced exposure to potential pathogens and decreased incidence of subclinical infections; 3) increased absorption of nutrients due to a thinner intestinal epithelium (Gaskins et al., 2002; Butaye et al., 2003; Dibner and Richards, 2005). These arguments were strengthened when AGP supplementation was studied in germ-free animals (Coates et al., 1963; Gaskins et al., 2002). These investigations revealed that the administration of sub-therapeutic doses of antibiotics did not affect these animals, suggesting that AGP play a pivotal role within the microbiota rather than exerting direct influence at the animal level.

However, this theory was questioned by other scientists who proposed that AGP may act by directly inhibiting the negative effects of intestinal inflammation on the host (Niewold, 2007; Costa et al., 2011). According to Niewold (2007), AGP are administered at doses less than the

minimum inhibitory concentrations (**MIC**), meaning they are not supposed to have bacteriostatic effects. In contrast, it seems that antibiotics can be accumulated in phagocytic inflammatory cells, enhancing the intracellular killing of bacteria, and inhibiting, to some extent, the innate immune responses. Phagocytic cells can accumulate antibiotics at 10- to 100-fold higher than the surrounding environment. This, in turn, results in more effective clearance of pathogens and a lower necessity of pro-inflammatory cytokine production than in untreated animals (Niewold, 2007). Since the activation of the immune system can divert dietary nutrients away from growth purposes in support of inflammatory-related processes (Broom and Kogut, 2018), treated animals have an advantage in getting closer to their maximum potential for growth.

Costa et al. (2011) conducted an experiment on mice challenged with *Citrobacter rodentium* to test this immunomodulatory theory. The mice given chlortetracycline (a common AGP used for mammal livestock) had up-regulation of transcription levels of Th1 and Th17 inflammatory cytokines in response to the *C. rodentium* infection. Also, they experienced less weight loss than non-AGP-supplemented mice. AGP supplementation of broiler chickens challenged with lipopolysaccharide (**LPS**) or co-infected with *Eimeria maxima* and *C. perfringens* increased performance and reduced levels of pro-inflammatory cytokine (IL-1β, IL-2, IL-6, IL-8, and IL-17A) transcripts compared to non-AGP-supplemented birds (Oh et al., 2019). The authors concluded that in-feed AGP may increase poultry growth, in part, through down-regulation of pathogen-induced inflammatory responses.

Later on, another theory suggested that the sub-inhibitory effects of antibiotics (i.e., effects when antibiotic concentrations are below MIC) on microbiota are responsible for the main effects of AGP (Broom, 2017a). It seems that the administration of antibiotics, even below the MIC, can affect bacterial growth characteristics, protein expression, and virulence factors (e.g., cell adhesion

capability, toxin production, biofilm formation) such that bacteria are rendered more susceptible to host immune responses (Broom, 2017a). Therefore, the direct effect on microbiota growth would allow the immune system to eliminate potential pathogens and prevent inflammatory processes that could hinder bird growth performance.

A more recent theory suggests that AGP can promote growth and prevent diseases in poultry by their hormetic effects on the mitochondria of intestinal epithelial cells (Fernández Miyakawa et al., 2024). Hormesis refers to a dose-response of an organism to a stressor agent (e.g., a chemical compound or environmental factor) in which a low dose promotes beneficial effects to the organism while high doses promote damage or toxicity (Mattson, 2008). This mechanism seems fundamental for developing stress resistance and for the evolution of most living organisms. In the proposed theory, the authors suggest that AGP exert a low level of stress on the mitochondria, stimulating an adaptive physiological response, which enhances the function and defence mechanisms of the cells to compensate for the disruption in homeostasis. These responses can be spread to distant cells via mitochondrial stress-induced cytokines or mitokines (soluble mediators), coordinating a range of adaptive responses, and enabling the organism to adjust to stress more effectively (Quirós et al., 2016). In addition, the authors suggest that the presence of AGP in the gut can stimulate certain microbial strains, such as *Lactobacilli*, due to a hormetic defensive tactic to increase their resilience, providing them with a competitive advantage (Storelli et al., 2011). While beneficial to the host in various ways, in the presence of AGP, symbiotic microbiota can produce metabolites and by-products (e.g., reactive oxygen species) that serve as stressors and function as a signalling agent to simulate an adaptive response at the mitochondrial level (Wentworth et al., 2011; Jones et al., 2013). Therefore, they hypothesized that direct and indirect sub-therapeutic AGP regulation of mitochondria functional output can regulate homeostatic

control mechanisms similarly to those involved with disease tolerance. Ultimately, these tolerance mechanisms promote a dynamic equilibrium in the intestine, reducing the energetic cost of gut inflammation and enhancing growth and poultry feed efficiency (Fernández Miyakawa et al., 2024).

Considering the evidence behind each proposed theory, it is clear that AGP are likely to have a complex and multifactorial mode of action that is not only limited to the microbiota or the host but is more likely to be a combination of both. Given the complexity amongst diet, microbiota, immune system, and host specificities, experiments to test the actual mode of action of AGP are challenging. In addition, administering different antibiotics as AGP can also result in different outcomes (e.g., favour different bacterial communities in the gut). According to Brown et al. (2017), a truly mechanistic approach would ideally include studies in animal models possessing a well-characterized microbiota and physiology, such as rodent models, with validation in livestock. In addition, it would be important to consider the variables involved in animal production (e.g., psychological and physiological stresses, changes in diet) and the class of antibiotics used (i.e., bactericidal vs bacteriostatic, broad vs narrow spectrum). Research in these areas would help us better understand the AGP mechanisms of action and create rationale-based alternatives to improve poultry gut health, welfare, and safe protein sources for human consumption.

1.4 IMPLICATIONS OF ANTIBIOTIC GROWTH PROMOTER REMOVAL FROM BROILER DIETS

The global human population is anticipated to reach 9.7 billion by 2050 (UN, 2022), driving an increased demand for high-quality livestock products. This necessitates the development of strategies to optimize livestock production while ensuring animal welfare and health. Poultry meat, with a worldwide per capita consumption of 32.1 kg/year, stands out as the most widely consumed

meat globally (OECD, 2023). Over the past 40 to 50 years, both poultry production and consumption have markedly risen and are projected to continue growing, particularly in developing countries (Wahyono and Utami, 2018). This trend positions chicken meat as an important and valuable protein source for the expanding global population.

The previous success in the increase of poultry production was partly due to the use of in-feed AGP. This practice helped to reduce production costs and meet the consumer demand for affordable protein sources while also playing an important role in maintaining poultry health. However, using sub-therapeutic doses of antibiotics in animal feed raised concerns about development of antimicrobial resistance (**AMR**) in pathogenic bacteria. This, in turn, could potentially undermine the therapeutic efficacy of antimicrobial agents in both veterinary and human medicine (Dibner and Richards, 2005; Castanon, 2007). According to Murray et al. (2022), it was estimated that in 2019, 4.95 million human deaths were associated with AMR globally. Other reports estimated that this number could reach 10 million deaths by 2050, surpassing the number of cancer deaths (O'Neill, 2014, 2016). Although there is a diversity of opinions about the use AGP in livestock production and their impact on the development of AMR in humans (Hao et al., 2014), various countries have banned or adopted restricted use of AGP in animal feed (Castanon, 2007; Wallinga et al., 2022; Da Silva et al., 2023). As the issue of AMR has escalated into a pressing public health concern, there has been a noticeable surge in consumer demand for poultry products raised without antibiotics. This exerted significant market pressure on grocery stores, restaurants, suppliers, and, ultimately, farmers, compelling them to address consumer expectations (Patel et al., 2020).

The decrease or elimination of the use of AGP is not a matter of debate anymore but is a reality of current poultry production. Although addressing an important One Health problem, it also brings

another challenge. The elimination of the preventive support of AGP from broiler production increases the exposure of birds to potentially harmful pathogens. This scenario has increased the risks of clinical and subclinical bacterial infections, which can divert of dietary nutrients away from growth or production purposes in support of intestinal or systemic inflammation (Broom and Kogut, 2018). Consequently, this also affects animal welfare, mortality rates, and sustainability (Casewell, 2003). This scenario exerts pressure on the poultry industry, which is responsible for providing sufficient, affordable, sustainable, and safe protein sources for the growing human population.

1.4.1 Implications of immune system activation on broiler performance and health

The primary role of the immune system is to safeguard the bird against both infectious and non-infectious challenges posed by external molecules or organisms. The immune system is divided into two primary elements: innate immunity and adaptive immunity (Korver, 2012). Innate immunity is the first line of defense against invading pathogens. It consists of antigen recognition, cell recruitment, phagocytosis, and antigen destruction, followed by antigen presentation to other immune cells and activation of the adaptive immune system when necessary (Lillehoj and Lee, 2012). The innate response recognizes generic patterns and non-self molecules in a non-specific manner and has a limited immunologic memory (Kumar et al., 2011; Boraschi and Italiani, 2018; Göbel and Smith, 2022). That is, when a specific pathogen is encountered, the innate response will consistently be triggered (Korver, 2012). Depending on the situation, the innate immune response can directly control the replication or spread of bacteria and viruses by inducing phagocytosis or antimicrobial products. However, when a specific immune response is required, the innate immune response can instruct the activation of adaptive immunity by inducing antigen presentation and co-stimulatory molecules (Kogut, 2009).

Unlike innate immunity, adaptive immunity is specific, heterogeneous, and has long-lasting memory. This arm of the immune system recognizes specific molecular features of pathogens through antigen receptor-antigen interactions, which allows for targeted, specific immune responses. Adaptive immunity involves two types of responses: humoral and cell mediated (Wigley, 2013). Cell-mediated responses are specialized in the elimination of intracellular pathogens. In this case, the cells infected with a pathogen are destroyed by an effector cell, such as an activated T cell.

On the other hand, humoral immunity is mediated by antibodies produced by B cells in response to extracellular antigens (Erf, 2004). The acquired immune response is initiated by recognizing antigens by specific receptors on T and B lymphocytes. This leads to the activation of adaptive immunity, resulting in antibody production, cytotoxic T cell-mediated killing of infected cells, and various helper T cell actions (Korver, 2006). The most important outcome of an adaptive immune response is the creation of memory B and T cells that provide long-term specific protection against subsequent infections with a pathogen bearing the same antigens (Kogut et al., 2020).

The activation of the adaptive immune system has minimal effect on the energy and nutrient requirements of the bird. Conversely, the activation of the innate immune system is more nutritionally demanding (Klasing, 2007; Korver, 2012). This is because, when activated, the innate response can trigger thermogenesis, effector cell invasion, heightened blood supply, and the generation of cytokines and chemokines, each imposing substantial energy and resource demands on the organism (Klasing, 1998). In addition, the production of acute-phase proteins by the liver (during acute inflammation) is considered the most nutrient-costly part of the immune response (Klasing, 1998).

Inflammation is a critical innate immune process that seeks to contain an infection, activate adaptive immunity, and repair damaged tissue in order to make the bird return to a homeostatic state (Broom and Kogut, 2018). Although this process is essential for developing immune resistance and keeping birds healthy, the constant activation of the innate immune system is not desirable. During inflammation, the innate immune cells release signals to recruit distant immune cells to the site of the infection and to change the metabolism of the bird (Korver, 2012). The metabolic changes aim to create an inhospitable environment for pathogens by increasing metabolic rate (resulting in fever), reducing feed intake, and breaking down skeletal muscle to synthesize acute-phase proteins in the liver. At the same time, a portion of the dietary nutrients is diverted to support the immune responses. Klasing (2007) estimated a diversion of around 10% of nutrients used during an acute phase immune response in chickens. Other authors have estimated the nutrient cost during inflammation to be 1.3x greater than the nutrients required for maintenance (Webel et al., 1998). Moreover, the requirements for specific amino acids such as threonine (part of mucins and acute phase proteins) and cysteine (part of acute phase proteins and glutathione) also increase during acute inflammation (Klasing, 2007; Corzo et al., 2007). This reallocation of nutrients and reduced feed intake during inflammatory processes directly affect broiler performance. Relative to non-challenged birds, chickens challenged with LPS had a 22% reduction in BW gain during the challenge period, with 59% of the loss due to a decreased feed intake and the remaining 41% attributed to immune response-related factors (Jiang et al., 2010).

In addition to the performance reductions, the increased pathogen exposure allowed with the removal of AGP, often combined with physical and physiological stress or dietary changes, can trigger shifts in the microbiota, leading to enteric disorders (Tsiouris et al., 2015a; Zaytsoff et al., 2020; Ye et al., 2023). These microbial shifts create favorable conditions in the gut (e.g., increase

in pH, substrate, and adhesion sites) for the overgrowth and colonization of opportunistic pathogenic bacteria, such as *C. perfringens* (Petit et al., 1999). This opportunistic bacteria can colonize the GIT of birds immediately after hatch, live without harming the host or other microorganisms, and can be tolerated by the immune system (Nagano et al., 2012; Divangahi et al., 2021; Abd El-Hack et al., 2022). However, in the presence of predisposing factors that alter the gut environment in their favor, they can rapidly activate virulence factors. This gives them a selective advantage over other bacteria and facilitates overgrowth, making it difficult for the immune system to mount an effective response in time to prevent the damage caused by the virulence factors (Shimizu et al., 2002; Fasina and Lillehoj, 2019; Mehdizadeh Gohari et al., 2021). This, in turn, can lead to subclinical and clinical enteric diseases (e.g., necrotic enteritis) that reduce the performance and welfare of broilers, increase mortality of the flocks, and also increase the risk of contamination of poultry products for human consumption (Dahiya et al., 2006; Wu et al., 2010; Grass et al., 2013). Although NE in broilers is not a novel challenge, removing in-feed AGP from broiler diets has increased the risk of NE development. As a result, this disease has become, arguably, the most important and costly poultry enteric disease (Williams, 2005; Wade and Keyburn, 2015).

1.5 NECROTIC ENTERITIS IN BROILER CHICKENS

NE is a multifactorial and complex enteric disease caused by the opportunistic pathogen *C. perfringens*, a Gram-positive, rod-shaped, spore-forming, anaerobic bacteria (Keyburn et al., 2008, 2010; Shojadoost et al., 2012). *C. perfringens* strains are currently classified into seven toxicogenic types: A, B, C, D, E, F, and G. The differences among these strains are related to their ability to produce different types of toxins, such as α, β, ε, ι, NetB, and enterotoxins (Table 1.2) (Rood et al., 2018). Not all strains induce NE in chickens, and some are part of the commensal microflora

of healthy birds. The pathogenicity of *C. perfringens* is related to the presence of host-specific virulence factors (Timbermont et al., 2011). Although it is still unclear which strains are involved in NE pathogenesis in broilers, it seems that NetB-positive strains (toxicogenic type G) are the most closely related to the disease occurrence (Keyburn et al., 2008, 2010; Rood et al., 2016).

Besides toxin production, *C. perfringens* has other virulence factors closely related to their capability to induce NE. These virulence factors are important for bacterial attachment to the mucosa (adhesins) and providing nutrients for their rapid proliferation (degradative enzymes) (Wade et al., 2015, 2016, 2020; Li et al., 2016; Lepp et al., 2021). However, the mere presence of these bacteria in the GIT does not indicate disease. Predisposing factors that cause intestinal disturbance are usually necessary to create a favorable environment for these bacteria to colonize, grow, replicate, and produce toxins that cause NE (Moore, 2016). The NE predisposing factors are discussed in more detail in the next section.

NE may occur as an acute clinical or subclinical (chronic) condition (Van Immerseel et al., 2004). The clinical disease is characterized by a sudden increase in flock mortality, often without premonitory signs. However, symptoms such as depression, dehydration, ruffled feathers, diarrhea, and reduced feed intake may accompany it. The subclinical form is the most common and mainly responsible for economic losses. In this condition, the chronic damage to intestinal mucosa caused by *C. perfringens* leads to intestinal inflammation and a decrease in digestion and absorption of nutrients, which reduces performance (Van Immerseel et al., 2009; Shojadoost et al., 2012). In broilers, NE typically occurs between 3 and 4 weeks of age, contributing to a global economic loss estimated at $6 billion annually (Wade and Keyburn, 2015).

1.5.1 Necrotic enteritis predisposing factors in broilers

Many predisposing factors increase the incidence and severity of the NE in broilers (Figure 1.1). Overall, any factor that changes the normal physical structure or environment of the GIT or causes immune suppression can be a potential NE trigger (Broom, 2017b). Among these factors, the induction of coccidiosis is most commonly used experimentally to cause NE in broilers because of its close relation with NE development in the field (Dierick et al., 2021). Coccidiosis is an enteric disease caused by various species of parasitic *Eimeria* (Mesa-Pineda et al., 2021). As a part of their life cycle, *Eimeria* oocysts invade the host enterocytes to mature and replicate. The damage caused to the host epithelium leads to the leakage of serum into the gut and stimulation of mucus production, providing nutrients and adhesion sites for *C. perfringens* proliferation (Collier et al., 2008; Wade et al., 2015).

Specific dietary components can also stimulate the occurrence of NE. The use of ingredients rich in soluble non-starch polysaccharides (**NSP**), such as wheat, barley, and oats, can increase the viscosity of the digesta, intestinal transit time, and mucus production (Annett et al., 2002; Kleessen et al., 2003). Therefore, enzyme diffusion is impaired, reducing the digestion and absorption of nutrients and providing time for *C. perfringens* to acquire the nutrients they need (Broom, 2017b). As part of their virulence factors, *C. perfringens* produces several mucolytic enzymes that allow them to degrade and use the nutrients present in the host mucus (Shojadoost et al., 2012).

On the other hand, *C. perfringens* lacks genes for the biosynthesis of many amino acids. Because of that, they need to acquire these nutrients from the environment or from the host (Shimizu et al., 2002). Therefore, high protein levels (especially animal protein sources) and poorly digestible protein in the diet are also risk factors for NE. Several models have used fish meal to increase dietary protein or poorly-digestible protein to increase protein fermentation in the hindgut,

inducing changes in the microbiota composition and favoring the growth of *C. perfringens* (Drew et al., 2004; Palliyeguru et al., 2010; Stanley et al., 2012; Wu et al., 2014). Although the preference for fish meal is not clear, this ingredient is richer in glycine and methionine than other protein sources (Drew et al., 2004). Glycine is among the amino acids that stimulate pathogen growth and production of alpha toxin and is positively correlated with the number of *C. perfringens* in the gut (Dahiya et al., 2005; Xue et al., 2017a). Methionine, while not a required nutrient for *C. perfringens*, is highly stimulatory to growth and is required for *C. perfringens* sporulation (Muhammed et al., 1975).

Factors related to immune suppression can also increase the incidence of NE in broilers. The peak risk of NE outbreaks (about 3 to 4 weeks of age) coincides with the time when maternal antibodies are disappearing from circulation (Patterson et al., 1962; Gharaibeh and Mahmoud, 2013), which increases susceptibility to infection or proliferation of *C. perfringens* (Moore, 2016). Other diseases such as Marek's disease, infectious bursal disease, chicken infectious anemia, and avian leukosis are major immunosuppressors and causes apoptosis and/or necrosis of lymphoid cells and induce the malfunction of immune response regulation (Hoerr, 2010; Li et al., 2023). This, in turn, increases the severity of NE. Moreover, additional stress on birds, such as high stocking density, heat or cold stress, feed mycotoxins, and others diminish innate and adaptive immune responses and cause shifts in the gut microbiota favoring *C. perfringens* proliferation and NE (Antonissen et al., 2014, 2015; Tsiouris et al., 2015a; b, 2018; Sersun Calefi et al., 2019).

1.5.2 The pathogenesis of necrotic enteritis in broilers

The presence of the predisposing factors is highly associated with NE pathogenesis. The enteric disturbance caused by the predisposing factors typically results in increased mucus production by epithelial cells to prevent bacterial colonization (Collier et al., 2003). However, *C. perfringens*

produces several mucolytic enzymes, including sialidase and zinc metalloprotease (mucinase). These enzymes break down the sialic acid and glycoprotein in the mucus and epithelial cell surfaces, generating nutrients for their growth (Li and McClane, 2014; Li et al., 2015; Wade et al., 2020). This allows them to form localized microcolonies on the mucosal surface and start their proliferation (Prescott et al., 2016). Once the population reaches a sufficient density, they send out a signal of small auto-inducing molecules that up-regulate the expression of the VirR-VirS two-component system and an accessory gene regulator (*agr*) quorum-sensing system that regulates virulence factors and related metabolism genes (Cheung et al., 2010; Ohtani and Shimizu, 2015). The VirR-VirS two-component signal transduction system governs the expression of the *netB* gene, which encodes the NetB toxin, and also several other *C. perfringens* virulence-associated genes (Cheung et al., 2010). This strategy is an efficient environmental adaptation to help the pathogen thrive since the NetB toxin will be produced only when *C. perfringens* reaches a high concentration in the gut. With a high *C. perfringens* population, the nutrients to keep these bacteria proliferating would be scarce. Thus, NetB toxin is produced as it causes necrotic damage to the host tissue, causing the leakage of cellular content from the host cells, providing nutrients to support the growth and proliferation of *C. perfringens* (Cheung et al., 2010). The *netB* gene is situated in large conjugative plasmids closely related to other toxin plasmids found in *C. perfringens*. The ability to transfer these plasmids through conjugation suggests that horizontal gene transfer is involved in spreading *netB* genes from *netB*-positive to *netB*-negative strains, effectively increasing the virulence of these strains (Rood et al., 2016). In addition to *netB*, a positive association of virulence genes such as *pfoA* (associated with cell membranes disruption), *cpb2* (unelucidated), *tpeL* (cytotoxicity), and *cna* (collagen adhesion) variants have also been linked to NE-inducing *C. perfringens* isolates (Kiu et al., 2019).

Another essential feature of the *C. perfringens* involved in the induction of NE is their capability to adhere to extracellular matrix proteins. The numerous extracellular matrix proteins exposed by the NetB-induced damage are the target of adhesins that allow the *C. perfringens* to adhere and continue the damage in the surrounding tissues (Hitsumoto et al., 2014; Wade et al., 2016). Wade et al. (2015) identified that virulent, NetB-positive *C. perfringens* strains adhere to collagen types IV and V and gelatin in vitro. The authors associated this ability with a putative fimbrial adhesin VR-10B operon. In addition, *C. perfringens* produces an adhesive pilus, which is required for adhering to the intestine, and encoded within the VR-10B chromosomal locus (Lepp et al., 2021). During *C. perfringens* infections, the changes caused in the intestinal environment also led to higher concentration of other pathogenic organisms (e.g., *Prevotellaceae*, *Escherichia-Shigella,* and *Salmonella* Typhimurium), enhancing disruption of the intestinal mucosal barrier, intestinal inflammation, as well as toxin and bacterial translocation to bloodstream (Shivaramaiah et al., 2011; Du et al., 2015; Li et al., 2017b; Emami et al., 2020). Bacterial toxins can reach other organs, such as the liver and brain, and eventually cause enterotoxemia (Li et al., 2016).

The ability of pathogenic strains of *C. perfringens* to produce each of these virulence factors makes them one of the fastest-growing bacterial pathogens known (Shimizu et al., 2002) and responsible for significant losses in the poultry industry. While NE remains a complex disease with a pathogenesis that is still not fully understood, there is an urgent need to develop non-antibiotic alternatives to prevent NE in broilers. A promising avenue for addressing this challenge involves a strategic focus on enhancing gut health (M'Sadeq et al., 2015). By prioritizing research and innovations in this area, we can pave the way for effective solutions to mitigate the impact of NE on broiler production and health.

1.5.3 Experimental induction of necrotic enteritis in broilers

In recent years, interest has increased in understanding the pathogenesis of NE and exploring non-antibiotic prevention methods. Many studies on NE involve experimentally inducing the disease. However, the multifactorial and complex nature of NE makes it challenging to reproduce (Shojadoost et al., 2012). Consequently, various methodologies have been used to induce NE, leading to varied results (Dierick et al., 2021). Different researchers have different reasons for reproducing NE, which impacts the design of the studies and the severity of the diseases (clinical vs subclinical) induced. The experimental induction of NE can be used to study its pathogenesis, *C. perfringens* virulence factors during NE, effects of antimicrobial drugs, test potential vaccines or AGP replacements, and study the effects of different predisposing factors on disease development (Shojadoost et al., 2012). The appropriate selection of the NE infection model and experimental design is crucial for obtaining reliable results. For example, if the research intends to test potential vaccines to prevent NE, the infection model should not incorporate immunosuppressive diseases as a predisposing factor, as it can result in confounding effects. Design considerations will also include the need for appropriate control groups and isolated facilities to ensure the lack of spread of infection between control and infected birds (Shojadoost et al., 2012). Preferably, all challenged animals should develop the characteristic necrotic lesions without manifestation of severe clinical disease or mortality to maintain experimental sample sizes and statistical power (Dierick et al., 2021).

The key distinguishing factor among the infection models lies in the extensive array of predisposing factors used to favour the conditions for *C. perfringens* growth and cause NE in broilers (Tables 1.3 and 1.4) (Dierick et al., 2021). As aforementioned, the list of confirmed predisposing factors is long and often includes infection with *Eimeria*, dietary changes (e.g., NSP,

animal protein, anti-nutritional factors), immunosuppression (e.g., stress, mycotoxins, viruses, high doses of vaccines), and management factors (e.g., stocking density, litter, feeding regimen) (Moore, 2016). These factors can be implemented alone or in combination, and they are often combined with the administration of pathogenic *C. perfringens* for consecutive days (Table 1.3).

The induction of coccidiosis is the most commonly used approach to induce NE (Dierick et al., 2021). Typically, virulent *Eimeria* species (e.g., isolated from field outbreaks) or high doses of live coccidiosis vaccine are used. Certain species, including *E. brunetti*, *E. maxima*, *E. necatrix*, and *E. tenella*, can elicit more severe diseases than others (Shojadoost et al., 2012). Consequently, some models prefer to use these specific species. Since *Eimeria* oocysts can cause initial damage to the intestinal epithelium, creating favorable conditions for *C. perfringens* proliferation, models usually administrate both *Eimeria* and *C. perfringens* to induce NE experimentally (Collier et al., 2008; Shojadoost et al., 2012). However, there is no consistency among studies regarding the time of administration, frequency, and doses used (Table 1.3). Dierick et al. (2021) studied the relationship between the timing and frequency of *Eimeria* infection and NE development. They observed pronounced subclinical NE lesions when the coccidia vaccine was administered 2 and 4 days before *C. perfringens* infection, compared to simultaneous administration of the vaccine and *C. perfringens*. Therefore, it is important that for models using coccidiosis as a predisposing factor for NE, the damage caused by *Eimeria* parasites occurs before the challenge with *C. perfringens* for successful induction of the diseases (Shojadoost et al., 2012). However, the optimal dosage of *Eimeria* oocysts and the concentration of *C. perfringens* necessary to be administrated to induce the disease in broilers are still unclear.

In contrast to most studies, some researchers were able to experimentally induce NE using only *C. perfringens* type G strains without the use of predisposing factors (Sarmah et al., 2021; Mohiuddin

et al., 2021). Mohiuddin et al. (2021) found that administrating *netB*-positive strains isolated from field outbreaks without predisposing factors caused moderate to severe NE lesions in broilers. When *C. perfringens* was provided along with *Eimeria* and fish meal, the disease severity did not change compared to the administration of *C. perfringens* alone. Although this model successfully induced NE, it is important to note that the authors intended to cause clinical NE in broilers. It may require high doses of virulent bacterial cultures and may be particularly useful to test vaccines or study the *C. perfringens* virulence factors associated with NE pathogenesis.

In the case of studies aiming to test the efficacy of potential AGP replacements in preventing or mitigating the NE in broilers, it is important to select a model of infection that closely mimics the field conditions under which NE occurs (He et al., 2022). However, many methodologies still use conditions that do not represent standard industry practices (Wilson et al., 2018). This is especially true when dietary changes are used as predisposing factors. For example, the use of diets with ingredients rich in NSP without the addition of NSP enzymes (e.g., xylanase and β-glucanase) or increasing crude protein using high levels of animal protein, such as fish meal (sometimes up to 50%) do not align with industry practices. In addition, the administration of single strains of virulent pathogenic *C. perfringens* for consecutive days does not represent the natural progression of NE in the field. As *C. perfringens* is ubiquitous, the natural infection may be caused by different strains that will become virulent according to changes in the environment (e.g., intestinal dysbiosis) (Prescott et al., 2016). Although NetB-positive strains of *C. perfringens* are the most correlated with NE occurrence, some studies were able to induce the disease with NetB-negative strains, indicating the possibility of other toxins being involved in the development of NE (Li et al., 2017b; Zhang et al., 2019; Shini et al., 2020). Therefore, putative AGP replacements aimed at

preventing NE using infection models that do not represent the field condition in which NE takes place can not guarantee efficacy in actual field conditions.

Models mimicking natural *C. perfringens* infection have been explored in an attempt to replicate closely the field conditions in which NE occurs (Table 1.4; Abildgaard et al., 2010; Fernando et al., 2011; Paiva et al., 2014; Emami et al., 2019; He et al., 2022). Natural infection approaches involve exposing birds to NE predisposing factors and relying on the spontaneous overgrowth of *C. perfringens* pathogenic strains in the gut. In addition to more closely simulating field conditions, these models can facilitate the disease induction protocol as the natural approach does not require the production of fresh *C. perfringens* colonies and application for consecutive days for the animals (He et al., 2022). Therefore, utilizing models to induce NE based on the natural infection of *C. perfringens* can be a valuable instrument for replicating the natural progression of the disease, simulating the field conditions in which NE occurs, and more effectively testing alternatives to AGP to prevent NE in broiler chickens.

1.6 POULTRY GUT HEALTH

The interest in gut health has increased following the pressure to remove in-feed AGP from animal production. Although the precise mechanism of action of AGP has still not been fully established, their withdrawal from poultry diets is associated with an increased incidence of intestinal disorders (Kogut et al., 2017). An example is the increase in the incidence of NE in poultry flocks following the phasing out of AGP. Before the removal of AGP from animal diets, NE was not considered a major enteric disease in poultry; however, it has grown in prominence and is currently one of the most important enteric disease in poultry (Van Immerseel et al., 2016). Owing to the significant losses NE causes to the poultry industry, attention is now being focused on preventing and controlling the disease using management and gut health strategies (Adhikari et al., 2020).

The GIT is the organ system with the largest exposed surface area, which is continually exposed to a wide variety of potentially harmful pathogens and substances. Because of that, the GIT must act as a selective barrier, reducing exposure to environmental toxins and pathogenic microorganisms while allowing nutrient absorption and waste secretion (Turner, 2009). In addition to the physical barrier, the gut also provides the ideal environment for the growth of a diverse microbiota that provides not only a second barrier against pathogen colonization but also regulates immune system development and provides metabolites to maintain intestinal homeostasis and host nutrition (Kogut et al., 2020). The GIT also acts as an active immune organ, containing a greater number of resident immune cells than any other organ in the host (Mowat and Viney, 1997; Kogut et al., 2020). The mucosal immune system has a highly regulated network of innate and adaptive elements, which can rapidly respond to foreign substances or insults (Honda and Littman, 2016). Lastly, the gut also exerts neuroendocrine function as it contains many neurons, gut hormones, and secondary messengers, so that it can regulate several physiological functions of the host. Thus, a healthy intestinal tract is crucial for animal health and productivity. Given the complexity of GIT, the definition of "intestinal health" or "gut health" is not yet clearly defined. Recently, Kogut et al. (2017) have proposed a meaningful definition of gut health as the ability of the gut to perform normal physiological functions and maintain homeostasis, thereby enhancing its resilience to both infectious and non-infectious stressors. In order to address gut health, a holistic approach that considers the interconnection between the diet, mucosa, microbiome, and immune system is necessary (Jha et al., 2019). A complex interrelationship exists among these areas, and a multidisciplinary approach is required to develop nutritional strategies that can enhance the resilience of farm animals against environmental and physiological challenges that they may face throughout their productive life (Celi et al., 2017).

1.6.1 Intestinal immune system

The intestinal immune system features a robust protective mucosal layer, interconnected epithelial cells, and immune components such as secreted IgA and antimicrobial peptides (**AMP**) (Kogut et al., 2017). The mucosal layer is loose on the outer face (lumen side), allowing colonization by microorganisms, while the inner face is compact and repels most bacteria. The mucus layer acts as a barrier to prevent gut microorganisms from entering the intestinal epithelium, serving as the first line of defense against infections (Hooper, 2009; Coleman and Haller, 2018). Connected with the inner face of the mucosal layer, a single layer of epithelial cells separates the lumen from the sterile subepithelial tissue (Kogut et al., 2020). The intestinal epithelium assists in the absorption of nutrients through the enterocytes (epithelial cells) while providing a physical barrier to protect against pathogen invasion and bacterial translocation from the lumen to the bloodstream (Snoeck et al., 2005; Miron and Cristea, 2012). In addition, the enterocytes are also considered part of the cellular component of innate immune responses, as they can express pattern recognition receptors (**PRR**), including Toll-like receptors and Nod-like receptors that sense conserved pathogen molecular signatures (e.g., genetic sequences, bacterial cell wall components, toxins) (Abreu, 2010; Keestra et al., 2013). These receptors remain non-responsive to commensal microbiota, but during infection or environmental insults, they can trigger a cascade of events, including the production of cytokines and chemokines to drive an inflammatory response (Keestra et al., 2013; Smith et al., 2022). The epithelial cells are linked to one another by a complex of proteins (claudins, occludins, and zona occludens protein 1) known as the tight junction complex (Alizadeh et al., 2022). This protein complex forms a continuous luminal surface and seals intercellular spaces from the external environment. Below the epithelial barrier lies the lamina propria, which is the area where professional immune cells reside, including macrophages, dendritic cells, and

lymphocytes (Abraham and Medzhitov, 2011). When a pathogen infection causes breaches in the epithelial barrier, the innate immune system identifies them through the PRR in the epithelial cells and lamina propria immune cells. The antigens are then presented to the naïve T cells that reside in the gut lymphoid tissues, such as Peyer's patches and cecal tonsils, where the differentiation of the effector T cell subset occurs (e.g., Th1, Th2, Th17, Treg) (Abraham and Medzhitov, 2011; de Geus and Vervelde, 2013; Smith et al., 2022). This cascade of immune responses is essential to eliminate invaders, maintain homeostasis, and protect against prolonged inflammation.

1.6.2 Intestinal microbiota

The chicken GIT is home to a complex microbial community, including commensal and symbiotic microbes. The host provides an ideal anatomical niche for bacteria attachment and growth, nutrients and substrates, and immune tolerance, allowing microbiota to thrive (Kogut, 2019). In turn, the microbiota contributes to the host by producing essential nutrients, promoting intestinal development, enhancing gut integrity, providing competitive exclusion of pathogens, modulating the regulation and function of the immune system, and sustaining homeostasis by controlling physiological inflammation (Lan et al., 2005; Choct, 2009; Rinttilä and Apajalahti, 2013; Oakley et al., 2014). Thus, the gut microbiome can be considered an additional complex organ within the host, containing about 40 to 50 times more genes than the entire chicken genome (Kogut, 2019).

In chicks, gut colonization starts after hatch, and the microbiota composition and diversity will be developed over time, influenced by environmental, dietary, and management factors. Adult birds have a more balanced and diverse microbiota, which increases resilience to enteric disturbances (Yeoman and White, 2014). While the microbiota maintains structural stability in a homeostatic state, it also exhibits remarkable plasticity. The microbial community can be readily changeable by diet, ingestion of antibiotics, infection by pathogens, and other environmental insults (Kogut,

2022). The diet performs a critical function in shaping and regulating the gut microbiota. As various bacterial species exhibit distinct substrate preferences and growth requirements, the microbial community in the GIT is largely influenced by the chemical composition of the digesta (Apajalahti et al., 2004). In homeostasis, the gut microbiota facilitates the digestion and fermentation of indigestible fractions of the diet, such as fibre, producing valuable nutrients for the host, such as amino acids, B and K vitamins, and short-chain fatty acids (**SCFA**; acetate, propionate, and butyrate) (Rinttilä and Apajalahti, 2013). Among the SCFA, butyrate seems to be the most related to the maintenance of intestinal homeostasis. Butyrate is the primary source of energy for colonic epithelial cells and is essential to the homeostasis of colonocytes, the development of intestinal villus morphology, and the strengthening of gut barrier function by promoting tight junction assembly (Rinttilä and Apajalahti, 2013; Onrust et al., 2015; Liu et al., 2021). In addition, butyrate can help maintain an anaerobic environment in the gut and suppress the expression of virulence factors of pathogenic bacteria (Polansky et al., 2016). The SCFA also have anti-inflammatory activities and can boost the immune response by stimulating the production of cytokines (such as TNF-α, IL-2, IL-6, and IL-10) by the host's immune cells. Additionally, SCFA aid in differentiating T cells into Tregs and promote their expansion by binding to receptors on immune cells, such as Toll-like receptors and G protein-coupled (**GPR**) receptors. Moreover, SCFA repair the intestinal mucosa and reduce intestinal inflammation by activating GPR, inhibiting histone deacetylases, and suppressing the expression of pro-inflammatory factors (Blacher et al., 2017; Liu et al., 2021).

In addition to the production of the important metabolites mentioned, the microbiota can degrade dietary tryptophan into indoles, which promotes epithelial cell barrier function. Likewise, the microbiota converts primary host-derived hepatic bile acids to secondary bile acids that inhibit

pro-inflammatory cytokines secretion by dendritic cells and macrophages (Postler and Ghosh, 2017). However, farm-to-farm differences influence microbiota and immune system development. It is unlikely that there is a single microbiota or microbiome related to optimal gut health, but rather a range of possibilities, which may be influenced by local conditions (Kogut et al., 2017).

1.6.3 Nutrition and dietary components

Host nutrition impacts many vital biological processes, including gene expression, protein synthesis, metabolism, signal transduction, and cellular proliferation (Klasing, 1998; Kogut et al., 2017). The diet composition (ingredients, nutrients, and additives) plays an important role in the development and function of the GIT, including the immune system and the microbiota (Celi et al., 2017). Although the development of the GIT initiates during the incubation period, it is in the period following the hatch when birds start to consume exogenous feed that the GIT and associated organs undergo a rapid development to digest a carbohydrate-rich feed effectively (Noy and Sklan, 1999; Noy et al., 2001). Birds show slower intestinal development and depressed performance when access to feed post-hatch is delayed (Maiorka et al., 2003; Proszkowiec-Weglarz et al., 2022). It is also during the post-hatch period that most of the microbiota colonization and immune system development will occur (Barshira and Friedman, 2006). Thus, feed access, quality of the dietary ingredients, and nutritional requirements according to each growth phase of the broiler are essential to assist intestinal development and gut health (Oviedo-Rondón, 2019). However, some factors related to the chemical composition and physical form of the diet, as well as environmental factors, can be negatively associated with the health of the GIT in poultry (Klasing, 1998). These factors include certain types of fiber, anti-nutritional factors (e.g., trypsin inhibitor, phytate, lectins, saponins), undigested protein, mycotoxins, unbalanced diets, oxidized oil, pathogen exposure, stress, water quality, and many others (Palliyeguru et al., 2010, 2011; Tellez et al., 2015; Tsiouris

et al., 2018; Zaytsoff et al., 2020; Ruhnau et al., 2020; Zhang et al., 2022). These factors can compromise the physiological, morphological, and, consequently, functional integrity of the gut to various degrees. More recently, an excess of nutrients has been discussed as a key dietary factor contributing to enteric disturbances and diseases in poultry (Oviedo-Rondón, 2019; Kogut, 2022; Ducatelle et al., 2023). This is because the excess of undigested nutrients in the hindgut, especially protein, serves as a substrate for the proliferation of pathogenic bacteria (Gilbert et al., 2018). For example, excess protein in the hindgut can be fermented by proteolytic bacteria and generate harmful metabolites to the host, such as ammonia, hydrogen sulfide, amines, phenols, and thiols, which have cytotoxic, genotoxic, and carcinogenic effects (Hughes et al., 2000; Toden et al., 2005). Therefore, it favors shifts in the microbiota and triggers low-grade chronic intestinal inflammation, which may lead to the development of enteric diseases (Oviedo-Rondón, 2019; Cardoso Dal Pont et al., 2020). An excess of nutrients in the hindgut may be due to either high nutrient levels in the diet or suboptimal digestion. Thus, formulating broiler diets closely to their nutritional requirements (reducing safety margins and using phase feeding) and high-quality ingredients are essential to ensure optimal nutrient digestibility and gut health.

The phasing out of AGP has led to a decline in broiler performance and an increased prevalence of subclinical and clinical diseases, primarily attributed to heightened disruptions in gut homeostasis (Kogut et al., 2017). In addition, dysbacteriosis and enteric diseases, such as NE, can increase digesta passage rate, reduce nutrient absorption, and increase both litter moisture and pathogenic bacterial loads in the litter. This results in irritation and ulcerative lesions in the footpads of broilers, thereby reducing their welfare (Dunlop et al., 2016). Due to this combination of factors, many studies have been conducted to identify functional feed additives or feedstuff that can modulate the immune system and the microbiota, providing gut health benefits similar to those

of AGP. Among these natural non-antibiotic alternatives, attention has been focused on the effects of prebiotics, probiotics, organic acids, enzymes, phytogenics, and bacteriophages. The potential benefits of some of these alternatives on broiler performance and health are discussed in the following sections.

1.7 NON-ANTIBIOTIC ALTERNATIVES TO MAINTAIN GUT HEALTH IN BROILERS

The ideal characteristic of a non-antibiotic AGP alternative is that it must improve broiler performance to at least at the same extent as AGP. Based on what is known about the mechanisms of action of AGP, components that can ameliorate feed conversion and growth rates by modulating gut health and the immune system have potential (M'Sadeq et al., 2015; Oh et al., 2019). There are several classes of AGP alternatives. Some of them have been extensively studied and tested in poultry production, such as prebiotics, probiotics, enzymes and organic acids, while others are emerging as potential approaches (Gadde et al., 2017). Although several compounds have been explored as potential AGP replacements, there is still a lack of consistency, and the results seem to vary from flock to flock. A common issue is that products often perform well in controlled research settings but fail to produce satisfactory results when used in the field. Thus, the research must be conducted appropriately when evaluating the potential of a product or multi-product strategy to replace AGP in order to make accurate conclusions about their effectiveness.

1.7.1 Evaluating replacements for antibiotic growth promotors

An often-used yet flawed method to evaluating potential AGP replacements involves running experiments that compare two types of diet: a basal diet containing an AGP and another containing the test product(s) but without AGP. A lack of difference in performance or health parameters between them is interpreted as evidence that the product works as well AGP. However, in

environments with low bacterial challenges, the inclusion or exclusion of AGP may not impact broiler performance since there is no chance for the AGP to act. There is little or no inflammation in animals raised in clean environments without bacterial challenges, even in the absence of in-feed AGP (Roura et al., 1992). Therefore, it cannot be concluded that a tested product will be effective when in the presence of an actual challenge (Korver, 2020).

Due to the impracticality of testing the replacements on commercial farms, it is essential to ensure the presence of a challenge in the research settings. Moreover, it is essential to use challenges that closely simulate field conditions. In this case, a more suitable experimental design for testing AGP replacements would involve comparing three types of diet: a positive control containing an AGP, a true negative control diet without any medication or test product, and a test diet containing the test product(s). To draw valid conclusions about the replacement, there must be a reduction in performance in the negative control group relative to the positive control. This would provide evidence of a challenge in the experimental environment, and the inclusion of an AGP mitigates this challenge. In such a case, if the performance of the test group is equal to that of negative control, it can be concluded that the test product has no effect. If the performance of the test product is intermediate to the controls, the product could be a partial AGP replacement in conjunction with other strategies. However, if the test product increases performance relative to the negative control and is equal to or greater than the positive control, under the conditions of the experiment, the product is effective as an AGP (Korver, 2020). To increase confidence in experimental outcomes, it is important to repeat the experiment as often as possible, either across different locations or over time.

1.7.2 Prebiotics as potential antibiotic growth promoter replacements

Prebiotics are defined as non-digestible compounds that, through their metabolism by microorganisms in the gut, modulate the composition and activity of the gut microbiota, conferring a beneficial physiological effect on the host (Pourabedin and Zhao, 2015). Non-digestible carbohydrates such as inulin, fructo-oligosaccharides (**FOS**), galacto-oligosaccharides, and mannan-oligosaccharides (**MOS**) are the most common examples of prebiotics studied in poultry. More recently, resistant starch and lactulose have also been shown to exhibit some prebiotic-like characteristics (Ricke et al., 2020). As non-digestible feed materials, prebiotics that reach the ceca can be fermented by a specific group of resident bacteria and generate SCFA as metabolites. This, in turn, helps to reduce the cecal pH and favor the growth of beneficial bacteria such as *Bifidobacteria* and *Lactobacillus* spp. (Pourabedin and Zhao, 2015). These bacteria can prevent the growth of pathogens by competitive exclusion (substrate and adhesion site), production of toxic compounds for microbes, and AMP. Prebiotics can also modulate immunity by interacting with PRR on immune cells, increasing cytokine production, lymphocyte proliferation, and natural killer cell activity (Suresh et al., 2018; Ricke et al., 2020).

MOS, a prebiotic derived from the outer cell wall layer of *Saccharomyces cerevisiae*, can stimulate immune responses by enhancing macrophage activity through mannose-specific receptors and also binding to mannose-specific type-I fimbriae on Gram-negative pathogens, preventing them from adhering to and colonizing the gut (Baurhoo et al., 2009; Chacher et al., 2017). MOS also increased villi height and performance of broilers compared to those on a basal diet (Hooge et al., 2003; Baurhoo et al., 2007; Teng et al., 2021).

Similarly, FOS, a prebiotic derived from plants, increased intestinal *Lactobacillus* community diversity while reducing pathogenic bacteria (e.g., *Escherichia coli* and *C. perfringens*) (Xu et al.,

2003; Kim et al., 2011), enhanced gut morphology and increased digestive enzyme activity (Xu et al., 2003) compared to non-AGP supplemented birds. It also modulated immune responses (Shang et al., 2015) and enhanced broiler performance (Yang et al., 2008). Other prebiotics, such as inulin (Li et al., 2018a) and lactulose (Cho and Kim, 2014; Calik and Ergün, 2015), can also favor the growth of beneficial bacteria in the GIT and improve gut morphology compared to non-supplemented birds; however, the effects on performance are variable.

1.7.3 Probiotics as potential antibiotic growth promoter replacements

Probiotics, or direct-fed microbials, are defined as "live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). The mechanism of action of probiotics is not always clear. The results can vary depending on the age and physiological state of the animal, the strain used, the dose of inclusion, and the frequency of administration (Huyghebaert et al., 2011). Some of the mechanisms observed in poultry include maintaining a healthy intestinal microflora by competitively excluding and antagonizing pathogens (Olnood et al., 2015; Ma et al., 2018). This involves limiting substrate availability and adhesion sites on epithelial cells, producing bacteriocins, and lowering the intestinal pH. Additionally, probiotics modulate the immune system by activating T lymphocytes through enhanced Toll-like receptor signalling (Bai et al., 2013; Bilal et al., 2021). Furthermore, the production of SCFA as metabolites by some probiotics contributes to regulating gut immunity and integrity (Blacher et al., 2017). Probiotics can also enhance gut morphology by stimulating mitotic cell division and promoting the proliferation of gut epithelial cells (Samanya and Yamauchi, 2002; Bai et al., 2013).

Bacillus and *Lactobacillus* spp. are the microorganisms most frequently investigated as probiotics due to their colonization ability (Spivey et al., 2014; Krysiak et al., 2021). Administration of *B.*

subtilis for broilers challenged with reused litter from flocks with coccidiosis increased both innate immune responses and broiler performance (Lee et al., 2015). Similarly, birds challenged with *S. enteritidis* and fed *B. coagulans* had enhanced performance, higher numbers of *Lactobacilli* and *Bifidobacterium* and lower numbers of *E. coli* and *Salmonella* in the ceca compared to birds on a basal diet (Zhen et al., 2018). Similar outcomes regarding the exclusion of pathogens, immune modulation and enhancements in growth performance were also observed with the supplementation of *L. acidophilus* for broilers challenged with *C. perfringens* (Li et al., 2018b) or *E. coli* (Wu et al., 2021) as well as with supplementation of *L. fermentum* for broilers challenged with *Campylobacter Jejuni* (Šefcová et al., 2020).

1.7.4 Exogenous enzymes as potential antibiotic growth promoter replacements

Enzymes are biological catalysts that increase the rate of reactions and act on specific substrates or reactants (Kiarie et al., 2013). The primary purpose of exogenous enzymes in poultry diets is to increase the nutritional value of feed ingredients (Adeola and Cowieson, 2011). The most common classes of enzymes used in poultry diets include phytase, carbohydrases (e.g., xylanase, β-mannanase, and α-amylase), and proteases (Adeola and Cowieson, 2011; Bedford and Cowieson, 2019). Several mechanisms of action are associated with the benefits of exogenous enzymes on gut health in poultry. The most important include: 1) increasing the digestibility of nutrients not entirely degraded by host enzymes, thereby reducing substrates for undesirable bacteria; 2) promoting beneficial shifts in the microbiota by reducing undigestible protein in the ceca and generating short-chain oligosaccharides from NSP cell-wall with potential prebiotic effects; 3) reducing intestinal inflammation and improving intestinal integrity (Bedford and Cowieson, 2012; Cowieson and Kluenter, 2019).

Most plant-based ingredients used in poultry diets contain anti-nutritional factors such as NSP and phytate. Excess of NSP (mainly arabinoxylans and β-glucans) in broiler diets is associated with reduced nutrient digestibility, increased gut permeability, and proliferation of pathogenic bacteria (Tellez et al., 2015; Latorre et al., 2015). In addition, some NSP can be recognized by PRR, triggering intestinal inflammation (Dal Pont et al., 2022). However, the use of dietary carbohydrases mitigates these effects. Supplementation of multi-carbohydrase to a wheat-based broiler diet in birds challenged with *C. perfringens* increased growth performance, reduced digesta viscosity and intestinal NE lesions compared to non-supplemented birds (Jia et al., 2009; Sun et al., 2015). Moreover, supplementation of xylanase and β-glucanase decreased ileal *E. coli* numbers and increased villus length in broilers on a wheat-based diet (Roofchaei et al., 2019).

Phytate is present in some plant-based ingredients that forms complexes with minerals, protein, and starch in the GIT of birds, reducing their availability (Selle and Ravindran, 2007). Supplementation of high levels of phytase (≥1,500 FTU/kg) enhanced the performance of broilers challenged with coccidiosis (Adedokun and Adeola, 2016) and NE (Zanu et al., 2020a). Phytase also enhanced the intestinal integrity of broilers by upregulating mucin gene expression and reducing IL-1β expression compared to non-supplemented birds (Jiang et al., 2018). Lysozyme (muramidase) has recently been suggested as a potential AGP replacement (Bedford and Apajalahti, 2022). This enzyme breaks down peptidoglycans from dead bacteria in the GIT, helping to eliminate bacterial debris from the gut and facilitate nutrient absorption. In addition, this enzyme may reduce intestinal inflammation caused by the recognition of peptidoglycans by PRR on immune cells (Goodarzi Boroojeni et al., 2019; Wang et al., 2021a).

1.7.5 Organic acids as potential antibiotic growth promoter replacements

Organic acids are naturally occurring carbon compounds with acidic properties (Broom, 2015). Chemically, organic acids used in food animal production can be divided into two categories: simple monocarboxylic acids (e.g., formic, acetic, propionic, and butyric acids) or carboxylic acids containing at least one hydroxyl group (e.g., lactic, malic, tartaric, and citric acids) (Dibner and Buttin, 2002). The observed benefits of organic acids in broilers are related to 1) altering the GIT microflora either by directly killing through cell-wall penetration or by modifying the pH, reducing the population of potential pathogens and benefiting the acid-tolerant species such as *Lactobacillus* spp.; 2) increasing nutrient digestibility by elevating protein and dry matter retention, increasing mineral absorption and phosphorous utilization, and 3) improving gut health through the direct effects on epithelial cells, as is the case of SCFA, which can serve as a direct energy source for epithelial cells and modulate the immune system (Broom, 2015; Khan and Iqbal, 2016; Gadde et al., 2017; Du et al., 2023). Due to differences in their nature, the results of studies evaluating organic acid supplementation in broilers vary (Gadde et al., 2017). A meta-analysis was conducted by Polycarpo et al. (2017) to evaluate the effectiveness of organic acids, used individually or in blends, on the performance of broilers. Organic acids reduced FCR by 5.67% compared to control birds fed a basal diet. However, they did not match the efficacy of AGP, which reduced FCR by 13.4% compared to the control. The study also revealed that blends of organic acids were more effective in enhancing broiler performance than individual organic acids (Polycarpo et al., 2017). Blends of organic acids modulated intestinal microbiota and enhanced gut morphology structure and nutrient digestibility in broilers (Ghazalah et al., 2011; Saleem et al., 2020; Ma et al., 2021a; b).

1.7.6 Phytogenics as potential antibiotic growth promoter replacements

Phytogenic feed additives, also known as phytobiotics or botanicals, are natural bioactive compounds derived from plants and incorporated into animal feed to increase productivity (Windisch et al., 2008). Different types of plants and their products are incorporated into this category; however, depending on their origin (part of the plant) and processing, they can be classified as herbs, spices, essential oils (volatile lipophilic compounds derived by cold expression or by steam or alcohol distillation), or oleoresins (extracts derived by nonaqueous solvents) (Windisch et al., 2008; Abdelli et al., 2021). The bioactive compounds of phytogenics are plant secondary metabolites, with polyphenols as the main group. Other bioactive compounds include terpenoids, phenolics (e.g., tannins, flavonoids), glycosides, and alkaloids (Yeoman and White, 2014). Due to the vast list of potential phytogenic products, their mechanisms of action on poultry remain not fully elucidated (Gadde et al., 2017). Despite that, the main benefits observed in poultry are related to their antioxidant and antimicrobial activity, especially in botanicals with phenolic compounds (Windisch et al., 2008). Other benefits include the reduction of oxidative stress (Zhang et al., 2013; Settle et al., 2014), stimulation of GIT functionality (increase intestinal and pancreatic enzyme production and activity and increase bile flow), and morphological structure (Hashemipour et al., 2013; Yarmohammadi Barbarestani et al., 2020; Su et al., 2021), and immune modulation (Pourhossein et al., 2015; Galal et al., 2015; Liu et al., 2019).

1.7.7 Other categories of AGP replacements

Several other categories and products have been studied as potential AGP replacements in broiler diets. Among them, bacteriophages are an emerging technology with the potential to help prevent bacterial infection in poultry (Wernicki et al., 2017). Bacteriophages are highly species-specific viruses that kill bacteria by producing endolysins and subsequently lyse the bacterial cells (Huff

et al., 2005). They bind specific receptors on bacterial cell surfaces, release their genetic material into the cell, and use the host cell machinery to synthesize multiple virion particles. Once the viruses have matured, the cell wall is lysed, thus releasing progeny phages (Johnson et al., 2008). Several studies have reported the antibacterial potential of bacteriophages against *S. enteritidis*, *C. jejuni*, and *C. perfringens* in poultry (Lim et al., 2012; Seal, 2013; Chinivasagam et al., 2020; Bae et al., 2021).

Another innovative approach gaining interest is administering egg yolk antibodies to develop passive immunity in broilers (Suresh et al., 2018). Egg yolk antibodies (**IgY**) are maternal antibodies the laying hens transfer to their offspring via the egg yolk (Yegani and Korver, 2010; Gadde et al., 2015). These antibodies are created by exposing the hens to a specific antigen, which stimulates their immune system to produce specific antibodies to this antigen, which are then transferred to the egg yolk. The antibodies are extracted and purified from the egg yolk and can be used as a feed additive (Yegani and Korver, 2010). The passive immunization of broilers with specific IgY has been demonstrated to prevent NE (Goo et al., 2023) and *Eimeria* infection (Lee et al., 2009) in broilers, as well as enhance performance and intestinal health in broilers challenged with *E. coli*, *S. enteritidis* and *S. typhimurium* compared to control birds (Chalghoumi et al., 2009; Mahdavi et al., 2010).

Although several products have demonstrated a potential to replace AGP in broiler diets, many studies have evaluated them in the absence of a challenge or failed to compare their effects against both a positive (diet with AGP) and negative control (basal diet). Thus, no accurate conclusions can be drawn about the product efficacy as an AGP. Furthermore, our understanding of the mechanisms of action of these products remains limited. This contributes to the wide variability in outcomes observed when the same product is used under different conditions. Therefore, there is

still a need to appropriately test the aforementioned products and better understand their mechanism in order to use them effectively in the field. Given the unlikely scenario that a single product will be able to compensate fully for the AGP benefits, it is still important to explore novel alternatives. The path forward to consistent success in AGP-free broiler production will likely depend on a combination of products with different mechanisms of action, complemented by effective management and biosafety practices.

1.8 NOVEL PRODUCTS AS POTENTIAL AGP REPLACEMENTS

1.8.1 Chitosan oligosaccharides

Chitosan oligosaccharides (**COS**) are deacylated derivatives of chitosan or chitin, which is the second most abundant polysaccharide in nature after cellulose. They can be obtained from the shells of crustaceans, exoskeletons of insects, and some fungi, algae, and yeast (Zou et al., 2016). Chitin is chemically inert, with over 90% acetylation degree, and is insoluble in both water and acid, which hinders its processing and limits its applications (Torres et al., 2019). COS are produced through the depolymerization and deacetylation of chitin by acid hydrolysis, physical hydrolysis, and enzymatic degradation (Figure 1.2; Lodhi et al., 2014). Unlike chitin, COS have low molecular weight, good solubility, and low viscosity, facilitating their application in agriculture, pharmaceuticals, and other industries (Naveed et al., 2019). COS exert a broad range of biological properties, including antimicrobial (Goy et al., 2009), anti-inflammatory (Ma et al., 2011; Moine et al., 2021), immunoadjuvant, and antioxidant activities (Je et al., 2004; Li et al., 2017a). These properties are closely related to their physicochemical structure. COS are formed by *N*-glucosamine units linked by β-1,4 glycosidic bonds. Each monomer contains three types of reactive functional groups: an amino/acetamido group, a primary hydroxyl group, and a secondary hydroxyl group (Figure 1.2; Zou et al., 2016; Naveed et al., 2019). However, no single type of

COS displays all the observed biological activities. The amino profile, deacetylation degree, and molecular weight (**Mw**) are the main reasons for the differences between their structures, which will also determine their biological functions (Zou et al., 2016).

The antimicrobial property of COS has been associated with more than one possible mechanism of action. COS bind to receptors on microbial cells and lead to the displacement of potassium (K^+) from the cell membrane, causing an efflux of K^+ and stimulation of extracellular acidification, enhancing the transmembrane potential difference between the cells and increasing the uptake of Ca^{2+}. This results in the impairment of bacterial cell functions, causing their death (Tan et al., 2013). Additionally, the positive charge at the C-2 position of the glucosamine unit facilitates COS binding to bacterial cell walls, inhibiting bacterial cell growth (Naveed et al., 2019). It is also suggested that COS can block bacterial RNA transcription due to its penetration into bacterial cell DNA (Kim et al., 2003). Although COS have non-pathogen-specific antibacterial activity, they can modulate the intestinal microbiota by stimulating the growth of beneficial bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Lee et al., 2002; Liu et al., 2008; Yang et al., 2012; Mateos-Aparicio et al., 2016). This is because COS can serve as a carbon source for bacteria that can digest these polymers in the gut. As a result, these bacteria produce short-chain fatty acids as metabolites that can contribute to an appropriate environment for the growth of beneficial ones (Altamimi et al., 2016; Moine et al., 2021). Beneficial bacteria can then prevent the pathogens from attaching to epithelial cells, making them free in the GIT and facilitating the COS to exert their antimicrobial activities (Altamimi et al., 2016).

Another important characteristic of COS is related to their adjuvant effects and immune recognition (Moine et al., 2021). COS have mucosal adjuvant properties, and when in contact with the mucus, the positive charges present in COS form mucoadhesive bonds with negatively charged

sialic groups of the glycocalyx on the epithelial cell surface. Depending on the Mw, COS can diffuse deeply (low Mw) into the mucus gel, crosslinking the mucin polymers, or only interact superficially (high Mw). The crosslink with mucin polymers can reinforce the mucosal barrier and stimulate the fitness of goblet cells, which could be relevant to restore intestinal homeostasis (Kootala et al., 2018; Moine et al., 2021). The COS adhered to the superficial mucus can be recognized by antigen-presenting cells such as dendritic cells and macrophages and enhance their ability to activate T lymphocytes and B lymphocytes, which are crucial for mounting an adaptive immune response and restoring homeostasis (Koppolu and Zaharoff, 2013; Moine et al., 2021). It is still unclear which receptors are involved in COS recognition; however, the mannose receptor was proposed as a chitosan receptor in macrophages (Feng et al., 2004). Because of their recognition by and stimulation of the immune system, several studies have evaluated the potential of COS to work as a vaccine adjuvant (Baudner et al., 2003; Zhang et al., 2017; Moran et al., 2018; Wei et al., 2020; Wen et al., 2022). It has been proposed that COS, as a component of the vaccine, can enhance antigen presentation and stimulate immunity (resistance) against viruses and bacteria by promoting T lymphocyte proliferation and initiating mixed Th1/Th2 responses (Zhang et al., 2017; Moran et al., 2018).

COS are potential alternative to AGP in poultry and pig production (Swiatkiewicz et al., 2015; Li et al., 2019; Guan et al., 2019). COS modulate the microbiota, enhance performance, stimulate GIT development and digestive enzyme activity, increase oxidative status, and attenuate intestinal inflammation in weaned pigs (Yang et al., 2012; Wan et al., 2017; Suthongsa et al., 2017; Yu et al., 2021). Specifically in broilers, COS alleviate heat stress-induced oxidative damage (Lan et al., 2020; Chang et al., 2022), alleviate stress induced by corticosteroids (Osho and Adeola, 2020) or pre-slaughter transportation (Lan et al., 2021), mitigate performance losses and pro-inflammatory

responses caused by coccidiosis (Osho and Adeola, 2019), and increase overall broiler performance (Huang et al., 2005; Li et al., 2007; Ayman et al., 2022). Despite these promising effects of COS on the performance and health of the animals, there is a lack of consistency among the studies on the COS Mw, deacetylation degree, and levels of inclusion used. Since these factors can dictate the COS mechanism of action and biological activity, it is important to understand how these differences impact the gut health, immune status, and performance of broilers. In addition, the physiological status of the birds and the type of challenge imposed to test the COS efficacy may also impact the outcome of the studies. Therefore, further studies are required to uncover the molecular mechanisms and the precise influences of the physicochemical properties of COS on broilers in order to determine their potential as an alternative to AGP.

1.8.2 Punicic acid

Punicic acid is an unusual polyunsaturated fatty acid (C18:3, 9 cis, 11 trans, 13 cis), an isomer of α-linolenic acid (Figure 1.3), mainly found in pomegranate seed oil (**PSO**), making up to 80% of the PSO composition (Pereira de Melo et al., 2014; Boroushaki et al., 2016). This conjugated fatty acid has gained wide attention for its range of bioactivities, including anti-diabetes, anti-obesity, antioxidant, and anti-inflammatory properties (Aruna et al., 2016; Shabbir et al., 2017; Holic et al., 2018). Among these, the anti-inflammatory and antioxidant properties are the most noteworthy, particularly when considering the maintenance of gut health. Conjugated fatty acids exert anti-inflammatory properties due to their potential to inhibit eicosanoid metabolism in the synthesis of prostaglandins from arachidonic acid (Aruna et al., 2016). Punicic acid mitigated pro-inflammatory responses and relieved intestinal inflammation in rats (Bassaganya-Riera et al., 2011; Yuan et al., 2015). In an experiment with induced colitis in rats, dietary punicic acid supplementation relieved colon inflammation by inhibiting TNF-α induced priming of NADPH

oxidase, an enzyme associated with the intestinal inflammatory response (Boussetta et al., 2009). PSO decreased the incidence and severity of necrotizing enterocolitis, increased intestinal integrity, and decreased mRNA encoding inflammatory cytokines in neonatal rats (Coursodon-Boyiddle et al., 2012). Punicic acid also modulated mucosal immune responses and reduced gut inflammation through peroxisome proliferator-activated receptor (**PPAR**)-γ and -δ-dependent mechanisms (Plourde et al., 2006; McFarlin et al., 2008; Bassaganya-Riera et al., 2011).

Oxidative stress reflects the imbalance between the production and accumulation of reactive oxygen species (**ROS**) in cells and tissues, and the ability of a biological system to detoxify these reactive products (Pizzino et al., 2017). This condition affects the progression of various clinical and chronic diseases (Yuan et al., 2014). Conjugated linolenic acid isomers have been associated with the reduction of ROS and antioxidant activity, ameliorating oxidative stress in humans and animal models (Banni et al., 1998; Suksatan et al., 2022). Similarly, punicic acid supplementation exerted antioxidant activities by enhancing the activity of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase), reducing nitric oxide synthesis, lipid peroxidation and alleviating oxidative stress in mice (Saha and Ghosh, 2009; Boroushaki et al., 2016). Therefore, punicic acid may have the potential as an AGP alternative for the prevention or attenuation of enteric diseases in broilers. However, limited research has been conducted on the effects of punicic acid on poultry. Since punicic acid can benefit human health, some studies have investigated its supplementation in poultry diets to increase the nutritional value of chicken meat and eggs by enhancing their fatty acid profile (Kostogrys et al., 2017; Białek et al., 2018; Ngo Njembe et al., 2021; Bölükbaşı et al., 2023). To the best of our knowledge, punicic acid has not yet been evaluated in broilers under challenging conditions. Moreover, the PSO, the most abundant natural source of punicic acid, is expensive and unsuitable for large-scale feeding (Holic et al.,

2018). Thus, efforts are ongoing to generate a biotechnological platform for punicic acid production through the metabolic engineering of plants and microorganisms (Holic et al., 2018; Wang et al., 2021b), which would facilitate the inclusion of punicic acid in broiler diets in case the product show effectiveness to be used as an AGP replacement.

1.8.3 Glucosamine-derived caramels

Glucosamine (2-amino-2-deoxy-D-glucose) is an amino monosaccharide, endogenously synthesized by the animal from glucose and utilized for the biosynthesis of glycoproteins and glycosaminoglycans. Glucosamine is present in almost all human and animal tissues and is highly concentrated in connective tissues, especially in the cartilage (Dahmer and Schiller, 2008). Within cartilage, glucosamine is essential for the formation of hyaluronic acid, chondroitin sulfate, and keratan sulfate, which are the most important components of the extracellular matrix of the articular cartilage and the synovial fluid along with the collagen fibers (Jerosch, 2011). Glucosamine can be exogenously supplemented as the sulfate, hydrochloride, *N*-acetylglucosamine, chlorohydrate salt, or as a dextrorotatory isomer (Dahmer and Schiller, 2008). Although its effectiveness is still controversial, glucosamine is mainly used as a dietary supplement to prevent and relieve osteoarthritis and articular joint disease (Block et al., 2010; Salazar et al., 2014; Conrozier and Lohse, 2022). During these conditions, the concentration of interleukin-1 β (IL-1β), a proinflammatory cytokine, increases locally, causing a cascade of events that leads to cartilage damage. These include the formation of ROS, metalloproteases, and extracellular matrix proteins absent in normal cartilage, the release of other inflammatory mediators, the inhibition of chondrocyte proliferation, and the induction of cell death (Largo et al., 2003). However, glucosamine supplementation seems to regulate the synthesis of nitric oxide and inflammatory mediators, including IL-1β in vitro and in affected cartilage in humans (Largo et al.,

2003; Chou et al., 2005; Nagaoka et al., 2011). In addition, glucosamine also inhibits TNF-α, an inflammatory mediator, via the suppression of signaling molecules such as p38 mitogen-activated protein kinases (**MAPK**) and nuclear factor kappa B (**NF-κB**) in rats with induced inflammatory bowel disease (Nagaoka et al., 2011; Bak et al., 2014; Roy et al., 2023).

As glucosamine has gained popularity, mainly for its potential to prevent or alleviate osteoarthritis, research on this compound has dramatically increased, aiming to understand and explore its properties (Hrynets et al., 2015). Glucosamine is an unstable amino sugar that can rapidly degrade, even under mild temperatures (as low as 25 °C), generating a plethora of compounds, including α-dicarbonyls, melanoidins, and non-volatile polyhydroxy alkyl pyrazines (Hrynets et al., 2015, 2016; Dhungel et al., 2018). The pyrazines produced by the self-condensation of glucosamine (e.g., caramelization) are known as fructosazine (**FR**) and deoxyfructosazine (**DOFR**; Figure 1.4). These compounds are recognized as flavoring agents and have been identified in roasted peanuts, caramel, and soy sauce (Henry et al., 2012). Besides being used as flavoring agents in the food and tobacco industries, these molecules are gaining interest for their pharmaceutical properties (Henry et al., 2012). FR and DOFR have a stronger capacity to alleviate joint inflammation than the native glucosamine (Zhu et al., 2007). They inhibit inflammatory mediators such as interleukins-2 and -1β and reduce the damage to proteoglycans and collagen in the connective tissue of joints (Giordani et al., 2006; Zhu et al., 2007). These can, in turn, prevent pathological cartilage degradation and inflammatory diseases. Furthermore, the caramelization of glucosamine produces melanoidins, which serve as substrate for commensal *Bifidobacterium* (Borrelli and Fogliano, 2005). Thus, melanoidins may act as a prebiotic, mitigating joint inflammation indirectly through promoting gut health.

Modern broilers selectively bred for rapid growth are particularly prone to leg problems due to excessive shear stress on immature cartilage (Wideman, 2016). This stress, in turn, can result in microfractures and clefts among the chondrocytes in susceptible bone growth plates, which expose the collagenous matrix that facilitates adhesion and infection by opportunistic bacteria (Wideman et al., 2012; Wideman, 2016). This infection can lead to bacterial chondronecrosis with osteomyelitis (**BCO**), the leading cause of broiler lameness and responsible for significant losses in poultry production (Ekesi et al., 2021). This condition occurs when opportunistic bacteria enter the blood via translocation from the respiratory system or gastrointestinal tract and spread hematogenously to vulnerable sites (i.e., microfractures or clefts). Subsequently, the overgrowth and the release of lytic substances by these bacteria cause gross abscesses and necrotic voids, ultimately leading to BCO (Wideman et al., 2012; Wideman, 2016). Among the bacteria isolated from BCO cases, the most common are *E. coli*, *Staphylococcus aureus*, *S. agnetis*, *Enterococcus cecorum*, and *S. enteritidis* (Wideman et al., 2012, 2015; Ekesi et al., 2021). As these bacteria are common inhabitants of the intestinal microbiota, it suggests that the translocation of bacteria from the gut to the joints due to failures in the tight junctional complexes is highly associated with the pathogenesis of BCO in broilers (Wideman et al., 2015; Rojas-Núñez et al., 2020). To the best of our knowledge, no studies have evaluated the potential of glucosamine-derived caramels to alleviate locomotor problems and promote gut health in broilers. Therefore, the supplementation of glucosamine-derived caramels containing FR, DOFR, and melanoidins may have the potential to improve gut health, alleviate locomotor problems, and improve the welfare of broilers. However, these effects still need to be elucidated.

1.9 RESEARCH APPLICATION

The phasing out of AGP from broiler diets has increased the exposure of birds to potentially harmful pathogens, resulting in a recurring activation of their innate immune system. This has raised the risks of clinical and subclinical enteric diseases in broilers. Among them, NE is now considered the major enteric disease in poultry, causing a significant economic impact on the poultry industry, reducing the performance and welfare of broilers, increasing mortality of the flocks, and also increasing the risk of contamination of poultry products for human consumption. Additionally, enteric disturbances can facilitate the translocation of opportunistic pathogenic bacteria from the lumen to the bloodstream, causing other diseases such as BCO in broilers. Therefore, there is an urgent need to develop AGP alternatives to enhance gut health and immune modulation in broiler production. The objective of this Ph.D. thesis was to evaluate the effects of COS, punicic acid, and glucosamine-derived caramels as potential AGP replacements in broiler diets.

1.9.1 Thesis objectives

The objectives of this Ph.D. thesis were:

1) To evaluate the effects of dietary shellfish COS as potential AGP replacement in broilers challenged with a natural, subclinical NE infection model. Two studies were conducted to address this objective. The objective of the first study was to determine the optimal COS Mw and levels of inclusion in the diet on performance, NE intestinal lesion scores, and body weight uniformity of broilers challenged with subclinical NE. The objective of the second study was to investigate further the most promising COS products selected from study one and their effects on performance, carcass traits, cecal microbial abundance, quantification of *C. perfringens*, and concentration of

short-chain fatty acid, immune modulation, intestinal morphology, and *Eimeria* oocysts count in the intestine of broilers challenged with a natural, subclinical NE infection model.

2) To assess the effects of punicic acid, provided through the dietary supplementation of PSO, as a potential AGP replacement on growth performance, intestinal NE gross lesion, litter quality, and footpad dermatitis of broilers challenged with subclinical NE.

3) To evaluate the effects of glucosamine-derived caramels as a potential AGP replacement on growth performance, femoral and tibial bacterial gross lesions, gait scores, and cecal concentrations of short-chain fatty acids in broilers raised in wire flooring cages as a model to induce lameness.

1.9.2 Thesis hypotheses

The hypotheses of this PhD thesis were:

1) The natural, subclinical NE infection model would be a suitable model to mimic field conditions in which NE takes place and effectively test the efficacy of the tested products in replacing AGP in broiler diets.

2) COS at a specific Mw and level of inclusion in the diet would partially restore the growth performance decreased by subclinical NE, compared to the AGP, through the modulation of gut health and immune responses.

3) Supplementation of punicic acid through PSO would partially restore the performance decreased by subclinical NE, compared to the AGP, and mitigate NE intestinal lesions in broilers. The results of this study would serve as a guide for developing new biotechnological sources of PA (i.e., genetically modified yeast enriched with punicic acid).

4) Glucosamine-derived caramels would enhance broiler performance and mitigate BCO lesions potentially caused by translocated bacteria from the lumen to the joints.

1.10 REFERENCES

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1.11 TABLES

Table 1.1. Classification of antibiotics according to the importance for human medicine in Canada.

Category	Category Criteria	Antimicrobial Family
I - Very High Importance	Essential for serious human infections and limited or no alternatives available	Cephalosporins Fluoroquinolones
II – High Importance	Essential for treating serious human infections and few alternatives available	Aminoglycosides Lincosamides Macrolides Penicillins Streptogramins Diaminopyrimidines
III - Medium Importance	Important for treating human infections and alternatives generally available	Bacitracins Sulphonamides Tetracyclines
IV – Low Importance	Not used for humans	Flavophospholipids Ionophores
Uncategorized		Orthosomycin

Adapted from Chicken Farmers of Canada, 2018.

Table 1.2. *Clostridium perfringens* toxicogenic type and toxin production scheme.

Toxicogenic type	Toxin production*					
	α - toxin	β - toxin	ε - toxin	ι - toxin	CPE	NetB
A	+	-	-	-	-	-
B	+	+	+	-	-	-
C	+	+	-	-	+/-	-
D	+	-	+	-	+/-	-
E	+	-	-	+	+/-	-
F	+	-	-	-	+	-
G	+	-	-	-	-	+

*The sign "+" indicates productions of that toxin, and the sign "-" indicates the lack of that toxin production (Adapted from Rood et al., 2018).

Table 1.3. Experimental models using diverse predisposing factors combined with the exogenous *Clostridium perfringens* infection to cause necrotic enteritis (NE) in broilers.

Predisposing factors	***C. perfringens* strains and dosage**	**Time of application**	**Severity of the disease induced**	**Purpose of the study**	**References**
10-fold dose of coccidiosis vaccine, and finisher feed with fish meal (30%)	NetB-positive strain CP56 administered orally 3x a day at a concentration of 4×10^8 CFU/mL	Coccidiosis vaccine on day 18, and *C. perfringens* at 18, 19, 20 and 21 d.	Subclinical	Study suitable models to cause subclinical NE in broilers	(Gholamiandehkordi et al., 2007)
High protein wheat-based feed containing 50% fishmeal	NetB-positive strain EHE-NE18 administered orally (1.5 mL at 10^9 to 10^{10} CFU) or via feed (1:10 (v/w) culture to feed)	High protein diet introduced on day 23, *C. perfringens* oral infection at 24 and 25 d, or in-feed infection at 26 and 27 d	Variable	Test the efficacy of a potential vaccine against NE in broilers	(Keyburn et al., 2013)
Eimeria infection (*E. acervulina*, *E. maxima* and *E. brunetti*) and fish meal (25% w/w)	NetB-positive strain EHE-NE18, 1 mL administered orally at a concentration of 10^8–10^9 CFU/mL	*Eimeria* infection on day 9, and *C. perfringens* at 14 and 15 d	Subclinical	Study shifts in the cecal microbiota during NE in broilers	(Stanley et al., 2014)
Feed contaminated with mycotoxins (fumonisins and deoxynivalenol), fish meal (30%) and IBD vaccine	NetB-positive strain CP56 administered orally at a concentration of 4×10^8 CFU/mL	Fish meal introduced from day 17 onwards, IBD vaccine administered on day 16, and *C. perfringens* at 17, 18, 19 and 20 d.	Subclinical	Study mycotoxins as potential predisposing factors for NE in broilers	(Antonissen et al., 2014, 2015)
10-fold coccidiosis vaccine, IBD vaccine, and high stocking density (30 birds/m^2)	NetB-positive strain CP56 administered orally 3x a day at a concentration of 4×10^8 CFU/mL	IBD vaccine administered on day 16, coccidiosis on day 18, and *C. perfringens* at 17, 18, 19 and 20 d	Subclinical	Study stocking density as a predisposing factor for NE in broilers	(Tsiouris et al., 2015a)

Eimeria infection (*E. acervulina*, *E. maxima* and *E. brunetti*)	*C. perfringens* type A strain EHE-NE36, 1 mL administered orally at a concentration of 1 × 10^8 CFU/mL	*Eimeria* infection on day 9, and *C. perfringens* at 14 and 15 d	Subclinical	Study potential AGP replacements in broiler diets	(Xue et al., 2017b)
Infection with *Salmonella* Typhimurium, and *E. maxima*	*C. perfringens* type A administered orally at a concentration of 1 × 10^8 CFU/bird/day	Infection with *Salmonella* on day 1, *Eimeria* on day 18, and *C. perfringens* at 23 and 24 d	Subclinical	Study a challenge model suitable to test AGP replacements in broiler diets	(Latorre et al., 2018)
Infectious bursal disease (IBD) vaccine, 10-fold dose of coccidiosis vaccine, and finisher feed with fish meal (30%)	NetB-positive strain CP56, 1 mL overnight culture administered orally (concentration not specified)	IBD vaccine on days 4 and 9; coccidiosis vaccine at day 14, and *C. perfringens* at 17, 18 and 19 d	Subclinical	Study broiler rapid growth as a potential predisposing factor for NE	(Dierick et al., 2019)
Eimeria infection (*E. acervulina*, *E. maxima* and *E. brunetti*)	NetB-positive EHE-NE18 and WER-NE36 strains 1 mL administered orally at a concentration of 1×10^8 CFU/mL	*Eimeria* infection on day 9, and *C. perfringens* at 14 and 15 d	Subclinical	Study the effects of *C. perfringens* strains on NE pathogenesis in broilers	(Gharib-Naseri et al., 2019)
Eimeria infection in broilers raised on fresh litter, broilers raised on recycled litter, or *Eimeria* infection in broilers raised in battery cages	NetB-positive strain administered via water or feed at a concentration of 1 × 10^8 CFU/mL	*E.maxima* infection on day 14*, and C. perfringens* at 19 and 20 d, or at 18, 19 and 20 d, or at 19, 20 and 21	Subclinical	Study different models of subclinical NE infection in broilers	(Bortoluzzi et al., 2019)
High dietary calcium, and *Eimeria* infection	NetB-positive strain EHE-NE18	*E.maxima* infection on day 9*, and C.*	Subclinical	Study the relation of high levels of	(Zanu et al., 2020b; c; d)

(*E. acervulina*, *E. maxima* and *E. brunetti*)	administered orally at a concentration of 10^8–10^9 CFU/mL	*perfringens* at 14 and 15 d.		Ca and phytase on NE development in broilers	
Administration of corticosterone hormone	NetB-positive strain CP1 administered orally at a concentration of 1-2 × 10^8	Corticosterone administered via feeding from day 11 to the end of the trial, and *C. perfringens* at 12 and 13 d.	Subclinical	Study the stress effects on the NE development in broilers	(Zaytsoff et al., 2020, 2022)
High protein diet (30% crude protein)	NetB-positive strain CP1, administered orally 2x a day at a concentration of 3 × 10^8 CFU/mL	High protein diet introduced on day 14, and *C. perfringens* at 21, 22 and 23 d	Subclinical	Study potential AGP replacements in broiler diets	(Shojadoost et al., 2022)
10-fold or 20-fold coccidiosis vaccine, IBD vaccine, water and food withdrawn (prior to vaccination)	*C. perfringens* type A strain NCTC 8798, 3 mL administered 2x a day at a concentration of 2.2 × 10^7 CFU/mL	Coccidiosis and IBD vaccines on day 14, and *C. perfringens* at 18, 19, and 20 d	Subclinical	Study potential AGP replacements in broiler diets	(Lee et al., 2022)
Infection with *E. maxima*	NetB and TpeL positive strains administered orally at a concentration of 1 × 10^8 CFU/mL	*Eimeria* infection on day 14 and *C. perfringens* at 19, 20 and 21 d.	Subclinical	Study potential AGP replacements in broiler diets	(Shah et al., 2023)

Table 1.4. Experimental models using diverse predisposing factors and based on the natural *Clostridium perfringens* uptake from the environment to cause necrotic enteritis (NE) in broilers.

Predisposing factors	**Time of application**	**Severity of the disease induced**	**Purpose of the study**	**References**
Wheat-based diet (620 g/kg) without xylanase, and coccidiosis vaccine	Diet rich in wheat and removal of xylanase from 11 to 35 d, and coccidiosis vaccine on day 0	Subclinical	Study potential AGP replacements in broiler diets	(Abildgaard et al., 2010)
Use of protein ingredient with low digestibility, and animal protein (fish meal)	Experimental diets (potato protein, fish meal or soybean meal) provided from day 20 to 36	Subclinical	Study different sources of protein in the NE induction in broilers	(Palliyeguru et al., 2010; Fernando et al., 2011)
Different levels of dietary calcium, phosphorus and phytase, and recycled litter from previous NE infected flocks	Exposure to the experimental diets and recycled litter for the entire experiment (0 to 35 d)	Subclinical	Study the involvement of Ca and P in NE pathogenesis in broilers	(Paiva et al., 2014)
High doses of live coccidiosis vaccine (10-fold)	Coccidiosis vaccine sprayed over the litter on day 0	Subclinical	Study potential AGP replacements in broiler diets	(Calik et al., 2019; Emami et al., 2019; Emami et al., 2020)
High doses of live coccidiosis vaccine (10 and 15-fold), inclusion or not of dietary fish meal (4%), and 24-hour feed withdrawn	Coccidiosis vaccine administered orally on day 12, and feed withdrawn at 18 d	Subclinical	Study models of natural subclinical NE infection in broilers	(He et al., 2022)

1.12 FIGURES

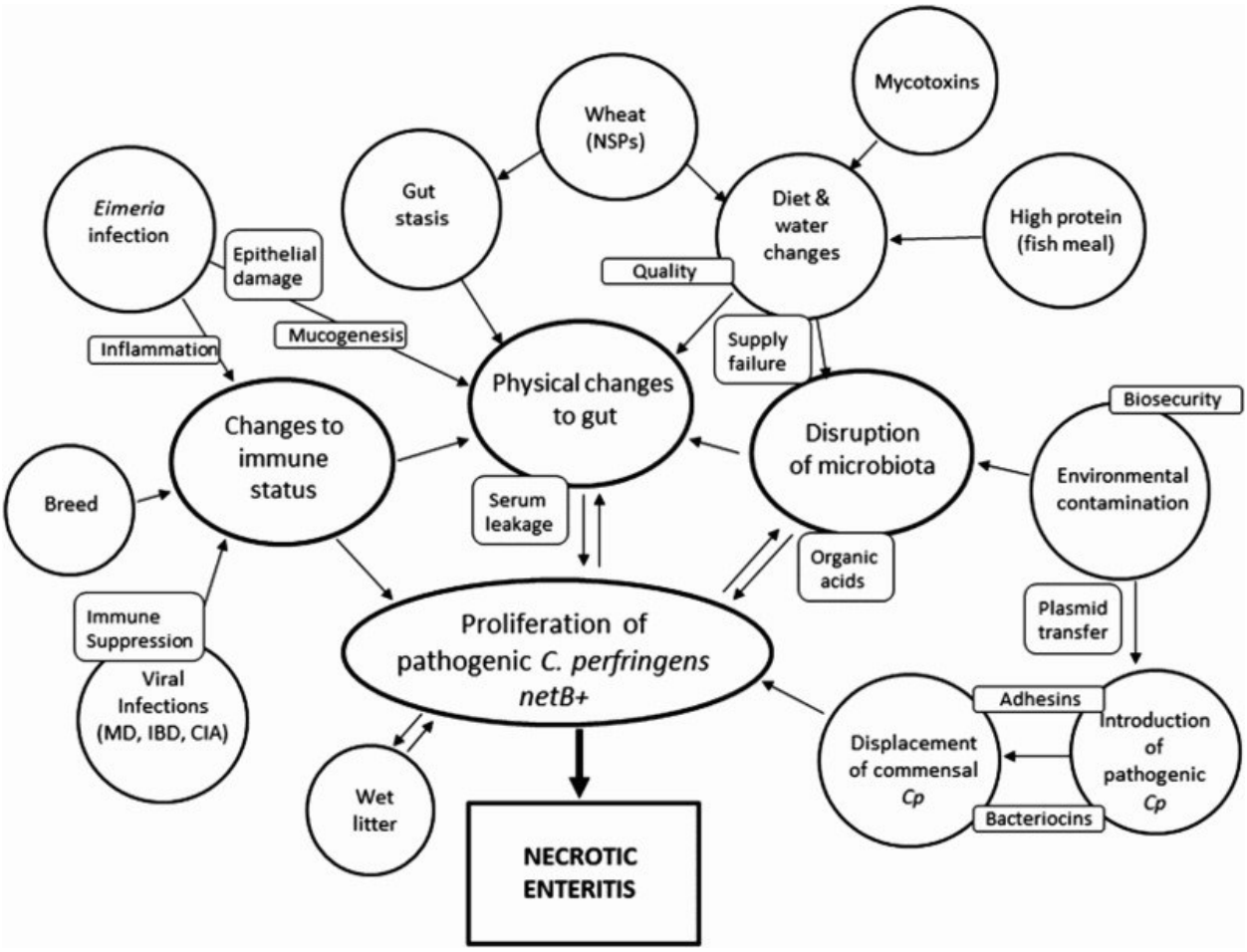

Figure 1.1. Summary of the complex and multifactorial relation of predisposing factors in the development of necrotic enteritis in broilers. Predisposing factors are shown in circles and the major effects of these factors are shown in the ovals. Important factors that may drive the influence of the predisposing factors are shown in the small rectangular boxes. Cp = *Clostridium perfringens*, IBD = infectious bursal disease, CIA = chicken infectious anaemia, MD = Marek's disease, NSPs = non-starch polysaccharides (Moore, 2016).

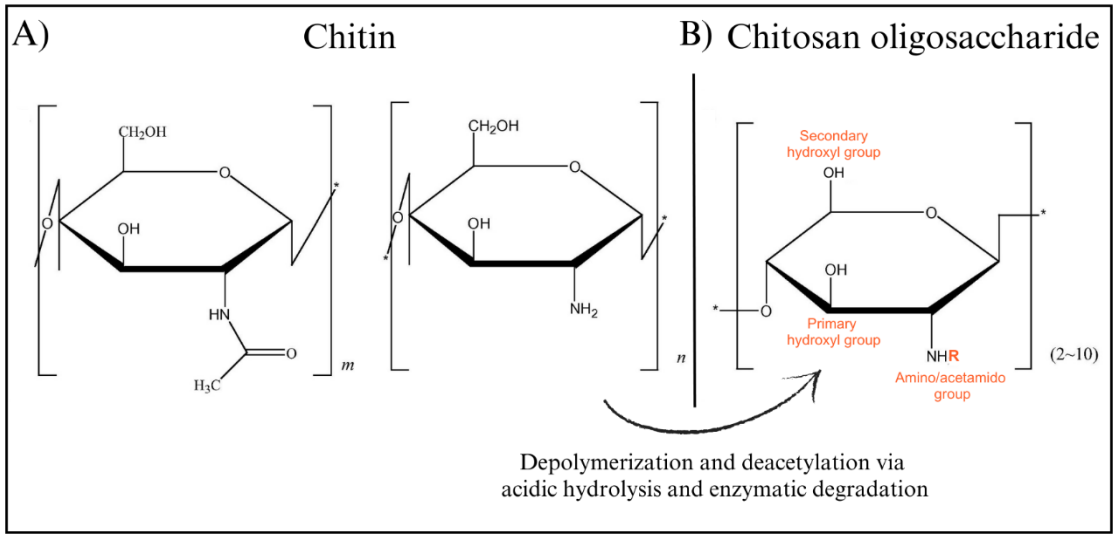

Figure 1.2. Chemical structure of chitin (A) and chitosan oligosaccharide (B). Chitin degradation by chemical and enzymatic degradation leads to the formation of chitosan oligosaccharides, which can have from 2 to 10 monomers of glucosamine linked by β-1,4 glycosidic bonds. Each monomer of glucosamine contains three reactive functional groups, including an amino/acetamido group ('R' can be either H or acetyl group depending on the deacetylation degree) and two hydroxyl groups (Adapted from Zou et al., 2016).

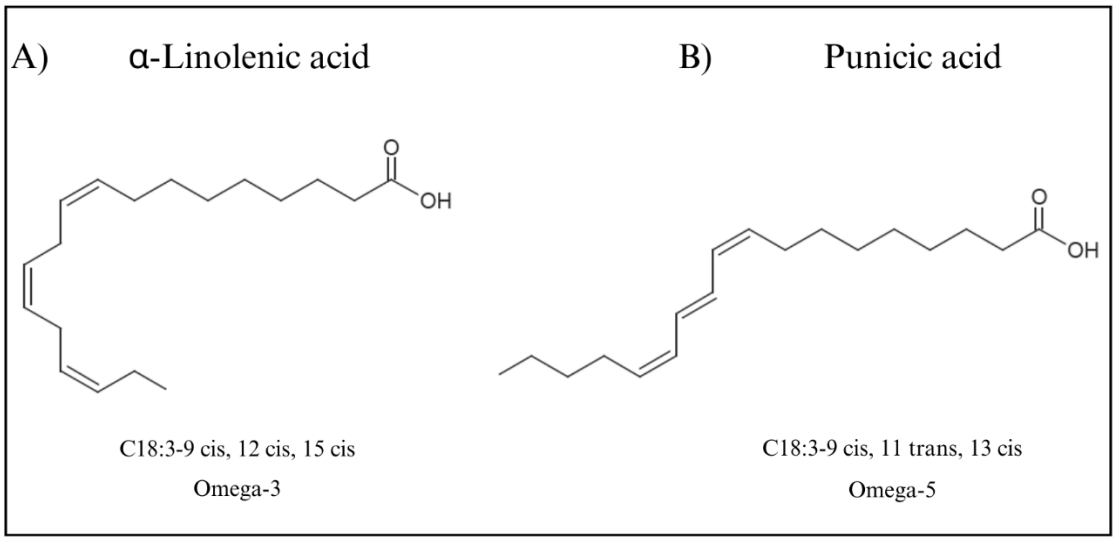

Figure 1.3. Chemical structure of A) α-linolenic acid and B) punicic acid (an isomer of α-linolenic acid) (Adapted from Pereira de Melo et al., 2014).

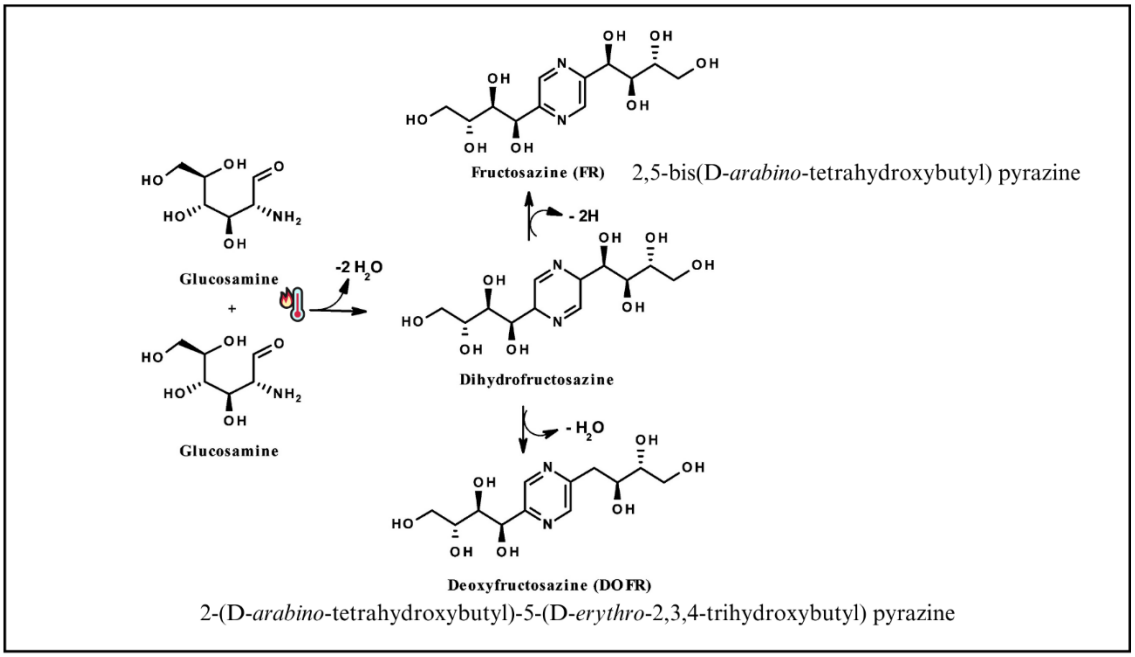

Figure 1.4. Molecular structure and the mechanism of glucosamine self-condensation to dihydrofructosazine, fructosazine (FR) and deoxyfructosazine (DOFR) (Adapted from Hrynets et al., 2016).

2. DIETARY CHITOSAN OLIGOSACCHARIDES IN BROILERS CHALLENGED WITH A NATURAL, SUBCLINICAL NECROTIC ENTERITIS CHALLENGE MODEL: EFFECTS ON PERFORMANCE, INTESTINAL GROSS LESION SCORES, UNIFORMITY, CARCASS TRAITS AND FOOTPAD DERMATITIS

OVERVIEW

The phasing out of antibiotic growth promoters (**AGP**) from broiler chicken diets has led to the re-emergence of necrotic enteritis (**NE**), and effective AGP replacements must be found. Dietary chitosan oligosaccharides (**COS**) of varying molecular weights were evaluated as potential AGP replacements on performance, BW uniformity, NE lesion scores, carcass traits and footpad dermatitis in broiler chickens. In Experiment 1, treatments were: Positive Control (**PC**; basal diet with antibiotic and coccidiostat), Negative Control (**NC**; basal diet without medications), and NC plus COS at 220, 180, 110, 95, 30, 25, 17 or 14 kDa at each of 0.2, 2 and 5 g/kg. In Experiment 2, the PC, NC, and NC plus COS 180 kDa at 0.2, 2, and 5 g/kg, 95 kDa at 0.2 and 5 g/kg, and COS 110 kDa at 5 g/kg treatments were used. A natural, subclinical NE challenge (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d) was applied to all birds. Performance and BW uniformity were measured at the end of the starter, grower, and finisher phases. Intestinal NE lesion scores were evaluated after the challenge and at the end of each experiment. In Experiment 1, PC increased BW gain after the challenge relative to NC, COS 110, COS 30, COS 25, or COS 14 kDa ($P < 0.01$), while birds fed COS 220, 180, 95 or 17 kDa maintained similar performance levels to PC. Following the challenge, birds fed COS 95 kDa at 2 or 5 g/kg had lower NE lesions ($P < 0.01$) than COS 180 at 2 g/kg, COS 14 or 17 kDa at 5 g/kg. In Experiment 2, no differences between PC and NC treatments were found. However, COS 95 kDa at 5g/kg had higher BW ($P = 0.03$) at 25 d than those fed COS 180 at 5 g/kg and tended to be the heaviest ($P = 0.08$) at 36 d. Dietary COS 95 kDa showed promise in enhancing performance and mitigating NE-associated

intestinal damage in broilers. Further mechanistic studies under a more robust subclinical NE challenge are needed to explore the protective effects of COS 95 kDa against NE in broilers.

Key Words: broiler chicken, necrotic enteritis, chitosan oligosaccharide, performance, antibiotic replacement.

2.1 INTRODUCTION

The phasing out of antibiotic growth promoters (**AGP**) from animal diets has increased the risk of enteric diseases and caused a reduction in broiler performance (M'Sadeq et al., 2015; Cardinal et al., 2019). Necrotic enteritis (**NE**) is a major enteric disease of poultry that causes annual losses estimated at US$ 6 billion worldwide (Wade and Keyburn, 2015). The primary causative agent of NE is the opportunistic pathogen *Clostridium perfringens*, a Gram-positive, spore-forming, anaerobic bacteria (Parish, 1961; Shojadoost et al., 2012). *C. perfringens* infections in poultry may occur as either an acute clinical or subclinical disease (Wu et al., 2010). The subclinical form is the most common and is usually only noticeable at the end of the cycle when birds fail to achieve the expected target body weight. In this form of NE, chronic damage to the intestinal mucosa (focal necrosis) hinders the digestion and absorption of nutrients, reducing weight gain and increasing the feed conversion ratio (Van Immerseel et al., 2009; Wu et al., 2010). In contrast, acute clinical NE is characterized by a sudden increase in flock mortality, often without premonitory signs (Van Immerseel et al., 2009).

The development of alternatives to prevent NE in broilers has been a challenge due to the multifactorial complexity of the disease and the difficulties in experimentally reproducing the field conditions that cause subclinical NE. In spite of these challenges, the use of feed additives or dietary modifications able to enhance broiler gut health may be an effective approach to control poultry NE (M'Sadeq et al., 2015; Pham et al., 2020; Emami et al., 2021).

Previous studies involving poultry and swine suggest that chitosan oligosaccharides (**COS**) are potential alternatives to AGP (Huang et al., 2007; Han et al., 2007; Liu et al., 2008; Li et al., 2019). COS are functional oligosaccharides, natural alkaline polymers of glucosamine obtained by chemical or enzymatic hydrolysis of chitosan or chitin (Xia et al., 2013; Hamer et al., 2015). COS have a broad range of biological properties, including antimicrobial (Kim et al., 2003; Benhabiles et al., 2012), anti-inflammatory (Ma et al., 2011; Moine et al., 2021), and antioxidant activities (Je et al., 2004; Li et al., 2017). It has also been reported that COS can increase broiler performance in normal conditions (Huang et al., 2005) or when broilers are challenged with coccidiosis (Osho and Adeola, 2019), glucocorticoid (Osho and Adeola, 2020), heat and cold stress (Chang et al., 2020; Lan et al., 2020; Fathi et al., 2023). However, no single type of COS displays all the observed biological activities. The versatile functions of COS are closely related to the compound characteristics, such as molecular weight (**Mw**) and degree of deacetylation (Zou et al., 2016). However, this broad versatility impacts the consistency of results among previous studies. To the best of our knowledge, no study has evaluated different COS Mw combined with different dietary levels of inclusion and their potential to prevent NE in broilers. Therefore, this study aimed to evaluate dietary shellfish-derived COS on performance, BW uniformity, NE gross intestinal lesions, footpad dermatitis and carcass traits of broilers challenged with a natural, subclinical NE infection model.

2.2 MATERIALS AND METHODS

The animal care procedures were approved by the Animal Care and Use Committee for Livestock of the University of Alberta and followed principles established by the Canadian Council on Animal Care (CCAC, 2009).

2.2.1 Pilot and large-scale experiment

Two experiments were conducted to test the effects of COS on broilers. Experiment 1 was a pilot study designed to determine the optimal combination of COS Mw (220, 180, 110, 95, 30, 25, 17 or 14 kDa) and levels of inclusion in the diet (0.2, 2 or 5 g/kg). In Experiment 2, the six most promising treatments from Experiment 1 were evaluated in a larger-scale (higher number of birds and replicates) experiment in floor pens.

2.2.2 Preparation of chitosan oligosaccharides

Shellfish chitosan samples with high deacetylation degree (> 86%; Golden-Shell Pharmaceutical Co. Ltd., Zhejiang, China) were utilized to produce COS with varying molecular weights. The chitosan was subjected to chemical hydrolysis using H_2O_2 to achieve the desired molecular weights, following the methodology described by Qin et al. (2002, 2006). Briefly, the chitosan samples were diluted in distilled water at 20 or 10% (weight/volume) in order to produce COS at medium-high and low Mw, respectively. Subsequently, H_2O_2 was added to the solutions at a concentration of 1.5 (to create medium-high Mw COS) or 3% (to create low Mw COS). The solutions were stirred and heated at varying temperatures and times to produce COS with different Mw. The first solution was heated at 22°C for 0.5 hours or at 50°C for 2 hours to produce COS at high and intermediate Mw, respectively. In contrast, the second solution was heated at 60°C for 2 hours to generate COS at low Mw (lower than 25 kDa). The molecular weights were subsequently determined through high-performance liquid chromatography equipped with a size exclusion column, as described by Chen et al. (2004). Chitosan hydrolysates with Mw of 14, 17, 25, 30, 95, 110, 180 and 220 kDa were generated.

2.2.3 Animals and housing

Experiment 1. A total of 910 one-day-old, mixed sex Ross 708 broilers with similar weight (43.1 ± 3.61 g) were obtained from a commercial hatchery and randomly assigned to 26 treatments with 5 replicates of 7 birds each (4 males and 3 females per cage). The animals were feather-sexed, individually identified by neck tag and weighed. The chicks were placed in Specht pullet cages (1.2 m [length] × 0.53 m [width] × 0.43 m [height]; Specht-Ten Elsen GmbH, Sonsbeck, Germany) equipped with nipple drinkers, a galvanized feeder trough, and metal wire mesh (2 × 2 cm) flooring. Initially, chick paper, and flat plastic mesh (1 × 1 cm) mats covering the entire floor area of each cage were laid on top of the metal flooring to prevent chicks' feet from going through and were removed on day 10. During the trial, feed in the troughs was pushed down by hand 2 to 3 times per day, ensuring birds had continuous access to feed.

Experiment 2. A total of 1,152 one-day-old, mixed sex Ross 708 broilers, with similar weights (45.9 ± 0.75 g), were randomly assigned to 8 treatments with 8 replicates of 18 birds (9 males and 9 females per pen) each. Birds were feather-sexed, individually identified by neck tag and weighed. They were then placed in floor pens (1.061 x 1.397 m), each equipped with a nipple drinker line, a hanging tube feeder, and 8 cm of new wood shaving litter. During the trial, feeders were shaken 2 to 3 times per day, ensuring birds had continuous access to feed.

For each experiment, the temperature and lighting programs followed commercial practices recommended by Ross 708 commercial management guidelines (Aviagen, 2018). The initial temperature was set to 32°C at placement and decreased gradually to 21°C by 24 days of age, remaining the same until the end of the trial. Twenty-four hours of light was provided for the 1st week, after that a 4-hour dark period was provided each day. Observation of bird health conditions, availability of feed, water, and environmental housing conditions were recorded twice daily.

2.2.4 Experimental diets

In each experiment, birds were fed a commercial-type, wheat-soy-canola basal diet typically fed to broiler chickens in Western Canada. Diets were made to meet or exceed Ross 708 nutritional recommendations (Aviagen, 2019) according to each growth phase (Table 2.1).

Experiment 1. The experimental diets were a Positive Control (**PC**), a commercial-type diet supplemented with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., Kirkland, QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, Mitchell, ON, Canada at 0.05% of the diet); a Negative Control (**NC**) that was the same commercial-type diet, except without any medications; and COS supplemented to the NC diet at each of 8 different molecular weights (220, 180, 110, 95, 30, 25, 17 or 14 kDa), and at each of three levels in the diet (0.2, 2 or 5 g/kg). Dietary treatments were fed in mash form during the 38-day growth cycle.

Experiment 2. The most promising COS treatments from Experiment 1 were used in Experiment 2. Treatments were PC, NC (both PC and NC had the same composition as described in Experiment 1), COS 95 kDa included at 0.2 or 5 g/kg, COS 180 kDa included at 0.2, 2 or 5 g/kg, and COS 110 kDa included at 5 g/kg of the diet. Dietary treatments were fed in mash form for the 38-day growth cycle.

2.2.5 Natural subclinical necrotic enteritis challenge model

In each experiment, a subclinical, natural exposure NE challenge model was used (He et al., 2022). At 12 days of age, each bird was gavaged with a 15X dose of a commercial coccidiosis vaccine (Coccivac®-B52; Merck Animal Health, Intervet Inc., Millsboro, DE) diluted in phosphate-buffered saline. The vaccine contained live, sporulated *Eimeria* oocysts (*E. acervulina*, *E. mivati*, *E. tenella*, and two strains of *E. maxima* at unspecified doses). The high vaccine dosage was used to cause an initial intestinal dysbiosis and mild lesions in the gut tissue. At 18 days of age, the feed

was withdrawn for 24 hours from all birds to serve as a stress factor, enhance the disruption in the intestinal microflora, and stimulate the gut leakage (Thompson et al., 2008; Kuttappan et al., 2015; Lacey et al., 2018) facilitating the conditions for a natural proliferation of *C. perfringens* in the gut. Every 4 hours during the 24-hour feed removal, a trained person walked through the pens and took notes of any clinical or subclinical signs of NE, distress, or any other abnormalities.

2.2.6 Broiler performance and uniformity

In each experiment, feed intake (**FI**), body weight gain (**BWG**), and feed conversion ratio (**FCR**) were measured on a pen basis at the end of the starter (10 d), grower (25 d) and finisher (38 d in Experiment 1, and 36 d in Experiment 2) phases. Mortality and weight of dead animals were recorded daily to calculate mortality and adjust FI and FCR within each period, and over the duration of the trial. Individual body weights measured at 10, 25 and 36 (Experiment 2) or 38 d (Experiment 1) and were also used to calculate the pen uniformity (CV%) according to the following equation:

$$Cv\% = (Pen\ BW\ Standard\ Deviation \div Pen\ Average\ BW)\ \times 100$$

2.2.7 Intestinal lesion scoring

In each experiment, the entire length of the jejunum was examined for NE gross lesion scores by an experienced assessor. Scores ranged from 0 to 4 and was adapted from the methodology described by Shojadoost et al. (2012). A score of 0 was assigned when there were no visible abnormalities in the exposed segment of the jejunum; a score of 1 when there were no apparent ulcers in the mucosa, but the entire mucosal surface was covered with a layer of loosely adherent fibrin; a score of 2 when there was an excavated ulcer of the mucosa with fresh red bleeding and had light crusting of fibrin around the edges; a score of 3 when there was an excavated ulcer of the mucosa with dark green-black pigment within the ulcer bed and light crusting of fibrin over the

surface or, excavated ulcers with the periphery covered by thick, tightly adherent layers of fibrin, necrotic tissue, and inflammatory cells. A score of 4 was given when large, merged fibrin plaques, necrotic tissue, and inflammatory cells covered extensive areas of the intestinal mucosa.

2.2.8 Footpad lesion scoring

Footpad dermatitis (**FPD**) was assessed at 36 d in both feet of each broiler in Experiment 2. Paw quality was evaluated using the four-point footpad scoring by an experienced assessor according to the Welfare Quality® Protocol for Poultry (2009). Score 0 was given when no detectable lesion was observed; Score 1 for minimal discoloration of the skin foot; Score 2 for a detectable lesion on the central pad; Score 3 when the lesion took over most of the central pad; Score 4 when lesions took over the entire central pad and lesions on toes were also observed.

2.2.9 Carcass traits

On day 38 of Experiment 2, two males per experimental unit were randomly selected for processing. Live BW was recorded prior slaughter, and birds were identified with wing bands. After 12 h of feed and water withdrawal, broilers were euthanized by electrical stunning, exsanguinated and then scalded, defeathered, and eviscerated (Schneider et al., 2012). Carcasses (without feathers, head, viscera, and feet) were blast chilled to 4°C. The carcasses were weighed, and then broken down into saleable cuts: breast including *Pectoralis* major and minor, thighs, drumsticks, and wings. Chilled carcass yield was calculated relative to day 38 live BW prior to processing. Carcass part yields were calculated as proportions of chilled carcass weights.

2.2.10 Statistical analyses

Bartlett's test was used to evaluate the homogeneity of variances, and Shapiro-Wilk test to verify the normality of residuals. The performance data from Experiment 1 were analyzed by two-way ANOVA considering an 8 (COS molecular weights) x 3 (inclusion levels) + 2 (positive and

negative controls) factorial arrangement. The analysis was conducted using R software and the *agricolae* (de Mendiburu and Yaseen, 2020) package. For Experiment 2, uniformity and carcass trait data were analyzed by one-way ANOVA procedures using Statistix 10 software (Analytical Software, Tallahassee, FL). Significant effects ($P \leq 0.05$) were further evaluated with Tukey's test for pairwise comparisons. As the data of intestinal and footpad lesion scores were not normally distributed, Kruskal-Wallis, a non-parametric statistical test, was used, and statistical significance was considered at $P \leq 0.05$.

2.3 RESULTS

2.3.1 Growth performance

Experiment 1. There were no significant differences between PC and NC groups for the average BW at 10, 25 or 38 d. However, at 25 d broilers fed the PC diet (896.2 ± 42.5 g) were heavier than those fed COS 110 (782.2 ± 13.7 g), COS 25 (762.9 ± 14.3 g), or COS 14 kDa (787.1 ± 12.9 g; $P = 0.01$; Table 2.2). There were no effects of COS level ($P = 0.39$) or interaction between the diet and COS level ($P = 0.26$) on average BW at 25 d (Table 2.2). Similarly, the average BW of the birds at 10 and 38 d were also not affected by COS Mw, COS level, or their interaction ($P > 0.05$; Table 2.2).

Birds fed the PC diet had higher daily BWG during the grower phase (45.0 ± 2.3 g; $P < 0.01$) than those fed NC (37.3 ± 1.6 g), COS 110 (37.0 ± 1.2 g), COS 30 (38.4 ± 0.9 g), COS 25 (36.9 ± 0.8 g), or COS 14 kDa (37.2 ± 1.2 g). COS 220, COS 180, COS 95, and COS 17 kDa helped to partially restore the BWG after the NE challenge, compared to NC birds (3.5%, 9.0%, 6.8%, and 6% greater than NC, respectively; Table 2.3). The interaction between the diet and COS level was nearly significant in the grower phase ($P = 0.08$, Table 2.3). Birds fed the PC diet had the greatest BWG

(45.0 ± 2.3 g), but when the lowest dose (0.2 g/kg) of COS 110 was fed, BWG was reduced by 27% compared to PC and 17% compared to NC (data not shown).

For the entire period (0 to 38 d), birds from the PC (43.0 ± 2.2 g), COS 220 (41.5 ± 1.2 g), COS 180 (42.6 ± 0.7 g), COS 95 (42.6 ± 0.8 g), and COS 30 kDa (42.1 ± 0.9 g) groups had greater BWG than birds from the COS 25 kDa group (37.9 ± 1.4 g; P = 0.01; Table 2.3). However, they had similar BWG compared to birds from the NC (38.1 ± 1.4 g), COS 110 (39.3 ± 1.2 g), COS 17 (40.8 ± 1.1 g), and COS 14 kDa (39.4 ± 1.0 g). The diet did not affect the BWG in the starter (P = 0.24) or finisher (P = 0.11) phases. The COS inclusion level did not affect the BWG of the birds during any phase (P > 0.05). Also, there were no interactions between diet and COS level during the starter (P = 0.64) and finisher (P = 0.31) phases, nor during the entire cycle (P = 0.15; Table 2.3).

There were no significant differences between the PC and NC groups for FI at any phase. However, dietary treatments tended to affect the FI during the starter phase (P = 0.08). For the entire period, birds in the COS 95 and COS 30 kDa groups had higher FI (76.0 ± 1.4 and 76.1 ± 1.6 g/day/bird, respectively) than those fed NC, COS 110, COS 25, and COS 14 kDa (68.8 ± 1.5, 71.4 ± 2.2, 69.7 ± 1.7, and 71 ± 1.5 g/day/bird, respectively; P = 0.02; Table 2.4). Across COS type, supplementation at 5 g/kg increased the FI of the birds (70.3 ± 1.1 g/bird/day) compared to the diets supplemented with COS at 0.2 g/kg (67.2 ± 0.9 g/bird/day; P = 0.03; Table 2.4). There were no interactions between diet and COS level on FI for any of the phases (P > 0.05).

There were no significant differences between the PC and NC groups for FCR at any phase. In addition, the FCR of the birds was not affected by the dietary treatments or the interaction between the diet and COS level at any phase. However, during the grower phase, COS included at 5 g/kg tended to increase the FCR of the birds (1.82 ± 0.03 g/g) compared to the other levels of

supplementation (COS at 0 g/kg = 1.65 ± 0.03, COS at 0.2 g/kg = 1.76 ± 0.02, and COS at 2 g/kg = 1.78 ± 0.02 g/g; P = 0.07; Table 2.5).

Experiment 2. The PC group had the lowest average BW at 10 d (209.9 ± 9.1 g; P < 0.01) and the lowest BWG (16.0 ± 1.3 g; P < 0.01) compared to all the other treatments (Table 2.6). However, there were no dietary effects on FI or FCR (P = 0.52 and P = 0.11, respectively). At the end of the grower phase, birds fed COS 95 kDa at 5 g/kg had a higher BW (904.8 ± 34.3 g) than those fed COS 180 kDa at 5 g/kg (839.0 ± 43.2 g; P = 0.03). The PC treatment resulted in higher BWG (44.4 ± 1.9 g/day/bird; P = 0.02) and lower FCR (1.57 ± 0.07; P = 0.05) than the COS 180 at 5 g/kg treatment (39.8 ± 3.0 g and 1.70 ± 0.13, respectively), but was similar to the other treatments. FI was not affected by dietary treatment (P > 0.05; Table 2.6). At 36 d, birds fed COS 95 kDa at 5 g/kg were nearly significantly heavier (1,838 ± 67.1 g; P = 0.08; Table 2.6) by 81.6 g than birds from the NC group. Dietary treatments did not affect the BWG, FI or FCR of broilers during the finisher phase (P > 0.05). From 0 to 36 d, no significant differences were observed among the treatments for BWG, FI or FCR (P > 0.05; Table 2.6).

2.3.2 Uniformity

The body weight uniformity (CV%) was not affected by dietary treatment at any phase in either of the two experiments (P > 0.05; Supplemental Tables 2.1 and 2.2). In Experiment 1, the overall uniformity (CV%) across treatments at 10, 25, and 38 d was 16.9 ± 0.5, 19.2 ± 0.7, and 17.3 ± 0.8%, respectively. In Experiment 2, the overall uniformity across treatments at 10, 17, 25, and 36 d was 14.6 ± 0.5, 16.7 ± 0.5, 15.6 ± 0.6, and 15.6 ± 0.5%, respectively.

2.3.3 Mortality and clinical necrotic enteritis signs

No signs of clinical necrotic enteritis were observed in either of the two experiments. In addition, there were no treatment effects on mortality in either experiment (P > 0.05; data not shown). In

Experiment 1, the total mortality rate was 7.36%, while in Experiment 2, the total mortality rate was 3.91%. Although there were no treatment effects on mortality rates and no clinical NE signs, we observed a slight increase in the overall number of deaths after the challenge in both experiments. In Experiment 1, 53.7% of the total mortality (N = 67) occurred between 10 and 25 d. Similarly, in Experiment 2, 69% of the total mortality (N = 45) occurred between 10 and 25 d.

2.3.4 Necrotic enteritis gross lesion scores

Experiment 1. At 20 d, birds fed COS 95 kDa at 2 or 5 g/kg had lower NE gross lesion scores in the jejunum (score 0 for all assessed birds; $P < 0.01$) than birds fed COS 180 kDa at 2 g/kg, and COS 17 kDa or COS 14 kDa at 5 g/kg (1.20 ± 0.37, 1.20 ± 0.20 and 1.20 ± 0.49, respectively; Table 2.7). Nonetheless, their scores were similar to those of birds from each of the other treatments, including the PC (0.75 ± 0.25) and NC (0.50 ± 0.29) groups. At 38 d, birds fed COS 95 kDa at 5 g/kg had the lowest NE lesion score (0.20 ± 0.20; $P = 0.02$; Table 2.7) compared to birds fed COS 17 kDa at 2 g/kg (1.40 ± 0.25) or COS 14 kDa at 5 g/kg (1.20 ± 0.20). Birds from COS 110 at 5 g/kg and COS 25 at 2 g/kg groups also had lower NE lesion scores (both with 0.40 ± 0.24 scores) than birds from the COS 17 kDa at 2 g/kg group. All the remaining treatments resulted in similar NE lesion scores, including PC and NC (Table 2.7).

Experiment 2. There were no significant treatment effects on NE gross lesion scores at 22 d or 38 d in the jejunum of the broilers ($P = 0.46$, and $P = 0.14$, respectively; Supplemental Table 2.3). The overall mean for NE lesion score across all treatments was 0.75 ± 0.05 at 22 d and 1.08 ± 0.04 at 38 d.

2.3.5 Footpad dermatitis

In Experiment 2, dietary treatments did not affect the FPD in broilers (P = 0.52; data not shown). A total of 988 birds were assessed for footpad lesions, but only 3 birds (0.3%) had a score of 1 (minimal discoloration of the skin foot), while all the other birds had healthy footpads (score 0).

2.3.6 Carcass traits

In Experiment 2, there were no dietary effects on live weight before processing (overall mean = 1,796.8 ± 21.42 g), carcass yield (63.51 ± 0.21%, the proportion of *Pectoralis* minor (4.94 ± 0.03%), thighs (18.29 ± 0.12%), wings (11.63 ± 0.06%) or inedible (27.86 ± 0.16%; P > 0.05; Table 2.8).

Birds from the NC group had a higher drumstick yield (16.03 ± 0.36%; P = 0.03) than those fed COS 110 kDa (14.56 ± 0.44%), but they were similar to the other treatments, including PC (14.93 ± 0.22%; Table 2.8). The proportion of *Pectoralis* major tended to be the greatest in birds from the COS 180 kDa at 5 g/kg group (21.67 ± 0.42%) and the lowest in those from the COS 180 kDa at 2 g/kg group (20.2 ± 0.39%; P = 0.06).

2.4 DISCUSSION

Although studies evaluating COS pharmaceutical properties have been conducted in the human biomedical field for over 60 years (Periayah et al., 2016), the exploration of COS as a potential feed additive in animal production increased in the 2000s, coinciding with the search for alternatives to AGP. In broilers, dietary COS alleviated heat stress-induced oxidative damage (Lan et al., 2020; Chang et al., 2022), alleviated stress induced by glucocorticoid (Osho and Adeola, 2020), mitigated performance losses and pro-inflammatory responses caused by coccidiosis (Osho and Adeola, 2019), and increased overall broiler performance (Li et al., 2007; Ayman et al., 2022). However, there is little consistency among the studies on the COS Mw used and their level of

inclusion in the diets. As the biological properties of COS depend on its Mw (Zou et al., 2016), it is important to understand the effects of different COS Mw on broilers. To the best of our knowledge, no studies have evaluated the effects of COS at different Mw on broilers challenged with subclinical NE. Therefore, the present studies offer a preliminary of COS to prevent or alleviate subclinical NE in broilers.

In Experiment 1, COS at medium to high Mw (i.e., 95 to 220 kDa) had a greater potential to partially restore performance after the challenge than diets supplemented with COS at low Mw, such as 30, 25, 17 and 14 kDa. This may be explained by the fact that COS at lower Mw are more easily absorbed from the intestine and less susceptible to bioactive interactions with the intestinal environment (Chae et al., 2005; Zeng et al., 2008). Like other birds, broilers produce acidic chitinase in the proventriculus (Tabata et al., 2017, 2018). Although the production of this enzyme is not stimulated by dietary chitin (Koh and Iwamae, 2013), chitinase may have partially degraded COS, facilitating their absorption in the gut. This may be especially true for diets containing COS at low Mw and inclusion levels (Chae et al., 2005).

In contrast, COS at medium to high Mw (e.g., 220 to 95 kDa) and at high levels of inclusion may be poorly absorbed and, therefore, have higher chances of exerting their bioactive properties (e.g., prebiotic, immunomodulation) at the microbiota and mucosal level, even after being subjected to endogenous chitinase. This is supported by birds fed COS 95 kDa (medium Mw) included at 2 or 5 g/kg having no NE gross lesion in the jejunum at 20 d. In addition, birds fed COS 95 kDa at 5 g/kg had lower NE scores at 38 d compared to some of the other treatments. These results may be related to the COS properties, which, in this case, may include 1) the ability of COS to interact with mucosal tissue and be recognized by immune cells, triggering immune responses that can, in turn, mitigate the proliferation of pathogenic bacteria (Moine et al., 2021); 2) the ability of COS

to serve as a substrate for beneficial bacteria, facilitating their growth and establishment in the gut (Lee et al., 2002), and 3) the ability of COS to bind negatively charged microbial cell membranes from free bacteria in the lumen, preventing adhesion to the intestinal wall and facilitating their washout (Kim et al., 2003). Thus, in Experiment 1, COS 95 kDa may have reduced gut dysbiosis during the challenge and stimulated intestinal homeostasis reestablishment, preventing performance losses.

In Experiment 1, significant differences in performance between the PC and NC groups were only observed during the grower period, following the NE challenge application. In this period, the 17% BWG reduction in birds from the NC relative to the PC group suggests that the NE challenge caused an enteric disturbance that might have triggered mild intestinal inflammation, decreasing broiler performance (Klasing, 2007; Broom and Kogut, 2018). In contrast, birds that received antibiotic and coccidiostat (i.e., the PC group) appeared to be more resistant to the NE challenge. Interestingly, in the subsequent period (26 to 38 d), the NC birds recovered the BWG to the same level as the other treatments, including PC. This indicates that the challenge may have caused mild subclinical NE, and NC birds were able to recover from it. The compensatory growth following the challenge period may be related to the acquired immunity against *Eimeria* and intestinal cell regeneration (Henken et al., 1994) that may have occurred during the finisher phase. Similar results are often observed after applying a non-attenuated coccidiosis vaccine in broilers. Birds tend to have compensatory growth following the initial setback from vaccination (Li et al., 2005; Ritzi et al., 2016). In addition, birds may have had limited *Eimeria* reinfection, which in this experiment, in particular, may have occurred due to animals being placed in battery cages with reduced exposure to the manure and, as a consequence, reduced contact with sporulated oocysts (Price, 2012). Similarly, in Experiment 2, although birds were placed in floor pens, the absence of FPD

and considering the dry weather conditions in Alberta, Canada, suggest that the litter was probably too dry for proper parasite cycling (Venkateswara Rao et al., 2015). Given that coccidiosis was used as a primary predisposing factor to induce NE, a reduction in coccidiosis severity leads to a corresponding decrease in the chances of NE persistency. Although the effects of the NE challenge were observed only during the grower period, this aligns with the risk period of NE occurrence in the field (Mot et al., 2014; He et al., 2022). However, the effects were subtle. We expected a more substantial performance reduction between the PC and NC groups. This suggests that refinements to the challenge model to yield a more potent, yet still subclinical NE episode might be necessary to understand more clearly the effects of COS in preventing this intestinal disease.

In the present studies, a natural *C. perfringens* infection model was used as an attempt to mimic field conditions that cause subclinical NE in broilers and, therefore, test the efficacy of COS as potential AGP replacements. This model is based on the exposure of birds to predisposing factors that can alter gut homeostasis and facilitate the natural proliferation of *C. perfringens* strains already present in the GIT environment (He et al., 2022). For this, birds are challenged with a 15x recommended dose of a coccidiosis vaccine, containing live *Eimeria* oocysts by gavage at 12 d and 24-hour feed withdrawal at 18 d. Coccidiosis was used as a predisposing factor because it is the most important risk factor associated with NE based on the strong correlation between the prevalence of both diseases in the field (Dierick et al., 2021). Coccidiosis is an enteric parasitic disease caused by various *Eimeria* spp. oocysts. As a part of their life cycle, these parasites invade the host enterocytes to complete maturation and replicate (López-Osorio et al., 2020). Upon exiting, they cause damage to the epithelium, including disruption in the extracellular matrix and plasma protein leakage, leading to increased mucus production. These changes create favourable conditions (nutrient substrate and adhesion sites) for the proliferation of *C. perfringens* (Collier et

al., 2008; Wade et al., 2015). The 24-hour feed withdrawal introduced on day 18 was important for enhancing microbiota disturbance, favoring the conditions for *C. perfringens* to thrive, and facilitating the timely onset of a subclinical disease outbreak (Lacey et al., 2018; He et al., 2022). As *C. perfringens* is an opportunistic pathogen, it gains a selective survival advantage over other bacteria during the feed withdrawal, using the host's mucus and tissues as a substrate for growth (Shimizu et al., 2002).

In Experiment 2, the effects of the PC, NC, and most promising COS treatments from Experiment 1 (COS 95 kDa at 0.2 and 5 g/kg, COS 110 kDa at 5 g/kg, and COS 180 kDa at 0.2, 2 and 5 g/kg) were further investigated in a larger-scale study conducted in floor pens. However, no significant differences between PC and NC were observed for most of the evaluated parameters. Since the main effect of subclinical NE is a reduction in broiler performance (Timbermont et al., 2011), we expected to see a decrease in performance in birds fed NC relative to those fed the PC. This would provide evidence of a challenge in the experimental environment, and the inclusion of AGP in the PC diet mitigated this challenge. In the absence of reduced performance in the NC group, it was not possible to accurately evaluate the effects of potential AGP replacements (Korver, 2020). Therefore, Experiment 2 highlighted the need to increase the robustness of our challenge model. A way to increase its robustness would be increasing the coccidiosis vaccine dosage (e.g., 20x recommended doses) on day 12. This might increase the epithelium disturbance by providing more oocysts invading enterocytes, creating more favourable conditions for *C. perfringens* proliferation. In addition, the relative low humidity in Alberta, Canada (~ 67% average in 2022; Alberta Climate Information Service) contributes to dry weather and dry litter, hindering the sporulation of *Eimeria* oocysts (Venkateswara Rao et al., 2015). To address this, spraying water over the litter could help maintain the right conditions for *Eimeria* re-cycling and bird reinfection. Another way to enhance

the challenge would be to use in-feed corticosterone (Zaytsoff et al., 2020) as a stress factor following the coccidia challenge. In regions with higher relative humidity, using a sanitation challenge, such as reused litter (Bortoluzzi et al., 2019), would be an option to increase the robustness of the challenge.

Although the same challenge model was used in both experiments, Experiment 1 showed a slightly more effective induction of subclinical NE than Experiment 2. This may be related to the different housing systems in which the broilers were placed. In Experiment 1, birds were raised in battery cages with wire mesh flooring, whereas in Experiment 2, they were reared on floor pens with wood shaving litter flooring. Typically, broilers housed in cages experience higher levels of stress due to space limitations, restricted expression of natural behavior like scratching and dustbathing, and increased susceptibility to leg disorders compared to birds placed in floor pens (Wideman et al., 2012; Shields and Greger, 2013). Furthermore, the litter, when of good quality, can serve as a habitat for commensal and beneficial bacteria, which can prevent the establishment of pathogenic bacteria. The litter can also aid in the development and proper functioning of the gizzard (Santos et al., 2011). It is possible that the caged broilers in Experiment 1 were exposed to a more stressful environment, potentially contributing to an enhanced challenge. Any factor that causes stress in broilers can lead to a suppressed immune system and disturb the balance of the intestinal ecosystem in such a way that the risk of a NE outbreak increases (Tsiouris et al., 2015a; b, 2018; Zaytsoff et al., 2020).

Although no differences were found between the PC and NC treatments in Experiment 2, COS 95 kDa at 5 g/kg tended to enhance BW at 36 d. However, compared to Experiment 1, COS treatments did not affect the NE gross lesion scores. The macroscopic examination of intestinal NE gross lesions seems to be a more effective diagnostic tool in cases of clinical NE, in which the lesions

are more aggressive and visible. Subclinical NE lesions are often mild and are more accurately detected by microscopic examination (Olkowski et al., 2006). The microscopic examination of the jejunum from broilers used in Experiment 2 was reported in a complementary study (Chapter 3). Birds from the NC group had shorter villi compared to those fed COS 95 kDa at 0.2 g/kg, and the deepest crypt, lowest villus height to crypt depth ratio, thickest epithelium, thickest lamina propria, and greater count of *Eimeria* oocysts in villi compared to all other treatments. On the other hand, birds in the COS 95 kDa treatments had intestinal morphology integrity similar to that of birds from the PC group (Chapter 3). Thus, although there were no differences in gross lesions among treatments, we observed a greater disturbance of the intestinal morphology integrity in NC birds at a microscopic level. This suggests that there was an ongoing enteric challenge which mainly affected NC birds. However, it may not have been strong enough to negatively affect the performance of NC birds compared to those fed the PC diet. It is important to note that this does not mean that the PC group was not affected by the challenge. We observed that all birds from both experiments, including those in the PC group, weighed less than expected based on the Ross 708 performance objectives (Aviagen, 2022). However, the birds in the PC group were better able to handle the challenge due to the presence of AGP and coccidiostat in their diets.

Although the potential of COS products in Experiment 2 could not be properly evaluated, COS 95 kDa demonstrated promising results in both experiments. While COS 95 kDa alone is unlikely to replace AGP, it may have the potential to be used as a part of a non-AGP strategy to prevent subclinical NE in broilers. Further exploration of its mechanism of action under a more potent subclinical NE challenge model should be considered, as well as investigating potential synergistic relationships with other proposed AGP replacements (e.g., probiotics, enzymes, organic acids).

In conclusion, COS at medium to high Mw (95 to 220 kDa) exhibited a greater capacity to partially restore performance following the NE challenge than COS at low Mw (30 to 14 kDa). In both experiments, COS 95 kDa demonstrated some potential to enhance performance and mitigate NE-associated damage in the jejunum of broilers. Adjustments of the natural subclinical NE challenge model are required to provide a more robust challenge (i.e., a more substantial decrease in the performance of birds fed the NC relative to the PC) and more accurately test the effects of COS in preventing NE in broilers.

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2.6 TABLES

Table 2.1. Ingredient and calculated nutrient composition of the basal diets provided to birds during the starter, grower, and finisher phases in Experiments 1 and 2.

Ingredients (%)	**Starter (Day 0 to 10)**	**Grower (Day 11 to 25)**	**Finisher (Day 26 to 38)**
Canola meal	7.5	10	12
Soybean meal	27.96	22.44	19.38
Wheat	59.18	61.46	61.37
Calcium carbonate	1.18	1.03	0.93
Monocalcium phosphate	1.00	0.75	0.57
NaCl	0.26	0.26	0.26
L-lysine	0.10	0.07	0.02
DL-methionine	0.30	0.25	0.23
L-threonine	0.05	0.01	-
Hy-D® premix[1]	0.05	0.05	0.05
Vitamin mineral premix[2]	0.5	0.5	0.5
Choline chloride premix[3]	0.05	00.5	0.05
Phytase[4]	0.01	0.01	0.01
Canola oil	1.86	3.11	4.63
Mycotoxin binder[5]	0.15	0.15	0.15
Xylanase[6]	0.05	0.05	0.05
Calculated nutrient composition			
Crude protein (%)	25.16	23.58	22.73
ME, kcal/kg (%)	3,000	3,100	3,200
Calcium (%)	0.96	0.87	0.81
Available phosphorus (%)	0.48	0.435	0.405

[1]Provided 69 µg 25-hydroxycholecalciferol per kg diet (DSM Nutritional Products Inc., Parsippany, New Jersey).
[2]Provided per kilogram of diet: vitamin A (retinyl acetate), 10,000 IU; cholecalciferol, 4,000 IU; vitamin E (DL-α-tocopheryl acetate), 50 IU; vitamin K, 4.0 mg; thiamine mononitrate (B_1), 4.0 mg; riboflavin (B_2), 10 mg; pyridoxine HCL (B_6), 5.0 mg; vitamin B_{12} (cobalamin), 0.02 mg; D-pantothenic acid, 15 mg; folic acid, 0.2 mg; niacin, 65 mg; biotin, 1.65 mg; iodine (ethylenediamine dihydroiodide), 1.65 mg; Mn ($MnSO_4 \cdot H2O$) , 120 mg; Cu, 20 mg; Zn, 100 mg, Se, 0.3 mg; Fe ($FeSO_4 \cdot 7H_2O$), 800 mg.
[3]Provided 100 mg choline per kg of diet.
[4]Provided 500 FTU phytase/kg diet (Phyzyme XP, Danisco Animal Nutrition, Marlborough, UK).
[5]Biomin II (Biomin Canada Inc., Mont-St-Hilaire, Québec, Canada).
[6]Econase XT 25 (AB Vista, Marlborough, UK) provided 80,000 BXU of endo-1, 4-beta-xylanase activity per kg of diet.

Table 2.2. The average body weight (g) of broilers at 10, 25 and 38 days of age fed with or without chitosan oligosaccharide (COS) in the diet in Experiment 1.

Item	**BW (g) at 10 d**	**BW (g) at 25 d**	**BW (g) at 38 d**
Diet effect means			
PC[1]	207.1	896.2[a]	1,718.0
NC[2]	194.5	809.5[ab]	1,694.2
COS 220 kDa	207.1	794.3[ab]	1,702.1
COS 180 kDa	202.5	891.4[ab]	1,756.9
COS 110 kDa	207.1	782.2[b]	1,697.1
COS 95 kDa	202.5	809.2[ab]	1,779.1
COS 30 kDa	207.1	796.8[ab]	1,746.5
COS 25 kDa	196.5	762.9[b]	1,593.3
COS 17 kDa	208.2	801.3[ab]	1,707.3
COS 14 kDa	197.6	787.1[b]	1,668.2
SEM	1.28	5.45	13.81
COS level effect means			
0 g/kg	200.8	852.2	1,701.1
0.2 g/kg	204.3	791.2	1,692.8
2 g/kg	200.0	790.5	1,714.0
5 g/kg	204.1	801.5	1,712.1
SEM	14.17	51.88	23.94
Source of variation		*Probability*	
Diet[3]	0.23	0.01	0.10
COS level[4]	0.87	0.39	0.61
Diet x COS level[5]	0.94	0.26	0.48

[1] PC: Positive Control (basal diet with antibiotic and coccidiostat).
[2] NC: Negative Control (basal diet without antibiotic and coccidiostat).
[3] Diet: Positive Control, Negative Control, COS 220 kDa, COS 180 kDa, COS 110 kDa, COS 95 kDa, COS 30 kDa, COS 25 kDa, COS 17 kDa, and COS 14 kDa.
[4] COS Inclusion: 0, 0.2, 2 and 5 g/kg.
[5] Effect of the interaction between Diet and COS Inclusion.
[a,b] Means with different superscripts within the same row are significantly different $P \leq 0.05$.
Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 2.3. The average body weight gain (BWG) of broilers in the starter (0-10 d), grower (11-25 d), finisher (26-38 d), and entire (0-38 d) phases fed with or without chitosan oligosaccharides (COS) in the diet in Experiment 1.

Item	**BWG (g/day/bird) 0-10 d**	**BWG (g/day/bird) 11-25 d**	**BWG (g/day/bird) 26-38 d**	**BWG (g/day/bird) 0-38 d**
Diet effect means				
PC[1]	16.1	45.0[a]	66.9	43.0[a]
NC[2]	15.2	37.3[b]	65.7	38.1[ab]
COS 220 kDa	16.3	38.6[ab]	70.0	41.5[a]
COS 180 kDa	16.1	40.6[ab]	71.0	42.6[a]
COS 110 kDa	15.9	37.0[b]	69.0	39.5[ab]
COS 95 kDa	16.2	39.8[ab]	71.7	42.6[a]
COS 30 kDa	15.5	38.4[b]	72.4	42.1[a]
COS 25 kDa	15.3	36.9[b]	61.7	37.9[b]
COS 17 kDa	16.5	39.5[ab]	66.0	40.9[ab]
COS 14 kDa	15.1	37.2[b]	67.0	39.4[ab]
SEM	0.13	0.35	0.87	0.38
COS level effect means				
0 g/kg	15.6	41.1	66.3	40.5
0.2 g/kg	15.9	38.1	68.1	40.4
2 g/kg	15.6	38.5	68.1	40.6
5 g/kg	16.1	38.9	69.6	41.4
SEM	1.68	3.35	7.25	4.32
Source of variation		*Probability*		
Diet[3]	0.24	< 0.01	0.11	0.01
COS level[4]	0.55	0.37	0.45	0.26
Diet x COS level[5]	0.64	0.08	0.31	0.15

[1] Positive Control: The basal diet with antibiotic (Bacitracin methylene disalycilate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2] Negative Control: The basal diet without any medications.

[3] Diets: Positive Control, Negative Control, COS 220 kDa, COS 180 kDa, COS 110 kDa, COS 95 kDa, COS 30 kDa, COS 25 kDa, COS 17 kDa, and COS 14 kDa.

[4] COS Inclusion: 0, 0.2, 2 and 5 g/kg.

[5] Effect of the interaction between Diet and COS Inclusion.

[a,b] Means with different superscripts within the same row are significantly different $P \leq 0.05$.

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 2.4. The feed intake (FI) of broilers in the starter (0-10 d), grower (11-25 d), finisher (26-38 d), and entire (0-38 d) phases fed with or without chitosan oligosaccharides (COS) in the diet in Experiment 1.

Item	FI (g/day/bird) 0-10 d	FI (g/day/bird) 11-25 d	FI (g/day/bird) 26-38 d	FI (g/day/bird) 0-38 d
Diet effect means				
PC[1]	23.3	73.3	124.2	73.8^{abcd}
NC[2]	22.0	62.3	132.7	68.8^{d}
COS 220 kDa	23.5	69.7	132.8	75.1^{abc}
COS 180 kDa	23.4	71.5	132.5	75.8^{ab}
COS 110 kDa	22.2	67.4	131.2	71.4^{bcd}
COS 95 kDa	21.9	69.2	139.1	76.0^{a}
COS 30 kDa	22.7	69.0	136.7	76.1^{a}
COS 25 kDa	22.1	65.7	125.4	69.7^{d}
COS 17 kDa	23.7	69.1	130.2	74.1^{abcd}
COS 14 kDa	21.4	68.3	125.3	71.0^{cd}
SEM	0.20	0.59	1.36	0.58
COS level effect means				
0 g/kg	22.7	67.8^{ab}	128.4	71.3
0.2 g/kg	22.6	67.2^{b}	132.5	72.9
2 g/kg	22.5	68.8^{ab}	131.3	73.3
5 g/kg	22.8	70.3^{a}	131.2	74.6
SEM	2.22	5.85	13.82	5.45
Source of variation		*Probability*		
Diet[3]	0.08	0.18	0.26	0.02
COS level[4]	0.66	0.03	0.74	0.23
Diet x COS level[5]	0.88	0.33	0.80	0.38

[1] Positive Control: The basal diet with antibiotic (Bacitracin methylene disalycilate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2] Negative Control: The basal diet without any medications.

[3] Diet: Positive Control, Negative Control, COS 220 kDa, COS 180 kDa, COS 110 kDa, COS 95 kDa, COS 30 kDa, COS 25 kDa, COS 17 kDa, and COS 14 kDa.

[4] COS Inclusion: 0, 0.2, 2 and 5 g/kg.

[5] Effect of the interaction between Diet and COS Inclusion.

[a,b,c,d] Means with different superscripts within the same row are significantly different $P \leq 0.05$.

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 2.5. The feed conversion ratio (FCR) of broilers in the starter (0-10 d), grower (11-25 d), finisher (26-38 d), and entire (0-38 d) phases fed with or without chitosan oligosaccharides (COS) in the diet in Experiment 1.

Item	**FCR (g/g) 0-10 d**	**FCR (g/g) 11-25 d**	**FCR (g/g) 26-38 d**	**FCR (g/g) 0-38 d**
Diet effect means				
PC[1]	1.44	1.64	1.86	1.72
NC[2]	1.45	1.67	2.05	1.81
COS 220 kDa	1.46	1.81	1.91	1.82
COS 180 kDa	1.45	1.74	1.86	1.78
COS 110 kDa	1.41	1.82	1.90	1.81
COS 95 kDa	1.35	1.73	1.95	1.80
COS 30 kDa	1.46	1.80	1.88	1.81
COS 25 kDa	1.45	1.78	1.94	1.86
COS 17 kDa	1.44	1.75	1.96	1.82
COS 14 kDa	1.41	1.84	1.88	1.81
SEM	0.01	0.01	0.02	0.01
COS level effect means				
0 g/kg	1.44	1.65	1.95	1.76
0.2 g/kg	1.42	1.76	1.94	1.81
2 g/kg	1.44	1.78	1.90	1.81
5 g/kg	1.42	1.82	1.89	1.81
SEM	0.15	0.20	0.22	0.14
Source of variation		*Probability*		
Diet[3]	0.57	0.23	0.64	0.77
COS level[4]	0.92	0.07	0.22	0.14
Diet x COS level[5]	0.72	0.52	0.63	0.38

[1] Positive Control: The basal diet with antibiotic (Bacitracin methylene disalycilate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2] Negative Control: The basal diet without any medications.

[3] Diets: Positive Control, Negative Control, COS 220 kDa, COS 180 kDa, COS 110 kDa, COS 95 kDa, COS 30 kDa, COS 25 kDa, COS 17 kDa, and COS 14 kDa.

[4] COS Inclusion: 0, 0.2, 2 and 5 g/kg.

[5] Effect of the interaction between Diet and COS Inclusion.

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 2.6. The performance of broilers in the starter (0-10 d), grower (11-25 d), finisher (26-36 d), and entire (0-36 d) phases fed with or without chitosan oligosaccharides (COS) in the diet in Experiment 2.

Item	Treatments								SEM	P-value
	PC[1]	**NC**[2]	**COS 95 kDa 0.2 g/kg**	**COS 95 kDa 5 g/kg**	**COS 110 kDa 5 g/kg**	**COS 180 kDa 0.2 g/kg**	**COS 180 kDa 2 g/kg**	**COS 180 kDa 5 g/kg**		
BW at 10 d (g)	209.9^{B}	234.1^{A}	239.9^{A}	239.0^{A}	237.7^{A}	232.8^{A}	238.5^{A}	229.7^{A}	3.67	< 0.01
BW at 25 d (g)	879.9^{ab}	866.8^{ab}	882.8^{ab}	904.8^{a}	880.7^{ab}	879.8^{ab}	872.5^{ab}	839.0^{b}	12.18	0.03
BW at 36 d (g)	1792.5	1756.4	1824.3	1838.0	1791.3	1765.5	1764.3	1733.4	25.43	0.08
BWG[3] 0-10d (g/d)	16.0^{B}	18.8^{A}	19.4^{A}	19.2^{A}	19.1^{A}	18.7^{A}	19.2^{A}	18.3^{A}	0.40	< 0.01
BWG[3] 11-25d (g/d)	44.4^{a}	42.1^{ab}	41.9^{ab}	43.4^{ab}	41.2^{ab}	42.7^{ab}	41.8^{ab}	39.8^{b}	0.85	0.02
BWG[3] 26-36 d (g/d)	83.0	80.8	85.3	84.8	81.4	80.7	81.1	81.4	0.75	0.54
BWG[3] 0-36d (g/d)	47.1	46.3	47.1	47.8	45.9	46.3	46.2	44.4	0.92	0.28
FI[4] 0-10d (g/d)	26.7	28.3	28.2	28.9	27.8	27.2	27.8	27.7	0.72	0.52
FI[4] 11-25d (g/d)	69.9	70.4	69.3	71.2	68.1	68.9	71.1	68.7	1.77	0.88
FI[4] 26-36d (g/d)	147.2	153.0	159.1	159.0	155.3	155.2	155.8	148.4	1.20	0.09
FI[4] 0-36d (g/d)	79.3	81.7	81.8	83.2	80.7	80.8	82.1	78.1	1.55	0.37
FCR[5] 0-10d (g/g)	1.68	1.52	1.47	1.51	1.46	1.45	1.44	1.51	0.05	0.11
FCR[5] 11-25d (g/g)	1.57^{a}	1.68^{ab}	1.65^{ab}	1.64^{ab}	1.65^{ab}	1.61^{ab}	1.70^{ab}	1.72^{b}	0.03	0.05
FCR[5] 26-36d (g/g)	1.80	1.90	1.88	1.87	1.91	1.94	1.92	1.86	0.02	0.67
FCR[5] 0-36d (g/g)	1.69	1.76	1.74	1.74	1.75	1.75	1.78	1.76	0.02	0.34

[1] Positive Control: The basal diet with antibiotic (Bacitracin methylene disalycilate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).
[2] Negative Control: The basal diet without any medications.
[3] BWG: Average body weight gain (g) per day (d) per bird.
[4] FI: Average feed intake (g) per day (d) per bird.
[5] FCR: Feed conversion ratio.
[a,b] Means with different superscripts within the same row are significantly different $P \leq 0.05$.
[A, B] Means with different superscripts within the same row are significantly different $P < 0.01$.
Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 2.7. Evaluation of necrotic enteritis (NE) lesion scores in the jejunum of broilers at 20 d and 38 d of age, fed with or without chitosan oligosaccharides (COS) in the diet in Experiment 1.

Treatment	COS Inclusion	Sample size	NE gross lesion scores[4]	
			20 d	38 d
PC[1]	----	5	0.75^{AB}	1.00^{abc}
NC[2]	----	5	0.50^{AB}	1.00^{abc}
COS 220 kDa	0.2 g/kg	5	0.80^{AB}	1.00^{abc}
	2.0 g/kg	5	0.80^{AB}	0.80^{abc}
	5.0 g/kg	5	0.80^{AB}	0.80^{abc}
COS 180 kDa	0.2 g/kg	5	0.80^{AB}	1.00^{abc}
	2.0 g/kg	5	1.20^{A}	1.00^{abc}
	5.0 g/kg	5	0.60^{AB}	0.80^{abc}
COS 110 kDa	0.2 g/kg	5	1.00^{AB}	1.00^{abc}
	2.0 g/kg	5	0.60^{AB}	1.00^{abc}
	5.0 g/kg	5	0.80^{AB}	0.40^{bc}
COS 95 kDa	0.2 g/kg	5	0.40^{AB}	0.80^{abc}
	2.0 g/kg	5	0.00^{B}	0.60^{abc}
	5.0 g/kg	5	0.00^{B}	0.20^{c}
COS 30 kDa	0.2 g/kg	5	1.00^{AB}	0.80^{abc}
	2.0 g/kg	5	1.00^{AB}	0.80^{abc}
	5.0 g/kg	5	0.80^{AB}	0.80^{abc}
COS 25 kDa	0.2 g/kg	5	0.80^{AB}	0.80^{abc}
	2.0 g/kg	5	0.80^{AB}	0.40^{bc}
	5.0 g/kg	5	0.80^{AB}	1.00^{abc}
COS 17 kDa	0.2 g/kg	5	0.20^{AB}	1.00^{abc}
	2.0 g/kg	5	1.00^{AB}	1.40^{a}
	5.0 g/kg	5	1.20^{A}	0.80^{abc}
COS 14 kDa	0.2 g/kg	5	1.00^{AB}	0.80^{abc}
	2.0 g/kg	5	0.80^{AB}	0.80^{abc}
	5.0 g/kg	5	1.20^{A}	1.20^{ab}
SEM			0.05	0.04
P-value[3]			< 0.01	0.02

[1] Positive Control: The basal diet with antibiotic (Bacitracin methylene disalycilate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2] Negative Control: The basal diet without any medications.

[3] P-value for Kruskal-Wallis One-Way Nonparametric AOV test.

[4] The lesion scoring system used ranged from 0 (no detected lesions) to 4 (extensive presence of fibrin, necrotic tissue, and inflammation covering mucosae).

[a,b,c] Means with different superscripts within the same column are significantly different $P \leq 0.05$.

[A, B] Means with different superscripts within the same column are significantly different $P < 0.01$.

Table 2.8. The carcass traits of broilers at 38 d fed with or without chitosan oligosaccharide (COS) in the diet in Experiment 2.

Treatment	COS Inclusion	Live BW[3] (g)	Chilled Carcass[4] (g)	Carcass Yield[5] %	*Pectoralis* major[6] %	*Pectoralis* Minor[6] %	Drumsticks[6] %	Thighs[6] %	Wings[6] %	Inedible[6] %
PC[1]	----	1,837.7	1,183.2	64.20	21.44	4.99	14.93[ab]	18.27	11.82	28.29
NC[2]	----	1,777.6	1,115.2	62.57	20.40	4.83	16.03[a]	18.20	12.11	27.94
COS 95 kDa	0.2 g/kg	1,808.7	1,156.3	63.88	21.47	4.90	15.30[ab]	18.23	11.70	26.99
COS 95 kDa	5.0 g/kg	1,865.6	1,183.2	63.35	21.41	4.99	14.96[ab]	18.40	11.55	27.90
COS 110 kDa	5.0 g/kg	1,822.6	1,164.0	63.69	21.51	4.97	14.56[b]	18.33	11.91	28.20
COS 180 kDa	0.2 g/kg	1,687.2	1,070.7	63.12	20.95	4.84	15.22[ab]	18.53	12.12	27.87
COS 180 kDa	2.0 g/kg	1,789.7	1,139.5	63.46	20.20	4.86	15.75[ab]	18.67	11.83	27.48
COS 180 kDa	5.0 g/kg	1,787.7	1,143.8	63.75	21.67	5.09	15.02[ab]	17.76	11.84	28.23
SEM		21.42	15.42	0.21	0.14	0.03	0.11	0.12	0.06	0.16
P-value		0.61	0.64	0.66	0.06	0.43	0.03	0.76	0.34	0.49

[1] Positive Control: The basal diet with antibiotic (Bacitracin methylene disalycilate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).
[2] Negative Control: The basal diet without any medications.
[3] Average live BW (g) of birds at 38 d prior to processing.
[4] Chilled carcass weight (g) without feathers, head, viscera, and feet.
[5] Carcass yield was calculated relative to the live BW (38 d) prior to processing.
[6] Carcass part yields were calculated as proportions of chilled carcass weights.
[a,b] Means with different superscripts within the same column are significantly different $P \leq 0.05$.

3. CHITOSAN OLIGOSACCHARIDES ON BROILERS CHALLENGED WITH A NATURAL, SUBCLINICAL NECROTIC ENTERITIS CHALLENGE MODEL: EFFECTS ON INTESTINAL HEALTH AND IMMUNE MODULATION

OVERVIEW

Dietary chitosan oligosaccharides (**COS**) have been tested as potential antibiotic (**AGP**) replacements. Previously, we observed that medium Mw COS (95 kDa) partially restored broiler performance after subclinical necrotic enteritis (**NE**) challenge to that of birds fed AGP and tended to increase BW compared to broilers not fed AGP. From that study, the most promising treatments were further evaluated to determine the effects of COS on immune modulation and broiler intestinal health. Treatments were a positive control (PC; with antibiotic and coccidiostat), negative control (NC; without medications), and NC plus COS 95 kDa at either 0.2 or 5 g/kg diet. A natural, subclinical NE challenge model (oral 15X coccidiosis vaccine dose at 12 d; 24-hour feed removal at 18 d) was applied to all birds. At 22 d, serum immune biomarkers and cecal microbial abundance were measured. At 22 and 38 d, cecal short-chain fatty acid concentrations were measured and jejunal tissue was collected for intestinal morphology and *Eimeria* oocyst count. NC had a greater abundance of *Clostridium sensu stricto 1* and *Erysipelatoclostridium* at the genus level than COS treatments. At the species level, COS treatments had a higher abundance of *Lactobacillus oris* than NC and PC, and a higher abundance of *Lactobacillus ingluviei* than PC. At 22 d, COS at 5 g/kg increased isobutyrate vs PC and NC. NC birds had 345% greater serum interferon-α than PC, while levels in birds fed COS were intermediate. COS at 0.2 g/kg increased interleukin-16 by 63.7% compared to PC. Macrophage Inflammatory Protein-3α was higher in COS at 0.2 g/kg and NC than PC. COS at 0.2 g/kg increased villus height by 9.0% compared to NC. The NC group had higher numbers of *Eimeria* oocysts, deeper crypts, and thicker epithelium and lamina propria than PC and COS groups. COS 95 kDa modulated cecal microbiota, enhanced

intestinal morphology, mitigated *Eimeria* infection, and shifted from innate to adaptive immune responses in broilers under subclinical NE challenge.

Keywords: Chitosan oligosaccharide, broiler chicken, necrotic enteritis, antibiotic alternative, gut health.

3.1 INTRODUCTION

Enteric diseases pose an important concern in the poultry industry as they cause reduced productivity, increased mortality, and an elevated risk of contaminated poultry products for human consumption (Dahiya et al., 2006; Grass et al., 2013; van der Klein et al., 2023). Antibiotic growth promoters (**AGP**) have been used to enhance growth rates and protect animals from enteric diseases since the 1940s (Castanon, 2007). Although different mechanisms of action have been proposed, it seems that the growth-promoting effects of AGP are related to the control of intestinal and systemic inflammation, enabling birds to come closer to expressing their genetic growth potential (Niewold, 2007; Oh et al., 2019). However, the phasing out of AGP from broiler diets has increased the risk of subclinical and clinical enteric diseases, which can divert dietary nutrients away from growth in support of inflammatory-related processes (Broom and Kogut, 2018).

Necrotic enteritis (**NE**) is a major enteric disease in poultry that is more prevalent with the withdrawal of AGP. NE is a multifactorial disease that results in estimated losses of $6 billion per year worldwide (Wade and Keyburn, 2015). This disease develops due to infection with pathogenic strains of *Clostridium perfringens* and the presence of predisposing factors (Parish, 1961; Annett et al., 2002; Wu et al., 2014; Antonissen et al., 2014). NE may present as a clinical or subclinical disease. The clinical form is characterized by a sudden increase in flock mortality, while subclinical NE leads to performance losses (Van Immerseel et al., 2009). Given the global shift towards antibiotic-free production and the great impact of NE on the poultry industry, there

is an urgent need to develop AGP alternatives to aid in NE control. Feed additives that promote gut health are potential candidates (M'Sadeq et al., 2015; Pham et al., 2020; Emami et al., 2021). Chitosan oligosaccharides (**COS**) have recently been explored as potential AGP replacements in animal diets (Huang et al., 2007; Han et al., 2007; Liu et al., 2008; Li et al., 2019). COS are deacetylated derivatives of chitosan or chitin, mainly found in the shells of crustaceans, exoskeletons of insects, and fungal cell walls (Zou et al., 2016). COS have a broad range of biological properties, including antimicrobial (Kim et al., 2003), anti-inflammatory (Ma et al., 2011; Moine et al., 2021), and antioxidant activities (Je et al., 2004; Li et al., 2017). While limited research exists evaluating COS in broilers under challenging conditions, some reports have indicated that COS can 1) enhance or maintain performance during coccidiosis, physical or physiological stress (Osho and Adeola, 2019, 2020; Chang et al., 2020); 2) preserve intestinal integrity, and reduce inflammatory responses during coccidiosis or heat stress (Osho and Adeola, 2019; Lan et al., 2020); 3) increase *Lactobacillus* concentrations while decreasing *Escherichia coli* in the ceca of broilers fed with fish meal (Li et al., 2007); 4) enhance nutrient digestibility (Li et al., 2007; Osho and Adeola, 2020), and 5) mitigate heat stress-induced intestinal oxidative stress compared to non-supplemented birds (Chang et al., 2020; Lan et al., 2020). Nevertheless, there is inconsistency among these studies regarding COS molecular weight (**Mw**), deacetylation degree, and levels of inclusion in the diet. As no single type of COS displays all the observed biological activities, it is likely that differences in the COS physicochemical structure and inclusion doses of the product act in different ways on broilers, depending on the challenge imposed. We have studied the effects of high deacetylation degree (> 85%) COS at various Mw (ranging from 14 to 220 kDa) and inclusion doses in the diet (0.2, 2 and 5 g/kg) on performance and NE intestinal lesions of broilers challenged with subclinical NE. Our preliminary findings suggested that COS 95 kDa

exhibited promising potential in sustaining growth rates while mitigating gross NE-associated intestinal lesions following the challenge (Chapter 2). Building upon these results, we selected samples from the COS 95 kDa treatments generated in that study for further laboratory investigation and comparison to PC and NC groups. Therefore, the objective of this study was to evaluate the potential of COS 95 kDa as a potential AGP replacement on cecal microbial abundance, quantification of *C. perfringens*, and concentration of short-chain fatty acids, immune modulation, intestinal morphology, and *Eimeria* oocysts count in the intestine of broilers challenged with a natural, subclinical necrotic enteritis infection model. We hypothesized that COS 95 kDa enhances gut health and modulates immune responses, mitigating NE in broilers.

3.2 MATERIALS AND METHODS

The animal care procedures were approved by the Animal Care and Use Committee for Livestock of the University of Alberta and followed principles established by the Canadian Council on Animal Care (CCAC, 2009).

3.2.1 Study approach and selection of COS treatments

The experimental design and animal husbandry procedures used in this study were originally described in detail in Chapter 2, Experiment 2. From this original experiment, in which different COS Mw and levels of inclusion in the diet were tested, a subset of treatments and their samples were selected for further laboratory investigation in comparison to the Positive Control (a diet with antibiotic and coccidiostat) and Negative Control (basal diet without any medication) treatments. These selections were based on the outcomes of the previous study, where diets supplemented with COS 95 kDa at 0.2 and 5 g/kg exhibited some potential to mitigate performance losses in broilers challenged with a natural, subclinical NE model (Chapter 2, Experiment 2). Thus, instead of conducting a new experiment, only the samples from COS 95 kDa, PC, and NC treatments

previously collected in Experiment 2 (Chapter 2) were selected for an in-depth evaluation of their effects on gut health and immune responses of broilers challenged with subclinical NE.

3.2.2 Chitosan oligosaccharides

Shellfish chitosan (Golden-Shell Pharmaceutical Co. Ltd, Zhejiang, China) with high deacetylation degree (94%) was used to produce COS at the desired Mw at the University of Alberta. Chemical hydrolysis was used to reduce chitosan into the desired molecular weights using H_2O_2, as described by Qin et al. (2002, 2006). Briefly, to produce COS with a targeted molecular weight of 95 kDa, a reaction mixture containing 1.5% H_2O_2 was utilized, and the reaction was carried out for 0.5 hours at 22°C. The resulting molecular weight was determined and validated using high-performance liquid chromatography equipped with a size exclusion column (Chen et al., 2004).

3.2.3 Animals and housing

Birds, housing, and experimental conditions were described in detail in Chapter 2, Experiment 2. In the original experiment, a total of 1,152 one-day-old, mixed-sex Ross 708 broilers were randomly assigned to 8 treatments with 8 replicates of 18 birds each. However, for the present study, only samples from (COS 95 kDa included at 0.2 and 5 g/kg, PC and NC) out of these 8 treatments were further investigated. In brief, birds were reared in floor pens (1.061 x 1.397 m) with fresh wood shaving litter and had free access to feed and water. The temperature and lighting programs followed practices recommended by Ross 708 commercial management guidelines (Aviagen, 2018). Observation of bird health, mortality, feed and water availability, and environmental housing conditions were recorded twice daily.

3.2.4 Experimental diets

Birds were fed a commercial-type, wheat-soy-canola basal diet typically fed to broiler chickens in Western Canada. The basal diet was formulated as described in Chapter 2 and made to meet or exceed Ross 708 nutritional recommendations (Aviagen, 2019) according to each growth phase. Although the original experiment had 8 dietary treatments as described in Chapter 2, Experiment 2, for the present study, we selected only samples from birds fed COS 95 kDa and Control diets for further laboratory investigation. Thus, for this study, samples from birds fed the: Positive Control (**PC**), a commercial-type diet supplemented with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., Kirkland, QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, Mitchell, ON, Canada at 0.05% of the diet); Negative Control (**NC**) that was the same commercial-type diet, except without any medications; and COS 95 kDa supplemented to the NC diet at either 0.2 or 5 g/kg were evaluated. All the diets were provided to the birds in a mash form through the 38 days of the experimental period.

3.2.5 Natural, subclinical necrotic enteritis challenge model

A subclinical NE, based on exposure of the birds to predisposing factors that disturb gut homeostasis and facilitate the natural proliferation of *C. perfringens*, was induced in all birds (He et al., 2022). Briefly, at 12 d of age, all birds were gavaged with 1 mL of a commercial coccidiosis vaccine containing 15x the recommended dose (Coccivac®-B52; Merck Animal Health, Intervet Inc., Millsboro, DE, USA) diluted in phosphate-buffered saline. At 18 days of age, the feed was withdrawn for 24 hours from all birds.

3.2.6 Sample collection

At 22 d (four days after the conclusion of the feed withdrawal challenge) and at 38 d of age, two birds per experimental unit were randomly selected for blood (only taken at 22 d), cecal content,

and intestinal tissue sample collection. For blood collection, birds were manually restrained, and 2.5 mL of blood was drawn by brachial venipuncture. The blood samples were kept on ice for approximately 3 h and then centrifuged at 1,500 × g for 15 min at 4°C, and the separated serum was stored at -20°C until used for immune biomarker quantification. For tissue and cecal content collection, birds were euthanized by cervical dislocation, and dissected. Approximately 3 cm of the proximal jejunum was collected and fixed in 10% formaldehyde for further preparation of the histological slides. Contents were collected from both ceca per bird and placed into 2 mL microcentrifuge tubes. To preserve the sample integrity, they were immediately placed in liquid nitrogen after collection. Afterward, the microcentrifuge tubes were transferred to -80°C storage until analysis for bacterial quantification and concentration of short-chain fatty acid (**SCFA**) analyses.

3.2.7 Bacterial DNA extraction

Total genomic DNA was extracted from the cecal content samples for further analysis of microbial abundance, and quantification of total and *netB*-positive *C. perfringens* strains. The DNA was extracted and purified as described by He at al. (2022). Briefly, 0.2 g of thawed cecal content was placed into a sterile tube with Zirconium beads and washed with and resuspended in 1 ml of TN150 buffer and then subjected to 3 minutes of bead-beating (Biospec Products, Bartlesville, USA). The DNA purification used the TE-saturated phenol and chloroform-isoamyl alcohol (24:1) method, followed by DNA precipitation with 100% ethanol at −20°C overnight for the formation of DNA pellets. After washing with 70% ethanol and air-drying, DNA pellets were dissolved in Nuclease-free water. DNA concentration and quality were assessed using a NanoDrop 2000 spectrophotometer at 260 and 280 nm (NanoDrop Technologies, Wilmington, DE, USA).

3.2.8 Cecal microbiota sequencing

Paired-end sequencing (2×300 bp) of amplicon DNA was performed using the Illumina MiSeq PE300 at Genome Quebec (McGill University, Montreal, QC, Canada). Briefly, the primers Bac9F (5'- GAGTTTGATCMTGGCTCAG) and Bac515R (5'- CCGCGGCKGCTGGCAC) were used to amplify the bacterial V1-V3 region of 16S rRNA genes. Two-step PCR was used to produce PCR amplicons and add barcodes separately. Specifically, the PCR program to produce bacterial amplicons using the following cycle program: initial denaturation at 94℃ for 2 min, followed by 33 cycles of 94℃ for 30 s, annealing at 58℃ for 30 s, elongation at 72℃ for 30 s, followed by a final elongation step of 72℃ for 7 min. Then, a second PCR was performed with the amplicons produced in the first step to add barcodes with the following cycle conditions: initial denaturation of 95℃ for 10 min, followed by 15 cycles of 95℃ for 30 s, 60℃ for 30 s, 72℃ for 60 s, followed by a final elongation step of 72℃ for 3 min. The bacteria sequencing data were analyzed using Quantitative Insights into Microbial Ecology 2 (QIIME 2) 2020.11 version. Specifically, the data were demultiplexed, then the sequence reads were filtered, denoised, and merged. After quality control, the Deficiency of Adenosine Deaminase 2 (DADA2) plugin in QIIME2 was used to remove chimeric sequences, and the amplicon sequencing variants (ASVs) table was generated. The representative sequences were aligned to the SILVA 138 Small Subunit rRNA Database for bacteria. The entire dataset was normalized, and alpha diversity and beta diversity were calculated. Pair-wise differential abundance analysis was performed to identify the significant phylotypes under each dietary condition using EdgeR. Data was expressed as relative abundance (%), indicating the proportion of each phylotype within the microbial community.

3.2.9 *Clostridium perfringens* quantification

The genomic DNA extracted from cecal samples was used to quantify the *C. perfringens* and *netB*-positive strains copy number by qRT-PCR targeting the 16s rRNA gene as described by He et al. (2022). For absolute quantification, a standard curve was established using serially-diluted commercial *C. perfringens* genomic DNA (ATCC strain 13124). For each experimental sample, triplicate reactions were set up on a 96-well plate, with a 20 µL of reaction mixture in each well, comprising the DNA template, forward (5'- GGGTTTCAACACCTCCGTG) and reverse (5'- GCAAGGGATGTCAAGTGTAGG) primers, Fast SYBR Green Master Mix, and Nuclease-free water. The qRT-PCR experiment was conducted using the QuantStudio™ 6 Flex System and data were analyzed with a QuantStudio rt-PCR Software v.1.3. The amplification process consisted of initial denaturation at 95°C for 20 s followed by 40 cycles of annealing including 95°C for 3 s and 62°C for 30 s. To ensure amplification specificity, a melting curve of PCR products was generated by collecting fluorescence data during slow heating from 60 to 95°C with a rate of 0.05°C/s.

To quantify the *netB*-positive strains in cecal digesta, the genomic DNA from a *netB*-positive strain (CA147, Arden Biotechnology Ltd., United Kingdom) was serially diluted, and included on each plate to generate a standard curve. The reaction mixture for each sample was prepared as aforementioned; however, using specific forward (5'- TGATACCGCTTCACATAAAGGTTGG) and reverse (5'- ATAAGTTTCAGGCCATTTCATTTTTCCG) primers to target the *netB* gene. The amplification process and analysis followed the same steps as previously mentioned. Copy numbers of target genes were calculated according to Li et al. (2009), and results were expressed as mean log-copies/g digesta.

3.2.10 Short-chain fatty acids

About 0.5 g of cecal content sample was weighed and transferred to a 5-mL Falcon tube and vortexed vigorously until it was fully dissolved in 25% phosphoric acid (4:1; v:v). The samples were then centrifuged at 19,000 × g at 4°C for 5 minutes. The supernatant was transferred to a microcentrifuge tube, and this process was repeated twice. The final supernatant (1 mL) was transferred to a GC vial and mixed with 200 µL of 25% phosphoric acid and 200 µL of isocaproic acid solution (Internal standard, 3 mg/mL). Concentrations of acetate, butyrate, propionate, caproate, isobutyrate, isovalerate, and valerate were measured using gas chromatography, as described by Guan et al. (2008). The SCFA concentrations were presented as µmol/ml fresh weight of digesta.

3.2.11 Serum concentration of cytokines, chemokines and growth factors

The Luminex xMAP technology was utilized to perform multiplexed quantification of 12 chicken cytokines, chemokines, and growth factors in serum samples. The analysis was conducted by Eve Technologies Corp. (Calgary, AB, Canada) and used the Luminex™ 200 system (Luminex, Austin, TX, USA) and the Chicken Cytokine 12-Plex Featured Assay (MilliporeSigma, Burlington, Massachusetts, USA) according to the manufacturer's protocol. The 12 markers simultaneously measured in the serum samples were Interferon alpha (**IFNα**), Interferon gamma (**IFNγ**), Interleukin – 2 (**IL-2**), Interleukin – 6 (**IL-6**), Interleukin – 10 (**IL-10**), Interleukin – 16 (**IL-16**), Interleukin – 21 (**IL-21**), Macrophage colony-stimulating factor (**M-CSF**), Macrophage inflammatory protein-1 beta (**MIP-1β**), Macrophage inflammatory protein-3 alpha (**MIP-3α**), Regulated on activation, normal T cell expressed and secreted (**RANTES**), and Vascular endothelial growth factor (**VEGF**). The sensitivity of the markers used in the 12-plex assay ranged

from 0.96 to 39.93 pg/mL. The MilliporeSigma MILLIPLEX® MAP protocol provided individual analyte sensitivity values.

3.2.12 Intestinal morphology

The fixed jejunal tissue samples were removed from the 10% formaldehyde solution, sectioned, and placed in identified slotted cassettes. The fixed tissue was dehydrated and embedded in paraffin wax, then sliced into 5 µm sections using a microtome (Leica RM2235, Leica Biosystems Inc, ON, Canada) and stained with hematoxylin and eosin. For intestinal morphology measurements, one slide per bird containing the tissue cross-sections was examined at 5X and 20X objective under light microscopy (Zeiss Axio Scope A1, Carl Zeiss Microscopy, NY, United States). Images from the tissues were collected using a SeBaCam digital microscope camera with SeBaView software (Thermo Scientific). A total of 16 villi per bird were analyzed and measured using ImageJ software (National Institutes of Health, MD, United States). In each analyzed villus, the villus height (µm), crypt depth (µm), villus to crypt ratio, epithelium thickness (µm), lamina propria thickness (µm), and *Eimeria* oocyst count (total number) were measured (Figure 3.1) (Belote et al., 2023).

3.2.13 Statistical analyses

Data were submitted to Bartlett's test to evaluate the homogeneity of variances, and Shapiro-Wilk test to verify the normality of residuals. The cecal concentration of SCFA, intestinal morphology measurements, and the quantification of immune biomarkers in the serum were analyzed by one-way ANOVA using Statistix 10 software (Analytical Software, Tallahassee, FL). Significant effects ($P \leq 0.05$) were further evaluated with Tukey's test for pairwise comparisons. As the data from *Eimeria* oocyst count and relative microbial abundance were not normally distributed, Kruskal-Wallis (comparison of all treatments) or Mann-Whitney (pairwise comparisons) non-

parametric statistical tests were used, and statistical significance was considered at $P \leq 0.05$. To determine the significance of beta diversity, a permutational multivariate analysis of variance (PERMANOVA) was conducted. For the quantification of *C. perfringens* and *netB*-positive strains, the copy numbers were transformed into mean-log copy numbers, and the data were then analyzed by one-way ANOVA.

3.3 RESULTS

3.3.1 Cecal microbial composition

At the phylum level, a total of five phyla were identified: Firmicutes, Cyanobacteria, Bacteroidota, Actinobacteriota, and Proteobacteria. The majority of the identified phylotypes belonged to Firmicutes (all > 90%) for all samples. At the genus level, 27 genera were identified, and *Lactobacillus* was the predominant genus in the cecum (35.23%). At the species level, 30 species were identified, and the most abundant species was *Lactobacillus crispatus* (16.32%).

An overall comparison was first performed to identify whether there was a treatment effect for the entire sample set. Afterwards, pairwise differential abundance analyses were performed (PC vs NC; PC vs COS 95 kDa; NC vs COS 95 kDa) to identify the amplicon sequence variant (**ASVs**). For cecum samples, 37 ± 10 ASVs were identified per sample. There were no significant differences for alpha-diversity ($P = 0.16$; data not shown); however, the beta-diversity was nearly significant ($P = 0.06$; Figure 3.2A) among treatments when analyzing all the cecum samples. For the pairwise comparison, NC was more beta-diverse than PC ($P < 0.01$; Figure 3.2B); however, no significant differences were observed for alpha-diversity ($P = 0.20$; data not shown). At the species level, the NC group had a higher relative abundance (%) of *Anaerotignum lactatifermentans* (0.0034 ± 0.0064; $P < 0.01$), *Lactobacillus ingluviei* (0.0047 ± 0.0073; $P < 0.01$), *Clostridium*

spiroforme (0.0108 ± 0.0108; P < 0.01), and uncultured bacterium (0.2272 ± 0.1252; P < 0.01) than PC (Table 3.1).

For the pairwise comparison between the PC and COS 95 kDa treatments, a significant difference for beta-diversity was observed (P = 0.05; Figure 3.3A). Moreover, significant differences were also observed for bacterial relative abundance at the phylum, genus, and species levels (Table 3.2). At the phylum level, COS 95 kDa treatment birds had a higher relative abundance of bacteria from the Bacteroidetes group (0.005 ± 0.0130) compared to PC (0.002 ± 0.0054; P < 0.01). At the genus level, COS 95 kDa treatments had a higher relative abundance of *Faecalibacterium*, *Tyzzerella*, *Romboutsia*, *Ruminococcaceae UCG*-005, and *Defluviitaleaceae UCG-011* than PC (Table 3.2). At the species level, the cecal content of birds fed COS 95 kDa treatments had the presence of *Anaerotignum lactatifermentans* (0.0013 ± 0.0042), *Lactobacillus oris* (0.0005 ± 0.0024), *Lactobacillus ingluviei* (0.0010 ± 0.0032), and *Clostridium spiroforme* (0.0002 ± 0.0007); however, the presence of these bacteria was not detected in birds fed the PC diet. In contrast, the PC treatment birds had higher relative abundance of *Agathobaculum sp* (0.0160 ± 0.021), *Eubacterium sp* (0.0034 ± 0.0040), and *Lactobacillus crispatus (*0.2592 ± 0.1975) than COS 95 kDa (0.0069 ± 0.0056, and 0.1153 ± 0.1005, respectively; Table 3.2).

No significant treatment effects on alpha or beta-diversity (P = 0.19; Figure 3.3B) were observed in the pairwise comparison between NC and COS 95 kDa treatments. At the genus level, birds fed the NC diet had a higher relative abundance of *Clostrodium sensu stricto 1* (0.0013 ± 0.0029) and *Erysipelatoclostridium* (0.0465 ± 0.0382) than those fed COS 95 kDa (0.0005 ± 0.0015, and 0.0230 ± 0.0223, respectively; Table 3.3). At the species level, the NC treatment had higher relative concentrations of *Massiliomicrobiota timonensis*, *Clostridium colinum*, and *Lactobacillus*

ingluviei than COS 95 kDa, while COS 95 kDa had a higher relative abundance of *Lactobacillus oris* (Table 3.3).

3.3.2 *Clostridium perfringens* quantification

Regardless of the assessment time point, *C. perfringens* was identified in the cecal content of all the sampled birds. The presence of the *netB*-positive strains was detected in 87.5 % of the sampled birds after the challenge, and in 78.1% of the sampled birds at 38 d of age. The density of the observed *netB*-positive strains had an average of 10^5 copies per gram of cecal content, while total *C. perfringens* had an average of 10^6 at 22 d and 10^5 copies per gram of cecal content at 38 d.

At 22 d (after the challenge), dietary treatment did not affect cecal content mean log-copies per gram of *C. perfringens* (overall mean log-copies across treatments = 5.369 ± 0.11; P = 0.44) or *netB*-positive strains (overall mean log-copies across treatments = 5.217 ± 0.07; P = 0.37; Table 3.4). Similarly, at 38 d, no treatment effects on the quantity of total *C. perfringens* (overall mean log-copies across treatments = 5.336 ± 0.05; P = 0.27) or *netB*-positive strains (overall mean log-copies across treatments = 5.264 ± 0.06; P = 0.20) were observed (Table 3.4).

3.3.3 Short-chain fatty acids

At 22 d, after the challenge, the cecal propionic acid content was higher in broilers fed the PC diet (4.79 ± 0.86 µ mol/ml) than those fed COS 95 kDa at 0.2 g/kg (2.63 ± 0.18 µ mol/ml; Table 3.5). The concentration of isobutyric acid was higher in broilers fed COS 95 kDa at 5 g/kg (0.58 ± 0.06 µ mol/ml) than those fed PC or NC (0.34 ± 0.04 and 0.37 ± 0.05 µ mol/ml, respectively). The cecal concentration of isovaleric acid in birds fed COS 95 kDa at 5 g/kg tended to be higher (0.44 ± 0.03 µ mol/ml) than in those fed the PC diet (0.20 ± 0.06 µ mol/ml; P = 0.07). No significant differences were observed for total SCFA, acetic acid, butyric acid, valeric acid or caproic acid in the ceca content of the broilers (P > 0.05; Table 3.5).

At 38 d, the concentration of isobutyric acid was higher in birds from the NC and COS 95 kDa at 5 g/kg treatments (0.65 ± 0.08 and 0.61 ± 0.08 μ mol/ml, respectively) than in birds from the PC group (0.33 ± 0.03 μ mol/ml; Table 3.5). Valeric acid was higher in birds from the NC group (0.81 ± 0.07 μ mol/ml compared to those from the PC group (0.52 ± 0.07 μ mol/ml) but similar to the other treatments. The PC group had the lowest concentration of isovaleric acid (0.29 ± 0.02 μ mol/ml), while NC and COS 95 kDa at 5 g/kg groups had the highest concentration of this SCFA (0.67 ± 0.13 and 0.64 ± 0.10 μ mol/ml). Birds fed the NC diet had a higher concentration of caproic acid (0.13 ± 0.01 μ mol/ml) than those fed COS 95 kDa at 0.2 g/kg (0.06 ± 0.01 μ mol/ml), but similar compared to the other treatments. No significant differences were observed for total SCFA, acetic acid, propionic acid, or butyric acid in the ceca content of the broilers ($P > 0.05$; Table 3.5).

3.3.4 Immune biomarkers

At 22 d, after the challenge, the concentration of IFNα was significantly higher in NC birds (6.81 ± 2.11 pg/mL) than in the PC birds (1.53 ± 0.39 pg/mL; $P= 0.03$; Table 3.6). Birds fed COS 95 kDa at 0.2 or 5 g/kg had intermediate levels, which were not significantly different from either of the controls. The concentration of IL-16 was higher in birds fed COS 95 kDa at 0.2 g/kg (84.79 ± 8.98 pg/mL) than those fed the PC diet (51.80 ± 3.38 pg/mL; $P < 0.01$), while birds from the NC treatment and those fed COS 95 kDa at 5 g/kg were not different from either of the other two treatments (64.17 ± 7.37 and 61.30 ± 5.66 pg/mL, respectively). The concentration of MIP-3α was higher in birds fed COS 95 kDa at 0.2 g/kg and NC (99.82 ± 9.40 and 96.02 ± 13.53 pg/mL, respectively) compared to those fed the PC diet (57.30 ± 10.12 pg/mL; $P = 0.05$). However, the concentration was similar to birds fed COS 95 kDa at 5 g/kg group (68.67 ± 13.42 pg/mL; Table 3.6). There were no significant differences in the concentrations of IFNγ, IL-2, IL-10, M-CSF, MIP-1β, RANTES, or VEGF ($P > 0.05$) among the treatments. The concentrations of IL-6 and IL-

21 in the majority of the samples analyzed were below the limit of detection for the multiplex assay, regardless of the treatment, and are not shown.

3.3.5 Intestinal morphology

At 22 d, after the challenge, villus height was 9.04% higher in birds fed COS 95 kDa at 0.2 g/kg (1,703.31 ± 24.89 µm) than those fed the NC (1,562.13 ± 355 µm; $P < 0.01$), while PC and COS at 5 g/kg resulted in similar villus height (1,623.27± 28.57 µm; 1,626.10 ± 24.50 µm, respectively) to COS 95 at 0.2 g/kg (Table 3.7). Birds in the NC group had the deepest crypts (241.20 ± 4.51µm; $P < 0.01$) compared to all the other treatments, while PC birds had the shortest crypts (174.66 ± 3.58 µm), 38.1% lower than NC. The same pattern occurred for the villus to crypt ratio. The NC diet resulted in the lowest villi to crypt ratio (6.62 ± 0.12; Table 3.7; Figure 3.4), whereas the PC group had the highest villi to crypt ratio (9.70 ± 0.21; $P < 0.01$). Birds fed NC also had the thickest epithelium (71.52 ± 2.46 µm) and lamina propria (124.21 ± 8.49 µm) compared to those fed PC (59.97 ± 2.66 µm and 83.79 ± 5.27 µm, respectively), COS 95 included at 0.2 (59.42 ± 1.50 µm and 76.70 ± 3.18 µm, respectively) and 5 g/kg (58.64 ± 1.78 µm, 69.64 ± 3.52 µm, respectively; Table 3.7). *Eimeria* oocyst count in the host enterocytes was the highest in birds fed NC ($N = 40$; $P < 0.01$; Table 3.7) compared to those fed any other treatment. Birds fed the PC diet had $N = 6$, COS 95 kDa at 0.2 g/kg had $N = 14$, and COS 95 kDa at 5 g/kg $N = 16$ oocysts.

At the end of the experiment (38 d), the villus height was 5.38% higher in birds fed COS 95 kDa at 0.2 g/kg (1,745.33 ± 17.19 µm) than those fed PC (1,656.21 ± 15.74 µm; $P < 0.01$) but was similar to those fed NC and COS 95 kDa at 5 g/kg (1,696.46 ± 18.70 µm; 1,704.34 ± 22.71 µm, respectively; Table 3.7). The crypt depth was greatest in birds fed NC (200.16 ± 3.63 µm; $P < 0.01$), whereas birds fed COS 95 at 0.2 and 5 g/kg had the shortest crypts (158.09 ± 2.76 µm; 149.53 ± 3.37 µm, respectively). For villus to crypt ratio, birds from COS 95 at 0.2 and 5 g/kg

treatments had the greatest ratio (11.42 ± 0.21 μm; 11.86 ± 0.22 μm; P < 0.01), while birds from the NC group had the smallest (8.73 ± 0.15 μm). The epithelium was 18.02% thicker in birds from the NC group (81.63 ± 3.07 μm; P = 0.05) than those from the COS 95 kDa at 5 g/kg (69.17 ± 3.02 μm) but similar compared to birds from the PC (77.16 ± 2.92 μm) or COS 95 kDa at 0.2 g/kg (75.81 ± 3.63 μm). In NC birds, the lamina propria was thicker (112.85 ± 6.46 μm; P < 0.01) by 27.31% compared to birds from the COS 95 kDa at 0.2 (88.63 ± 4.75 μm) and by 42.62% compared to birds at 5 g/kg (79.13 ± 4.36 μm) treatments, respectively. There was no significant difference among the treatments for the number of *Eimeria* oocyst counts in the enterocytes of the birds (P = 0.49; Table 3.7).

3.4 DISCUSSION

Consistent with previous studies, we observed that at 22 d, Firmicutes were the predominant phyla in the cecal content of the birds (Wei et al., 2013; Proctor and Phillips, 2019). Initially, we were not expecting the presence of Cyanobacteria in the cecal content of broilers because this phylum is believed to be composed primarily of photosynthetic bacteria mostly found in plants (Di Rienzi et al., 2013). However, recurring identification of this phylum in the gut of animals (including broilers) and humans led to the discovery of non-photosynthetic cyanobacteria (class Melainabacteria), suggesting a need for reclassification within this phylum (Di Rienzi et al., 2013; Xiao et al., 2017; Shi et al., 2019; Hu and Rzymski, 2022). Bacteria from the *Lactobacilli* genus, and *Lactobacillus crispatus* were also predominant habitants of the cecal content of broilers in this study. These bacteria have also been previously identified as common members of the intestinal microbiota of healthy poultry (Wang et al., 2014; Dec et al., 2018). The differences in the distribution of the microbiota between treatments, although subtle, were more evident at the genus and species levels. As expected, the PC diet resulted in a less beta-diverse cecal microbiota than

NC due to the presence of the sub-therapeutic dose of the antibiotic bacitracin methylene disalicylate (**BMD**). BMD is a relatively narrow spectrum antibiotic that targets primarily gram-positive bacteria, impairing protein synthesis, cell wall production and inducing cell lysis (Butaye et al., 2003). Although a sub-therapeutic dose of this antibiotic should not cause bacterial death, it can still diminish their proliferation (Broom, 2017).

In the pairwise comparison between NC and COS 95 kDa treatments, birds fed COS products had lower relative abundance of *Clostridium sensu stricto 1* and *Erysipelatoclostridium* genera. Furthermore, dietary COS 95 kDa led to a decrease in the relative abundance of *Clostridium colinum* and an increase in the relative abundance of *Lactobacillus oris* in the ceca of the broilers, as compared to the NC group. *Clostridium sensu stricto 1* is the genus to which *C. perfringens* belongs, and its abundance increases during NE development in broilers while the abundance of *Lactobacillus* species decreases (Yang et al., 2019, 2022). Increased abundance of this genus is also associated with intestinal inflammation and decreased SCFA production (Yang et al., 2019). Similarly, the increase of *Erysipelatoclostridium* in NC birds might also be associated with a potential gut dysbiosis as it is an opportunistic pathogen, and high abundance is linked to metabolic disorders (Zhang et al., 2021). Thus, in the present study, birds fed NC had a slight shift in their gut microbiota, favoring the increase in the relative abundance of *Clostridium sensu stricto 1* and *Erysipelatoclostridium* in response to enteric disturbances caused by the NE challenge model. In contrast, under the same challenging conditions, COS 95 kDa slightly mitigated the relative growth of these genera in the ceca of birds.

In addition, birds fed COS 95 kDa had increased relative abundance of *Lactobacillus ingluviei*, *Lactobacillus oris*, and *Anaerotignum lactatifermentans* compared to those fed PC. *Lactobacillus* species play an important role in maintaining poultry gut health, and are often used as probiotics

(Dalloul et al., 2003; Lin et al., 2007; Li et al., 2018; Xiang et al., 2022). The lactic acid produced by *Lactobacillus* strains can serve as a substrate for lactate-fermenting bacteria, which in turn produce SCFA as metabolites (Meimandipour et al., 2010; Stanley et al., 2016). Although we observed an increase in the relative abundance of *A. lactatifermentans*, a lactate-fermenting bacteria, in birds fed COS 95 kDa, the production of total SCFA or specific concentrations of acetic, propionic, or butyric acid were not affected. On the other hand, COS 95 kDa at 5g/kg increased the concentrations of isobutyric acid and tended to increase isovaleric acid in the ceca of broilers at 22 d. The production of branched-chain fatty acids (e.g., isobutyric and isovaleric acid) is often related to the fermentation of branched amino acids from undigested protein that reaches the hindgut (Heimann et al., 2016; Lee et al., 2017). This would, in turn, favour the growth of undesirable proteolytic bacteria in the ceca (Drew et al., 2004); however, this was not observed in the COS 95 kDa cecal microbiota profile results (Tables 3.2 and 3.3). The physiological role of branched-chain fatty acids in the gut is still not fully understood. However, based on our findings, the slight increase in the concentrations of isobutyric and isovaleric acids in birds fed COS 95 kDa may have helped to slightly decrease the luminal pH and created a more favourable conditions for *Lactobacillus* species colonization (Jacobsen et al., 1999; Hajati, 2018).

Other researchers have also observed increased *Lactobacilli* counts as a consequence of the presence of COS in vitro (Lee et al., 2002), or supplemented in the diet of broilers (Li et al., 2007), weaned pigs (Yang et al., 2012), and humans (Mateos-Aparicio et al., 2016). Since COS can serve as a carbon source for bacteria that can digest these polymers in the gut, its supplementation may have favoured the growth of this beneficial group of bacteria (Altamimi et al., 2016; Moine et al., 2021). Apart from being a source of carbon, COS contains positively charged groups that enable it to attach to the cell walls of bacteria, affecting their function, growth, and facilitating their

excretion (Kim et al., 2003; Benhabiles et al., 2012). Thus, the pathogens that could not attach to the host cells due to the competitive exclusion with beneficial bacteria and are free in the lumen are more exposed to COS binding and more susceptible to excretion. Altamimi et al. (2016) found that chitosan inhibited the attachment of bacteria from the *Clostridia* group to HT-29 human epithelial cells while increasing the attachment of those from the *Lactobacilli* group. The ability of COS to increase the abundance of beneficial bacteria such as *Lactobacilli*, and decrease potential pathogens suggests that COS may have the potential to exert both antimicrobial and prebiotic activities modulating the gut microbiota of poultry. However, no specific effects on *C. perfringens* absolute abundance were observed, as the copy numbers in the cecal content were similar among the treatments.

In the present study, COS 95 kDa maintained the intestinal morphology architecture of broilers and reduced *Eimeria* oocyst infection to the same extent as the PC (i.e., a diet containing BMD and coccidiostat) following the challenge. Birds fed NC were the most vulnerable to *Eimeria* infection. This was evident not only from the high number of oocysts present in the villi, but also from the low villus height to crypt depth ratio, thicker epithelium, lamina propria, and deeper crypts. The vaccine used to induce poultry coccidiosis as the main NE predisposing factor contained live sporulated oocysts (infective form) of *Eimeria acervulina*, *E. mivati*, *E. tenella*, and two strains of *E. maxima*. The oocysts in their infective form pass throughout the mucus layer and invade the enterocytes of the birds in order to complete their multi-stage replication process (Collier et al., 2008; Mesa-Pineda et al., 2021). This process results in pathological changes in the intestine of the host, which includes epithelial extracellular matrix disruption, nutrient and plasma protein leakage, impaired digestion and absorption, increased mucus production, and local recruitment of immune cells (Mesa-Pineda et al., 2021). In the present study, the jejunum of birds

from the NC group showed higher levels of histopathological changes characteristic of *Eimeria* infection as compared to the other treatments. The deeper crypts, and the lower ratio of villus height: crypt depth indicates that birds in the NC group had to spend more energy and nutrients to accelerate intestinal epithelial cell turnover as an attempt to restore homeostasis and expel parasites from the intestine (Cliffe et al., 2005; Teng et al., 2020). The thicker epithelium in NC birds may be related to an increase in the number of goblet cells due to the higher demand for protective mucus, enterocyte proliferation, inflammatory cells infiltration, and presence of oocysts in enterocytes (Kraieski et al., 2017). Similarly, the thicker lamina propria suggests an increase in immune cell infiltration to control a local inflammation, and potentially due to the presence of edema and hyperemia (Lillehoj and Trout, 1996). Previous studies evaluating the intestinal morphology of broilers challenged with *Eimeria* alone or coinfected with *C. perfringens* also observed thicker epithelium and lamina propria compared to unchallenged or AGP-supplemented animals (Kraieski et al., 2017; Belote et al., 2018, 2019). These results were related to the demand for immune cells to control pathogen infection and inflammatory processes. Thus, in the present study, the histopathological changes caused by the challenge may have caused mild gut dysbiosis and local intestinal inflammation, especially in the NC birds.

The activation of the immune system to control inflammation may have undesirable consequences, including tissue damage and diversion of nutrients away from productive purposes (Broom and Kogut, 2018). Although inflammatory processes can diminish performance, we observed that birds fed NC had a similar performance compared to those fed PC or COS products (Chapter 2), that apparently had lower levels of intestinal inflammation. This suggests that despite the histopathological alterations and the shift in microbiota observed in birds from the NC group, these changes were mild enough to avoid significant performance losses. The results of the present study

clearly demonstrate that there was an ongoing enteric disturbance, with a particular impact on birds from the NC group. However, subclinical NE in broilers is typically characterized by reduced performance, particularly in terms of growth rates and feed efficiency (Skinner et al., 2010; Timbermont et al., 2011). Interestingly, our studies (Chapter 2) did not clearly show reductions in performance in the NC birds. Therefore, adjustments of the natural, subclinical challenge model used in this study are required in order to capture more substantial decrease in the performance of birds fed the NC relative to the PC. This would help us to test the potential of COS 95 kDa more accurately as an AGP replacer to prevent or mitigate subclinical NE in broilers.

Although we did not observe clear differences in performance between PC and NC, COS 95 kDa tended ($P = 0.08$) to enhance BW from 0 to 36 d compared to the NC birds (Chapter 2, Experiment 2). This potential may be related to the results observed in the current study, in which COS 95 kDa increased villus height, villus to crypt ratio, decreased crypt depth, lamina propria and epithelium thickness, and prevented *Eimeria* infection to the same level (at 22 d) or greater (at 38 d) than the PC. Similarly, broilers challenged with coccidiosis and supplemented with 1 g/kg of COS in the diet had an increase in jejunal villus height, and villus height to crypt depth ratio, a decrease in crypt depth, and a lower count of *Eimeria* oocysts in the excreta compared to birds not fed with COS (Osho and Adeola, 2019). These authors suggested that COS protection against *Eimeria* could be linked to its capacity to penetrate and adhere to the intestinal mucosa, enhancing the physical barrier structure, and its ability to induce goblet cell hyperplasia. Dietary supplementation of COS also increased villus height and villus to crypt ratio of broilers at an early age (Li et al., 2019), broilers challenged with glucocorticoid (Osho and Adeola, 2020) or heat stress (Lan et al., 2020), as well as in weaning pigs (Liu et al., 2008; Walsh et al., 2012; Suthongsa et al., 2017). Villus height and villus to crypt ratio are useful criteria to estimate the relative nutrient digestion

and absorption capacity of the intestine, with higher values indicating greater capacity (Xu et al., 2003). Moreover, the reduced lamina propria and epithelial thickness indicate lower maintenance costs and more efficient absorption of nutrients, helping to maintain intestinal health (Cardinal et al., 2019). Thus, COS 95 kDa seemed to mitigate the effects of *Eimeria* infection, reducing intestinal inflammation and maintaining intestinal health of broilers. As coccidiosis is the most important risk factor associated with NE disease development (Dierick et al., 2021), its prevention can, in turn, reduce the risk of NE.

Although the antimicrobial and prebiotic properties of COS may play a role in maintaining gut health following the challenge, immune regulation might also be involved in this process. When COS contacts the mucus, positive charges present in its structure can form mucoadhesive bonds with negatively charged sialic groups of the glycocalyx on the epithelial cell surface (Moine et al., 2021). Depending on the COS Mw, they can diffuse deeply (low Mw) into the mucus gel, crosslinking the mucin polymers, or only interact superficially (high Mw). The crosslink with mucin polymers can reinforce the mucosal barrier and stimulate the fitness of goblet cells (Kootala et al., 2018; Moine et al., 2021). The COS adhered to the superficial mucus can be recognized by antigen-presenting cells such as dendritic cells and macrophages and, as a result, trigger the release of endogenous defensins, IgA and cytokines that help to maintain intestinal homeostasis (Moine et al., 2021). Because of this recognition and stimulation of the immune system, COS has been studied as a potential vaccine adjuvant. It enhances immunity against viruses and bacteria by promoting T lymphocyte proliferation and initiating a mixed Th1/Th2 response (Zhang et al., 2017; Moran et al., 2018; Wei et al., 2020; Wen et al., 2022).

In the present study, broilers fed COS 95 kDa at 0.2 g/kg had increased serum concentrations of MIP-3α and IL-16 compared to those fed PC. However, COS 95 kDa included at 5 g/kg kept MIP-

3α and IL-16 at intermediate levels between PC and NC groups and decreased the production of IFNα in the serum after the challenge. IFNα is a component of the innate immune response, secreted especially in response to viral infection (Jiang et al., 2011). It can activate macrophages and immature dendritic cells, increasing their ability to stimulate T cells and present antigen during infections. This contributes to the regulation of the local immune response, guiding activated T cells to site of injury or infection (Sekellick et al., 1998; Brassard et al., 2002). Although the role of IFNα in poultry is still not well explored, in the present study, the reduction in IFNα serum levels in birds fed COS at 22 d may have resulted from the initiation of specific immunity, with innate immunity playing a secondary role during that period.

On the other hand, the chemokine MIP-3α and the cytokine IL-16 are involved in pro-inflammatory responses led by the adaptive immune system. MIP-3α participates in the activation and recruitment of specialized immune cells toward the infected site to control the infection and restore the homeostatic state (Ranasinghe and Eri, 2018). MIP-3α is an important chemokine in mucosal immune response in humans and mice, and upregulated in response to microbial stimulation in the gut (Papadakis and Targan, 2000; Ranasinghe and Eri, 2018). Along with the increase of MIP-3α, we also observed an increase in the concentration of IL-16 in broilers fed with COS 95 kDa. IL-16 is a pro-inflammatory cytokine that is primarily produced by CD8+ T cells, in response to mitogenic or antigenic stimulation (Folwaczny et al., 2005; Xue et al., 2009). While few studies have looked at the role of IL-16 in poultry, levels of this cytokine increased following an *Eimeria* infection (Hong et al., 2006) and in the process of immunization against coccidiosis after vaccination (del Cacho et al., 2012). Since in the present study a high dose of coccidiosis vaccine was used as a predisposing factor to cause subclinical NE, signs of coccidiosis were expected. Birds fed PC and COS diets showed protective effects against *Eimeria* infection when

compared to the NC group. Since the PC diet contained a coccidiostat, and the vaccine used had *Eimeria* strains sensitive to coccidiostats, we anticipated stronger protection against coccidiosis in the PC group than in the NC group. Interestingly, birds fed COS appeared to have enhanced mucosal barrier protection in the gut, which could have hindered oocyst access to intestinal cells. However, when some oocysts managed to breach the mucosal barrier and invade the enterocytes, triggering immune responses, COS seemed to facilitate the detection of pathogens and assist in a rapid transition from an innate to an adaptive immune response. The increase in MIP-3α and IL-16 at 22 d may confirm that at the serum level, birds were overcoming the enteric challenge with the help of T-cells to develop resistance and immune memory against the stressors and restore homeostasis.

Cardoso Dal Pont et al. (2023) reported increases in MIP-3α and IL-16 levels in broilers fed with a diet high in non-starch polysaccharides and suggested that MIP-3α and IL-16 may serve as biomarkers for low-grade chronic intestinal inflammation in broilers. In the present study, the immune biomarkers were only measured at 22 days of age, that is, 10 days after the coccidia challenge and 3 days after the feed withdrawal, where the peak of NE effects was expected. Since we do not have other time points of immune biomarker measurement, and the model of the challenge applied was dynamic (it did not involve the same controlled stressor for the whole experimental period), we can not conclude that this increase in MIP-3α and IL-16 is related to a low-grade chronic intestinal inflammation. However, interestingly, among the serum biomarkers evaluated, only increases in concentration of MIP-3α and IL-16 were observed, which suggests that they may be involved in the control of intestinal inflammation. In addition, COS may have contributed to a vigorous local immune response, helping the birds shift from innate to adaptive immune responses more effectively, reducing the overall impact of immune activation. In our

study, obtaining blood samples at various time points following the challenge could have provided us with more precise insights into the impact of COS on immune modulation. Despite that, our data suggest that the increase in pro-inflammatory effects in birds fed COS was probably for a short period and did not cause losses in performance or negatively impact intestinal health.

In summary, the present study showed that the challenge caused intestinal dysbiosis and mild intestinal inflammation, especially impacting NC birds. COS 95 kDa had higher relative abundance of *Lactobacillus* species in the ceca and enhanced the intestinal morphology integrity of broilers following the challenge. Additionally, the mucoadhesive nature of COS may have played a role in enhancing the intestinal mucosal barrier, thus mitigating *Eimeria* infection. COS recognition by the immune system may have further enhanced the local immune response, facilitating the resolution of inflammation while preserving performance, microbiota composition, and intestinal health. Given these promising attributes, COS 95 kDa should be further explored as an AGP replacement to prevent or mitigate subclinical NE in broilers.

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3.6 TABLES

Table 3.1. Microbial average relative abundance in the ceca of broilers at 22 d fed with a Negative Control or a Positive Control diet.

Item	Average relative abundance (%)					
	Positive Control[1]	SEM[2]	**Negative Control**[3]	SEM[2]	P-value[4]	FDR[5]
Genus						
Fusicatenibacter	0	----	0.0018	0.0019	< 0.01	< 0.01
Alistipes	0.0045	0.0085	0.0028	0.0059	< 0.01	< 0.01
Streptococcus	0.0017	0.0040	0	----	< 0.01	0.01
Faecalibacterium	0.2067	0.1258	0.0074	0.0889	< 0.01	0.01
Bacteroides	0.0006	0.0012	0	----	< 0.01	0.02
Tuzzerella	0.0009	0.0020	0.0063	0.0082	< 0.01	0.02
Ruminococcaceae UCG-005	0.0007	0.0015	0	----	< 0.01	0.02
Erysipelatoclostridium	0.0382	0.0331	0.0082	0.0068	< 0.01	0.02
Clostridia UCG-014	0.0135	0.0117	0.0051	0.0087	<0.01	0.02
Lachnoclostridium	0.0110	0.0098	0.0027	0.0029	0.01	0.03
Subdoligranulum	0.0947	0.0449	0.2398	0.1599	0.01	0.03
Defluviitaleaceae UCG-011	0.0028	0.0040	0.0024	0.0044	0.01	0.03
Eubacterium brachy-group	0.0006	0.0011	0.0002	0.0005	0.01	0.04
Species						
Anaerotignum lactatifermentans	0	----	0.0034	0.0064	<0.01	< 0.01
Lactobacillus ingluviei	0	----	0.0047	0.0073	<0.01	< 0.01
Agathobaculum sp	0.0195	0.0252	0.0027	0.0057	<0.01	< 0.01
Clostridium spiroforme	0.0013	0.0025	0.0108	0.0108	<0.01	< 0.01

[1]Positive Control: The basal diet with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2]Standard Error of the Mean.

[3]Negative Control: The basal diet without any medications.

[4]P-value for Mann-Whitney non-parametric test.

[5]FDR: false discovery rate (adjusted P-value).

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 3.2. Microbial average relative abundance in the ceca of broilers at 22 d fed a Positive Control or Chitosan oligosaccharide (COS) 95 kDa diets.

Item	Average relative abundance (%)					
	Positive Control[1]	SEM[2]	**COS 95 kDa**[3]	SEM[2]	P-value[4]	FDR[5]
Plylum						
Bacteroidota	0.0020	0.0054	0.0053	0.0130	< 0.01	0.02
Genus						
Tuzzerella	0.0048	0.0055	0.0004	0.0010	< 0.01	< 0.01
Fusicatenibacter	0.0025	0.0029	0.0001	0.0003	< 0.01	< 0.01
Romboutsia	0.0042	0.0099	0.0048	0.0211	< 0.01	< 0.01
Ruminococcus	0.0010	0.0020	0.0002	0.0005	< 0.01	< 0.01
Faecalibacterium	0.0742	0.0857	0.1850	0.1457	< 0.01	< 0.01
Tyzzerella	0	----	0.0022	0.0042	< 0.01	0.01
Ruminococcaceae UCG-005	0	----	0.0017	0.0038	< 0.01	0.02
Species						
Anaerotignum lactatifermentans	0	----	0.0013	0.0042	< 0.01	< 0.01
Lactobacillus oris	0	----	0.0005	0.0024	< 0.01	< 0.01
Lactobacillus ingluviei	0	----	0.0010	0.0032	< 0.01	< 0.01
Clostridium spiroforme	0	----	0.0002	0.0007	< 0.01	< 0.01
Bacterium ic1311	0.0005	0.0009	0.0009	0.0023	< 0.01	< 0.01
Agathobaculum sp	0.0160	0.0212	0.0043	0.0096	0.01	0.03
Eubacterium sp	0.0034	0.0040	0.0069	0.0056	0.02	0.04
Lactobacillus crispatus	0.2592	0.1975	0.1153	0.1005	0.02	0.04

[1]Positive Control: The basal diet with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2]Standard Error of the Mean.

[3]COS 95 kDa: Combination of the results from birds fed the NC diet supplemented with chitosan oligosaccharide 95 kDa included at 0.2 or 5 g/kg of the diet.

[4]P-value for Mann-Whitney non-parametric test.

[5]FDR: false discovery rate (adjusted P-value).

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 3.3. Microbial average relative abundance in the ceca of broilers at 22 d old fed a Negative Control or Chitosan oligosaccharide (COS) 95 kDa diets.

Item	---------- Average relative abundance (%) ----------					
	Negative Control[1]	SEM[2]	**COS 95 kDa**[3]	SEM[2]	P-value[4]	FDR[5]
Genus						
Clostridium sensu stricto 1	0.0013	0.0029	0.0005	0.0015	< 0.01	0.02
Erysipelatoclostridium	0.0465	0.0382	0.0230	0.0223	< 0.01	0.02
Species						
Massiliomicrobiota timonensis	0.0244	0.0213	0.0087	0.0105	< 0.01	< 0.01
Clostridium colinum	0.0009	0.0018	0	----	< 0.01	< 0.01
Bacterium ic1311	0.0244	0.0213	0.0087	0.0105	< 0.01	< 0.01
Lactobacillus ingluviei	0.0036	0.0057	0.0008	0.0029	< 0.01	0.01
Lactobacillus oris	0	----	0.0012	0.0043	0.01	0.03

[1]Negative Control: The basal diet without any medications.

[2]Standard Error of the Mean.

[3]COS 95 kDa: Combination of the results from birds fed the NC diet supplemented with chitosan oligosaccharide 95 kDa included at 0.2 or 5 g/kg of the diet.

[4]P-value for Mann-Whitney non-parametric test.

[5]FDR: false discovery rate (adjusted P-value).

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 3.4. Mean log-copies of total *Clostridium perfringens* and its pathogenic strain expressing the *netB* gene in cecal content of broilers at 22 and 38 d fed with or without chitosan oligosaccharides in the diet.

	---------- 22 d ----------		---------- 38 d ----------	
Treatment	***C. perfringens* log-copies**	***netB*-positive log-copies**	***C. perfringens* log-copies**	***netB*-positive log-copies**
Positive Control[1]	5.080	5.046	5.144	5.005
Negative Control[2]	5.623	5.258	5.402	5.364
COS 95 at 0.2 g/kg[3]	5.424	5.176	5.405	5.418
COS 95 at 5 g/kg[4]	5.350	5.385	5.393	5.288
SEM[5]	0.12	0.07	0.06	0.06
P-value[6]	0.44	0.37	0.27	0.20

[1]Positive Control: The basal diet with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2]Negative Control: The basal diet without antibiotic and coccidiostat.

[3]COS 95 at 0.2 g/kg: The NC diet supplemented with chitosan oligosaccharide 95 kDa and included at 0.2 g/kg.

[4]COS 95 at 5 g/kg: The NC diet supplemented with chitosan oligosaccharide 95 kDa and included at 5 g/kg.

[5]Standard Error of the Mean.

[6]P-value for one-way ANOVA parametric test.

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 3.5. Broiler cecal concentrations of short-chain fatty acids (SCFA) at 22 d fed with or without chitosan oligosaccharides in the diet.

Item	Short-chain Fatty Acids (μ mol/ml)							
	Total SCFA	**Acetic Acid**	**Propionic Acid**	**Butyric Acid**	**Isobutyric Acid**	**Valeric Acid**	**Isovaleric Acid**	**Caproic Acid**
22 d								
Positive control[1]	64.27	52.49	4.79[a]	6.17	0.34[b]	0.20	0.20	0.07
Negative control[2]	67.36	55.57	2.82[ab]	7.90	0.37[b]	0.30	0.32	0.07
COS 95 at 0.2 g/kg[3]	67.43	56.95	2.63[b]	6.79	0.45[ab]	0.24	0.30	0.07
COS 95 at 5 g/kg[4]	67.26	54.91	3.32[ab]	7.58	0.58[a]	0.33	0.44	0.10
SEM[5]	3.19	2.62	0.30	0.59	0.03	0.03	0.03	0.005
P-value[6]	0.98	0.95	0.03	0.75	0.01	0.38	0.07	0.21
38 d								
Positive control[1]	85.77	67.89	4.73	11.93	0.33[B]	0.52[b]	0.29[b]	0.08[ab]
Negative control[2]	81.87	61.11	5.71	12.79	0.65[A]	0.81[a]	0.67[a]	0.13[a]
COS 95 at 0.2 g/kg[3]	82.22	63.52	5.27	12.02	0.40[AB]	0.57[ab]	0.37[ab]	0.06[b]
COS 95 at 5 g/kg[4]	68.79	52.47	5.79	8.49	0.61[A]	0.70[ab]	0.64[a]	0.07[ab]
SEM[5]	3.41	2.67	0.17	0.85	0.04	0.04	0.05	0.009
P-value[6]	0.31	0.22	0.12	0.29	< 0.01	0.03	0.01	0.03

[1]Positive Control: A basal diet with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2]Negative Control: A basal diet without antibiotic and coccidiostat.

[3]COS 95 at 0.2 g/kg: The NC diet supplemented with chitosan oligosaccharide 95 kDa and included at 0.2 g/kg.

[4]COS 95 at 5 g/kg: The NC diet supplemented with chitosan oligosaccharide 95 kDa and included at 5 g/kg.

[5]Standard Error of the Mean.

[6]P-value for one-way ANOVA parametric test.

[a,b]Means with different superscripts within the same row are significantly different $P \leq 0.05$.

[A,B]Means with different superscripts within the same row are significantly different $P < 0.01$.

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 3.6. Broiler serum concentrations of cytokines, chemokines, and growth factors at 22 d fed with or without chitosan oligosaccharides in the diet.

Treatment	Concentration (pg/mL)									
	IFNα	**IFNγ**	**IL-2**	**IL-10**	**IL-16**	**M-CSF**	**MIP-1β**	**MIP-3α**	**RANTES**	**VEGF**
Positive control[1]	1.53[b]	44.94	89.15	70.55	51.80[B]	685.71	10.41	57.30[b]	2.05	1.52
Negative control[2]	6.81[a]	65.31	59.60	23.15	64.17[AB]	762.65	14.57	96.02[a]	8.08	0.43
COS 95 at 0.2 g/kg[3]	4.24[ab]	49.11	47.67	43.00	84.79[A]	744.00	14.49	99.82[a]	6.20	1.31
COS 95 at 5 g/kg[4]	1.83[ab]	40.14	87.60	18.58	61.30[AB]	566.48	12.52	68.67[ab]	2.37	0.58
SEM[5]	0.75	3.86	9.76	15.1	3.69	46.6	0.73	6.42	1.19	0.41
P-value[6]	0.03	0.10	0.44	0.28	<0.01	0.46	0.14	0.05	0.20	0.74

Abbreviations: IFNα (interferon alfa), IFNγ (interferon gamma), IL-2 (interleukin-2), IL-6 (interleukin 6), IL-10 (interleukin-10), IL-16 (interleukin-16), IL-21 (interleukin-21), M-CSF (macrophage colony-stimulating factor), MIP-1β (macrophage inflammatory protein-1 beta), MIP-3α (macrophage inflammatory protein-3 alpha), RANTES (regulated on activation, normal T cell expressed and secreted), VEGF (vascular endothelial growth factor).

[1]Positive Control: The basal diet with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2]Negative Control: The basal diet without antibiotic and coccidiostat.

[3]COS 95 at 0.2 g/kg: The NC diet supplemented with chitosan oligosaccharide 95 kDa and included at 0.2 g/kg.

[4]COS 95 at 5 g/kg: The NC diet supplemented with chitosan oligosaccharide 95 kDa and included at 5 g/kg.

[5]Standard Error of the Mean.

[6]P-value for one-way ANOVA parametric test.

[a,b]Means with different superscripts within the same row are significantly different $P \leq 0.05$.

[A,B]Means with different superscripts within the same row are significantly different $P < 0.01$.

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 3.7. Intestinal morphology (proximal jejunum) of broilers at 22 and 38 d fed with or without chitosan oligosaccharides in the diet.

Item	Villus height (µm)	Crypt depth (µm)	Villi: Crypt (ratio)	Epithelium thickness (µm)	Lamina propria thickness (µm)	*Eimeria* oocysts (ranking)[6]	*Eimeria* oocysts (number)[7]
22 d							
Positive control[1]	1623.27^{AB}	174.66^{C}	9.70^{A}	59.97^{B}	83.79^{B}	277.3^{B}	6
Negative control[2]	1562.13^{B}	241.20^{A}	6.62^{D}	71.52^{A}	124.21^{A}	317.8^{A}	40
COS 95 at 0.2 g/kg[3]	1703.31^{A}	202.67^{B}	8.66^{B}	59.42^{B}	76.70^{B}	282.1^{B}	14
COS 95 at 5 g/kg[4]	1626.10^{AB}	211.00^{B}	7.97^{C}	58.64^{B}	69.64^{B}	293.5^{B}	16
SEM	12.80	2.21	0.08	1.06	2.92	0.02	----
P-value[5]	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01¥	----
38 d							
Positive control[1]	1656.21^{B}	179.08^{B}	9.59^{B}	77.165^{ab}	108.44^{AB}	244.00	2
Negative control[2]	1696.46^{AB}	200.16^{A}	8.73^{C}	81.626^{a}	112.85^{A}	249.90	5
COS 95 at 0.2 g/kg[3]	1745.33^{A}	158.09^{C}	11.42^{A}	75.809^{ab}	88.63^{BC}	243.90	2
COS 95 at 5 g/kg[4]	1704.34^{AB}	149.53^{C}	11.86^{A}	69.173^{b}	79.13^{C}	244.20	2
SEM	9.42	1.84	0.11	1.60	3.22	0.007	----
P-value[5]	< 0.01	< 0.01	< 0.01	0.05	< 0.01	0.49¥	----

[1] Positive Control: The basal diet with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2] Negative Control: The basal diet without antibiotic and coccidiostat.

[3] COS 95 at 0.2 g/kg: The NC diet supplemented with chitosan oligosaccharide 95 kDa and included at 0.2 g/kg.

[4] COS 95 at 5 g/kg: The NC diet supplemented with chitosan oligosaccharide 95 kDa and included at 5 g/kg.

[5] P-value for one-way ANOVA parametric test (except for *Eimeria* oocysts count).

[6] *Eimeria* oocysts mean ranking refers to the data transformation in which numerical or ordinal values are replaced by their rank when the data are sorted. The higher the mean ranking, the higher the count of *Eimeria* oocysts in the villi analyzed.

[7] *Eimeria* oocysts number refers to the total number of oocysts encountered in the villi analyzed.

¥ P-value for Kruskal-Wallis non-parametric test.

[a,b] Means with different superscripts within the same row are significantly different $P \leq 0.05$.

[A,B,C,D] Means with different superscripts within the same row are significantly different $P < 0.01$.

3.7 FIGURES

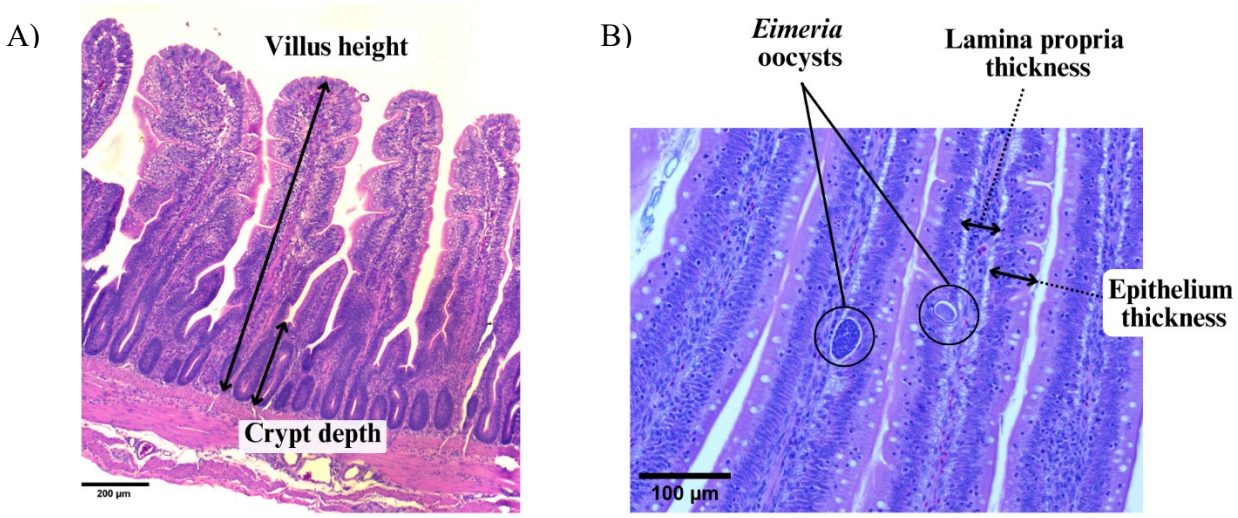

Figure 3.1. Photomicrographs of hematoxylin and eosin-stained chicken jejunum representing the morphological measurements performed. (A) Photomicrography (5x) representing the measurement of villus height and crypt depth; (B) Photomicrography (20x) representing the measurement of lamina propria and epithelium thickness, and the *Eimeria* oocysts count.

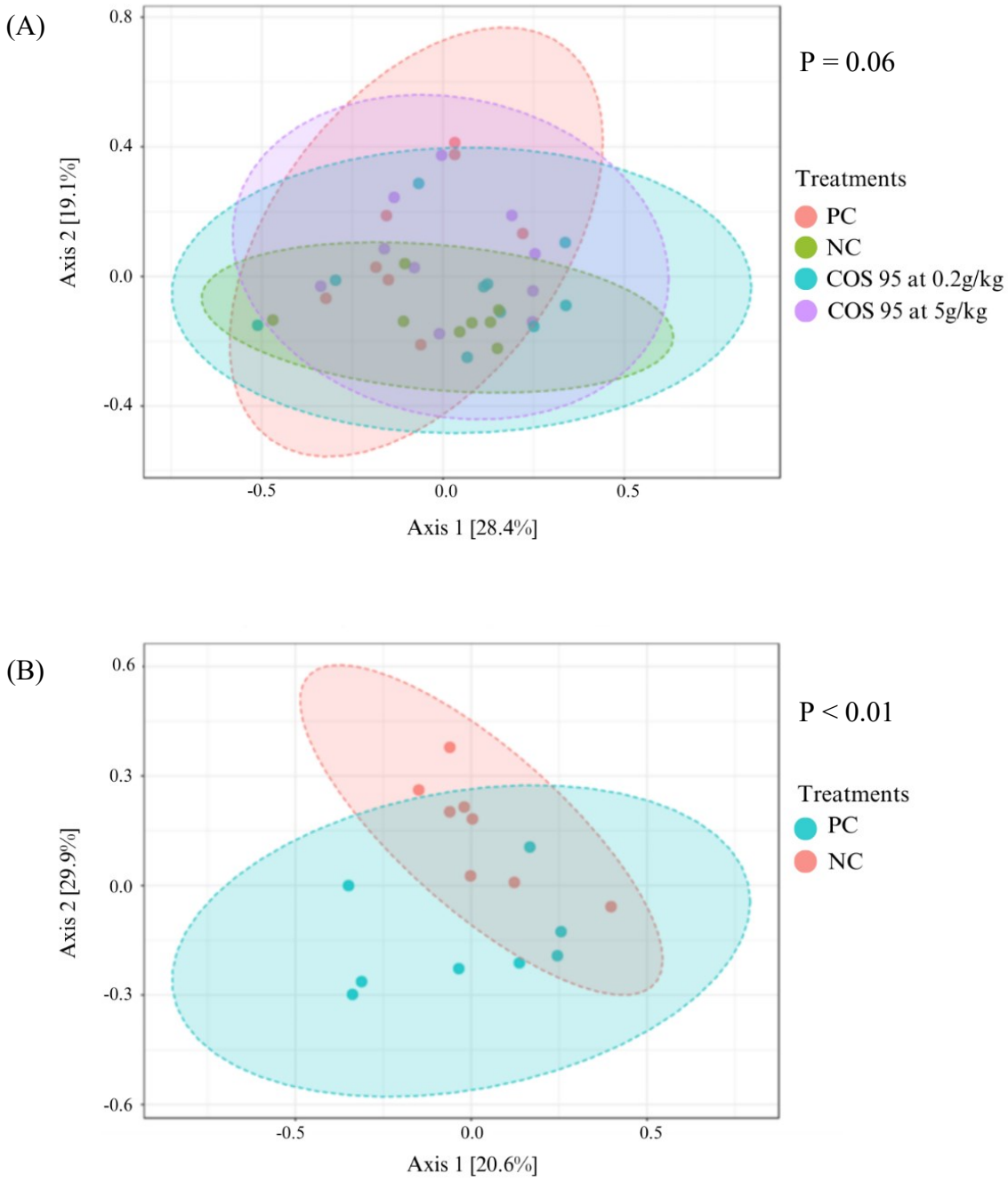

Figure 3.2. Beta diversity plot of cecal microbiota of broilers at 22 d. (A) Comparison of microbiota beta diversity of broilers fed Positive Control diet (PC; basal diet plus antibiotic and coccidiostat), Negative Control diet (NC; basal diet without medications), COS 95 kDa at 0.2 g/kg (NC plus COS 95 kDa included at 0.2 g/kg of the diet), or COS 95 kDa at 5 g/kg (NC plus COS 95 kDa included at 5 g/kg of the diet). (B) Pairwise comparison between the beta diversity of the cecal microbiota of birds fed PC and NC.

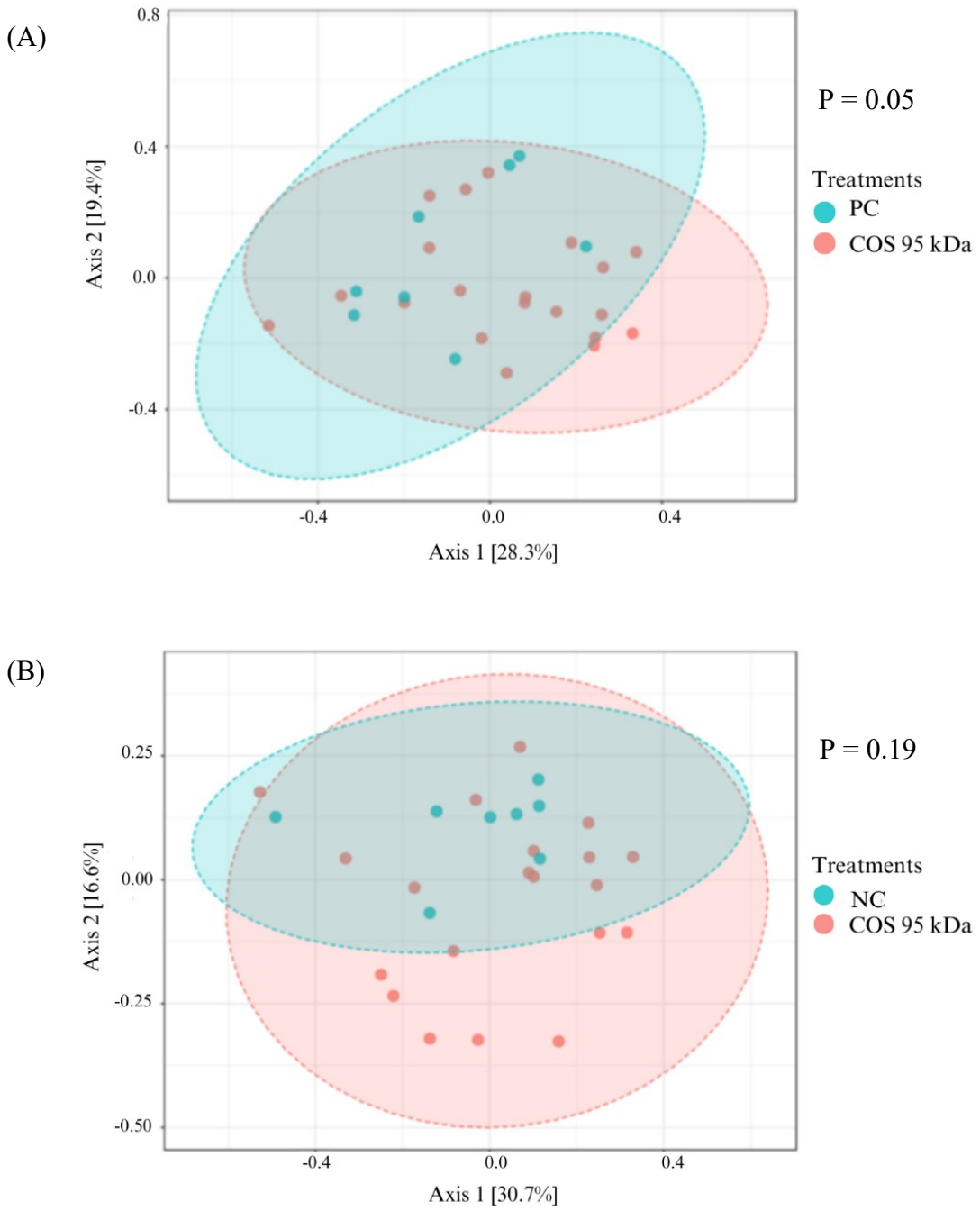

Figure 3.3. Beta diversity plot of cecal microbiota of broilers at 22 d. (A) Pairwise comparison between the beta diversity of the cecal microbiota of birds fed with Positive Control diet (PC; basal diet plus antibiotic and coccidiostat) and COS 95 kDa treatments (regardless of the level of inclusion). (B) Pairwise comparison between the beta diversity of the cecal microbiota of birds fed Negative Control diet (NC; basal diet without medications) and COS 95 kDa treatments (regardless of the level of inclusion).

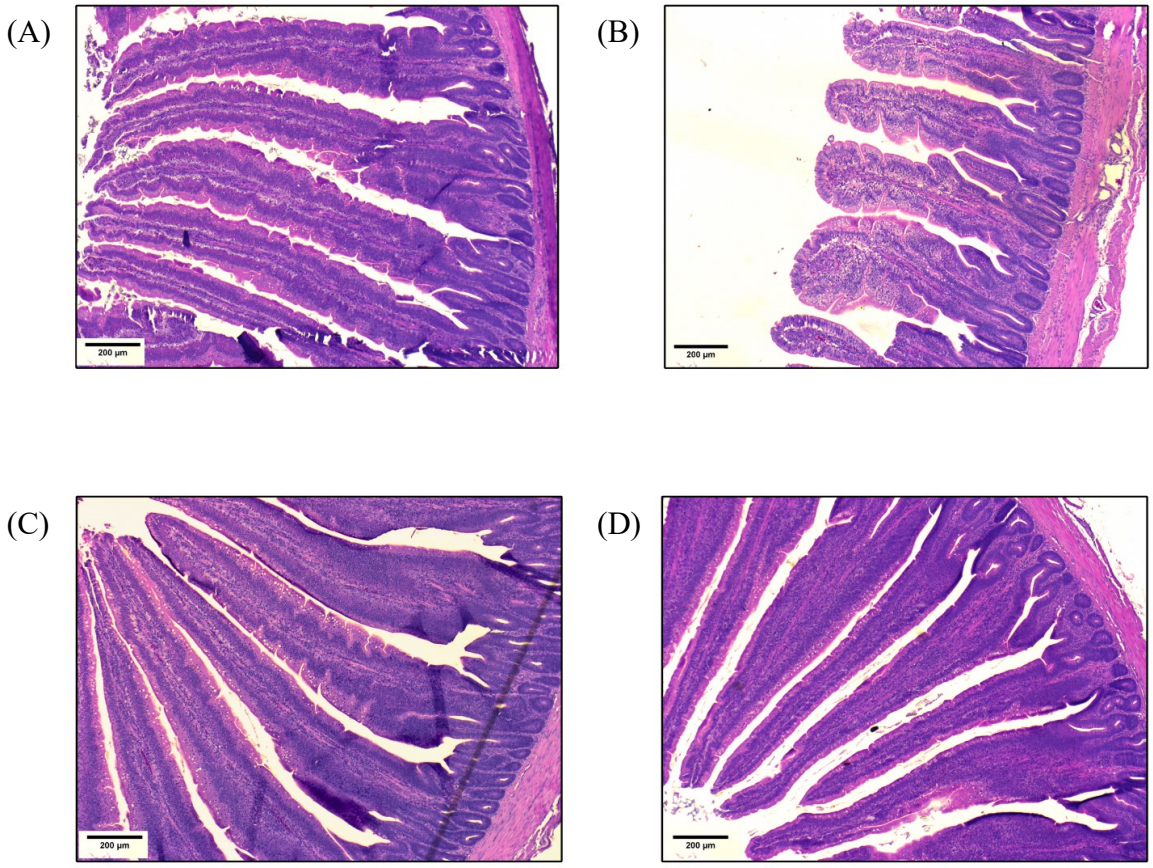

Figure 3.4. Photomicrographs of hematoxylin and eosin-stained jejunum of the broilers at 22 d (5x; scale bar positioned in the lower left corner: 200 µm). (A) Representative photomicrograph of the villus of birds fed with the Positive control diet (PC; basal diet plus antibiotic and coccidiostat); (B) Villus of the birds fed with the Negative Control diet (NC; basal diet without medications); (C) Villus of birds fed COS 95 kDa included at 0.2 g/kg of the diet; (D) Villus of birds fed COS 95 kDa included at 5 g/kg of the diet.

4. PUNICIC ACID AS A POTENTIAL ANTIBIOTIC REPLACEMENT IN BROILER DIETS: EFFECTS ON PERFORMANCE, NECROTIC ENTERITIS GROSS LESION SCORES, LITTER QUALITY AND FOOTPAD DERMATITIS

OVERVIEW

The removal of dietary antibiotic growth promoters (**AGP**) increases the risk of necrotic enteritis (**NE**) in broilers, and effective replacements must be found. Dietary punicic acid (**PA**) from pomegranate seed oil (**PSO**) was screened as a potential AGP replacement on performance, NE intestinal gross lesions, and footpad dermatitis (**FPD**) of broilers. A total of 1,280 birds were distributed across 8 treatments with 8 replicates of 20 birds each. The treatments were: Positive Control (**PC**; basal diet with a commercial antibiotic and coccidiostat program), Negative Control (**NC**; basal diet without medications), and six increasing doses of PA (NC diet + PA at either 0.1, 0.25, 0.5, 1, 1.5, or 2% of the feed). A subclinical NE challenge model (15 X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d) was applied to all birds. BW, feed intake (**FI**), BW gain (**BWG**), and feed conversion ratio (g feed:g gain; **FCR**) were measured at the end of the starter, grower, and finisher phases. NE lesions in sample birds were measured at 22 and 40 d, while FPD scores were measured at 41 d. No significant differences in performance between the PC and NC groups were observed. Increasing doses of PA reduced BWG and FI and increased FCR ($P<0.01$) in the starter and grower phases. For the entire period (0 to 41 d), dietary treatment had no effect on BWG or FCR; however, PA 2% had the lowest FI ($P=0.03$). Birds fed PC had lower NE gross lesions than those fed PA 2% at 20 d ($P=0.04$). The NC group had higher FPD scores ($P<0.01$) than other treatments. The lack of difference between the PC and NC groups indicates that the challenge may not have been severe enough to detect differences in performance and test PA efficacy. In the study conditions, PA supplemented through PSO did not mitigate NE or enhance broiler performance, and it may be related to the properties of PSO. Therefore, PA

should be tested under a stronger NE challenge, and under conditions that might avoid the confounding effects of high PSO inclusion.

Key words: Broiler chickens; antibiotic replacers; punicic acid; necrotic enteritis; performance.

4.1 INTRODUCTION

Necrotic enteritis (**NE**) is a multifaceted enteric disease of broilers caused by pathogenic strains of *Clostridium perfringens*, often in conjunction with predisposing factors (Parish, 1961; Annett et al., 2002; Wu et al., 2014). NE can occur in either clinical or subclinical forms, resulting in estimated annual losses of $6 billion in the poultry sector (Wade and Keyburn, 2015). The clinical form is characterized by a sudden increase in flock mortality, often without premonitory signs (Van Immerseel et al., 2009; Wu et al., 2010). In contrast, the subclinical form is silent and causes a reduction in broiler growth performance due to the chronic intestinal damage caused by *C. perfringens* toxins (Olkowski et al., 2008). The focal intestinal lesions caused by this disease condition impair the proper digestion and absorption of nutrients by the birds and also trigger intestinal inflammation (Van Immerseel et al., 2009). In addition to reduced nutrient absorption, activation of the immune system to control inflammatory-related processes will demand energy and nutrients from the host, diverting nutrients away from growth and also contributing to reductions in performance (Broom and Kogut, 2018).

For decades, NE was prevented in broilers through the use of in-feed antibiotic growth promoters (**AGP**) (Williams, 2005). Although the mechanisms of action by which AGP prevent diseases and promote growth in animals are still not fully understood, they appear to be linked to control of intestinal and systemic inflammation, as well as inhibiting bacterial growth and virulence factors (Niewold, 2007; Broom, 2017; Oh et al., 2019). Therefore, the reduced use of AGP in poultry diets has led to a rising prevalence of NE disease in commercial flocks worldwide (Van Immerseel et

al., 2016). The increasing incidence of NE in broilers emphasizes the need for effective disease control strategies to mitigate its impact on bird health and production. Addressing the NE challenge will likely involve a strategic focus on enhancing poultry gut health (M'Sadeq et al., 2015).

Punicic acid (**PA**) is an unusual long-chain polyunsaturated fatty acid (C18:3, 9 cis, 11 trans, 13 cis) mainly found in pomegranate seed oil (**PSO**), making up to 80% of the PSO composition (Aruna et al., 2016). This conjugated fatty acid has gained attention due to its pharmacological properties, including anti-diabetes (Hontecillas et al., 2009), anti-obesity (Yuan et al., 2021), antioxidant (Saha and Ghosh, 2009), and anti-inflammatory functions (Boussetta et al., 2009; Bassaganya-Riera et al., 2011). Among these, the anti-inflammatory properties are the most explored to alleviate enteric diseases in animal models. PA relieved colitis-induced intestinal inflammation in rats by inhibiting tumor necrosis factor α (TNF-α)-induced elevation of NADPH oxidase (Boussetta et al., 2009), enhancing intestinal integrity and reducing mRNA encoding inflammatory cytokines (Coursodon-Boyiddle et al., 2012). Similarly, PA mitigated inflammatory bowel disease in mice by modulating T-cell and macrophage function through peroxisome proliferator-activated receptors gamma (**PPAR-γ**) and delta-dependent mechanisms (Bassaganya-Riera et al., 2011). PA also enhanced the activity of antioxidant enzymes (superoxide dismutase, catalase, and glutathione peroxidase), reduced nitric oxide synthesis and lipid peroxidation, and alleviated oxidative stress in mice (Saha and Ghosh, 2009; Boroushaki et al., 2016). Hence, PA may have potential as an AGP alternative for the prevention or attenuation of enteric diseases in broilers. However, to the best of our knowledge, no studies have evaluated the potential of dietary PA in mitigating NE in broilers. Therefore, the objective of this study was to evaluate the potential of PA, provided through the supplementation of PSO in diet, in preventing or mitigating NE in broilers. For this, the effects of PA were assessed on the performance, intestinal NE gross lesion,

litter quality, and footpad dermatitis of broilers challenged with a natural, subclinical NE infection model. We hypothesized that PA provided through PSO would enhance performance and reduce intestinal lesions associated with subclinical NE in broilers.

4.2 MATERIALS AND METHODS

The protocol was approved by the Animal Care and Use Committee for Livestock of the University of Alberta and followed principles established by the Canadian Council on Animal Care (CCAC, 2009).

4.2.1 Pomegranate seed oil

As PSO is the main natural source of PA, a commercially available PSO (Jedwards International, Inc. Braintree, MA, USA) was used to provide the appropriate amount of PA in each diet.

4.2.2 Animals and housing

A total of 1,280 one-day-old male Ross 708 broilers, with similar weights (31.6 ± 0.09 g), were obtained from a commercial hatchery. Upon arrival at the research facility, they were individually weighed, identified by neck tag, and randomly assigned to one of 8 treatments, with 8 replicates of 20 birds each. The chicks were placed and reared in each of 64 floor pens (1.061 x 1.397 m) equipped with a nipple drinker line, a hanging tube feeder, and 8 cm of new wood shavings litter. The temperature and lighting program followed commercial practices recommended by the Ross 708 commercial management guidelines (Aviagen, 2018) as previously detailed in Chapters 2 and 3. During the trial, feeders were shaken 2 to 3 times per day, ensuring birds had ad libitum access to feed. Observations of bird health condition, availability of feed, water, and environmental housing conditions were recorded twice daily.

4.2.3 Experimental diets

Birds were fed a wheat-soy-canola meal-basal diet similar to what is commonly fed to commercial broiler chickens in Western Canada. Diets were made to meet or exceed Ross 708 nutritional recommendations (Aviagen, 2019a) according to each growth phase (Table 4.1). The experimental diets were: Positive control (**PC**), a commercial-type diet supplemented with an antibiotic growth promotor (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet); Negative Control (**NC**), that was the same as the PC, except without any medications, and the NC plus pomegranate oil added to a total final PA concentration of 0.1, 0.25, 0.5, 1, 1.5, or 2% of the feed (**PA 0.1%**, **PA 0.25%**, **PA 0.5%**, **PA 1%**, **PA 1.5%** and **PA 2%,** respectively). For the calculation of oil inclusion in the experimental diets, the concentration of PA in PSO was considered at 55% (Pereira de Melo et al., 2014, 2016). Thus, the PSO inclusion in diets containing PA was 0.18, 0.45, 0.91, 1.82, 2.73, and 3.64%, respectively. Canola oil was added as needed to provide a consistent total oil content across diets within each dietary phase (Table 4.2). Diets were fed in mash form during the 41-day experiment.

4.2.4 Punicic acid recovery

The concentration of PA was measured in the complete feed of each treatment to confirm the level of PA in each diet (Table 4.3). The total lipid extraction and PA analysis in the complete diets and PSO were determined as described previously (Xu et al., 2020; Wang et al., 2021). Briefly, samples were homogenized at 10,000 × g for 5 minutes using a Fisher Scientific Power Gen 1000 homogenizer (Fisher Scientific, Pittsburgh, PA) in a screw cap glass tube with 3 mL of chloroform: isopropanol (2:1, v/v) with 0.01% BHT as the antioxidant. The tube was flushed with nitrogen, placed on ice, and kept from light during homogenization. Then, another 3 mL of chloroform:

isopropanol (2:1, v/v) with 0.01% BHT and 1 mL of 0.9% NaCl solution were added to the mixture. After vortexing for 1 minute and centrifugation, the organic phase from each sample was transferred to a clean new tube. The samples were re-extracted twice. The organic phases were combined and evaporated under nitrogen. The total lipid extracts were transmethylated in screwcap tubes with 1 mL of 5% sodium methoxide in methanol for 30 min at room temperature. The resulting fatty acid methyl esters (**FAME**) were extracted twice with HPLC-grade hexane and dried under nitrogen. The FAME were then resuspended with 1 mL of HPLC-grade hexane and then analyzed on an Agilent 6890N Gas Chromatograph equipped with a 5975 inert XL Mass Selective Detector and flame ionization detector (Agilent Technologies). The FAME were separated on a capillary column DB-23 (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, Wilmington, DE, USA) using the following temperature program: 165 °C for 4 min, increased to 180 °C at 10 °C/min and held for 5 min and increased to 230 °C at 10°C/min and held for 5 min.

4.2.5 Broiler performance

Feed intake (**FI**), average BW, average body weight gain (**BWG**), and feed conversion ratio (**FCR**) were measured on a pen basis at the end of the starter (10 d), grower (25 d), and finisher (41) phases, and the entire experimental period (0 to 41 d). Mortality and weight of dead birds were recorded daily to calculate mortality and adjust FI and FCR within each period, and over the duration of the trial.

4.2.6 Subclinical necrotic enteritis challenge

Birds were challenged with a natural, subclinical necrotic enteritis infection model (He et al., 2022). Briefly, at 12 days of age, all birds were gavaged with a 15X dose of a commercial coccidiosis vaccine (Coccivac®-B52; Merck Animal Health, Intervet Inc., Millsboro, DE, USA) diluted in phosphate-buffered saline. The vaccine contained live sporulated *Eimeria* oocysts (*E.*

acervulina, *E. mivati*, *E. tenella*, and two strains of *E. maxima* at unspecified doses). At 18 days of age, the feed was withdrawn for 24 hours from all birds. Every 4 hours during the 24-hour feed removal period, a trained person walked through the pens and took note of any clinical or subclinical signs of NE, distress, or any other abnormalities.

4.2.7 Intestinal lesion scoring

At 22 (three days after the conclusion of the feed withdrawal challenge) and at 40 days of age, two birds per pen were randomly selected for intestinal lesion scoring evaluation. The birds were euthanized by cervical dislocation, and the entire length of the jejunum was examined for gross lesion scoring by an experienced assessor. The scoring system used was adapted from Shojadoost et al. (2012) and was described in detail in Chapter 2. Briefly, the scores ranged from 0 to 4, in which a score of 0 represented the absence of gross lesions in the tissue, while a score of 4 represented higher severity of the lesions (mucosae extensively covered by thick layers of fibrin, necrotic tissue, and inflammatory cells, spreading widely over the intestinal mucosa).

4.2.8 Litter measurements

On day 41, litter samples were collected from four specific locations (comprising four peripheral spots and one central spot) within each pen for moisture analysis. Steel scoops were used to punch out a representative sample of approximately 100 grams from each area to ensure comprehensive sampling throughout the litter depth. The samples were weighed and dried in a forced-air oven to constant weight for 160 hours at 65°C to determine moisture content from the loss in weight of the sample.

4.2.9 Footpad lesion scores

The occurrence and severity of footpad lesions were determined at 41 d in both feet of all the remaining birds (average of 113 birds per treatment). Paw quality was evaluated using the four-

point footpad scoring by an experienced assessor according to the Welfare Quality® Protocol for Poultry, 2009. A score of 0 was assigned when there was no noticeable lesion present. A score of 1 indicated minimal discoloration of the central footpad (< 10% of the central footpad). A score of 2 was given for superficial discoloration of the central footpad (> 10% of the central footpad). A score of 3 was assigned for deep lesions and ulceration (< 50% of central footpad and toes). Lastly, a score of 4 was recorded when deep lesions and ulceration (> 50% of central footpad and toes) were observed.

4.2.10 Statistical analyses

Bartlett's test was used to evaluate the homogeneity of variances, and the Shapiro-Wilk test was used to verify the normality of residuals. After checking the normality, the performance data were analyzed by one-way ANOVA procedures using Statistix 10 software (Analytical Software, Tallahassee, FL). Significant differences between means ($P \leq 0.05$) were further separated with Tukey's test for pairwise comparisons. To analyze the relationship between the inclusion dose of PA and the variables of interest, the data were analyzed by linear and polynomial regression, considering the significance level, coefficient of determination, and bird biological response.

As intestinal and FPD lesion score data were not normally distributed, Kruskal-Wallis, a non-parametric statistical test, was used, and significant effects ($P \leq 0.05$) were evaluated with Dunn's test for pairwise comparisons.

4.3 RESULTS

4.3.1 Fatty acid composition of pomegranate seed oil

The PSO used in the experimental diets (PA 0.1%, PA 0.25%, PA 0.5%, PA 1%, PA 1.5%, and PA 2%) contained the following fatty acid concentrations: 2.65% palmitic acid, 1.89% stearic acid, 4.5% oleic acid, 4.8% linoleic acid, 0.70% eicosenoic acid, and 85.4% punicic acid.

4.3.2 Grower performance

Increasing doses of dietary PA decreased BW of broiler at 10 d (BW= 235.40 – 22.27*Dose + 1.44*Dose²; $R^2 = 0.61$; Table 4.4). Animals fed the NC or PA 0.1% had the highest BW (235.8 ± 3.4 g and 234.9 ± 3.6 g/bird, respectively) at 10 d, while those fed PA 2% had the lowest BW (197.0 ± 4.9 g/bird; $P < 0.01$). Similarly, BWG from 0 to 10 d also decreased in response to increasing dose of PA in the diet (BWG = 20.2473 - 2.24849*Dose + 0.145585*Dose2; $R^2 = 0.60$). The FCR increased with the level of PA in the diets ($P < 0.01$; FCR = 1.24 + 0.12*Dose – 0.007*Dose^2; $R^2 = 0.32$; Table 4.4). The animals fed PA 2% had the highest FCR (1.45 ± 0.19 g), whereas animals fed NC or PA 0.1% had the lowest FCR (1.24 ± 0.02 g and 1.24 ± 0.01 g, respectively). There were no treatment effects on FI during the starter phase ($P > 0.05$).

In the grower phase, the period in which the NE challenge was applied, there were no significant differences between the PC and NC treatments for any of the performance parameters analyzed (Table 4.4). However, high doses of PA (1, 1.5 and 2%) in the diet caused a reduction in BW at 25d ($P < 0.01$; BW = 1007.36 – 72.50*Dose + 4.58*Dose^2; $R^2 = 0.57$). Birds fed PC, NC, PA 0.1%, or PA 0.25% were heavier (1,006.3 ± 9.6 g, 1,003.4 ± 15.1 g, 1,011.4 ± 12.5 g, and 986.0 ± 13.2 g) at 25 d than those fed PA 0.5%, PA 1%, PA 1.5%, or PA 2% (976.2 ± 10.64 g; 925.7 ± 13.8 g; 951.4 ± 10.1 g and 886.8 ± 13.0 g, respectively). Similar effects were observed for BWG (BWG = 50.72 -3.78*Dose + 0.24*Dose^2; $R^2 = 0.56$; Table 4.4). However, at this age, birds fed PC had a superior BWG (51.2 ± 0.51 g/day/bird) than those fed PA 1, 1.5 and 2% ($P < 0.01$). The highest inclusion of PA in the diet resulted in the lowest BWG (44.12 ± 0.65 g/day/bird). FI of birds fed PA 2% was lower compared to the other treatments (FI = 80.50 – 4.63*Dose + 0.30*Dose^2; $R^2 = 0.47$; $P < 0.01$; Table 4.4), including NC. Birds from the PA 2% group had a FI of 71.79 ± 0.51 g/day/bird, while birds fed PC and NC diets had a FI of 80.22 ± 1.11 g/day/bird

and 78.06 ± 0.90 g/day/bird each. The FCR increased in response to PA inclusion in the diet ($P < 0.01$; FCR = 1.587 + 0.03*Dose – 0.002*Dose^2; $R^2 = 0.13$). The NC group had lower FCR (1.55 ± 0.01 g) than those from the PA 0.5%, PA 1%, PA 1.5%, and PA 2% groups (1.63 ± 0.03 g; 1.62 ± 0.02 g; 1.66 ± 0.02 g and 1.63 ± 0.02 g, respectively). However, the FCR in NC was statistically similar to the other treatments, including the PC.

In the finisher phase, the BW of birds at 41 d dropped with the highest inclusion of PA in the diet (BW = 2478.71 – 99.44*Dose + 6.47*Dose^2; $R^2 = 0.14$; P-quadratic < 0.01; Table 4.4). However, there were no treatment effects on BWG, FI or FCR ($P > 0.05$).

For the entire experimental period (0 to 41 d), there were no differences between the PC and NC groups for any of the performance parameters analyzed (Table 4.5). However, the highest dose of PA in the diet reduced the FI compared to all the other treatments ($P < 0.01$; FI = 94.18 – 4.05*Dose + 0.27*Dose^2; $R^2 = 0.18$). The FI in birds fed with PA 2% was 85.54 ± 1.96 g/day/bird, while in those fed PC and NC was 94.0 ± 1.74 and 92.18 ± 1.60 g/day/bird, respectively. There was no significant difference ($P > 0.05$) among treatments for BWG or FCR considering the entire period.

4.3.3 Observations of subclinical or clinical signs of NE

Experienced personnel walked through the pens every 4 hours during the 24-hour feed withdrawal period at 18 d, evaluating the condition of the birds. During these observations, diarrhea and bloody feces were observed, specifically in 5 out of 8 pens of NC birds (Figure 4.1). However, no signs of clinical NE, such as increased mortality, depression, dehydration, or ruffled feathers, were observed in any of the treatments.

4.3.4 Intestinal NE gross lesions

At 22 d, birds fed PC had milder NE lesions (average score of 0.12 ± 0.08) than those fed with the highest inclusion of PA in the diet (0.69 ± 0.12; $P = 0.04$; Table 4.6). In the PC group, 87.5% of

the birds had no lesions, and only 12.5% had a score of 1. While the PA 2% had 31.25% of animals with no lesions, the other 68.75% of birds had lesions scored as 1. However, they were not different from the other treatments, including NC. None of the birds from any of the treatments developed lesions higher than score 2.

At 40 d, there was a nearly significant difference in the severity of NE gross lesions among the treatments ($P = 0.07$; Table 4.6). Birds fed with PA 2% tented to have more pronounced lesions (average score of 1.44 ± 0.13) than those fed with PC and PA 0.1% (average scores of 0.93 ± 0.11 and 0.94 ± 0.11, respectively). None of the birds from any of the treatment groups had lesions higher than score 2.

4.3.5 Litter quality and footpad dermatitis

The NC treatment resulted in higher litter moisture (41.23 ± 1.69%; $P = 0.01$) than the PA 2% group (31.96 ± 1.73%). However, NC was not different from the other treatments, including the PC group (33.87 ± 1.84%; Table 4.7).

Birds fed the NC diet had higher FPD scores (0.96 ± 0.11) on the right foot than all other treatments ($P < 0.01$; Table 4.7; Figure 4.2). Within this group, 57.02% of the birds showed no visible lesions (score 0), 8.77% exhibited mild lesions (score 1), 15.79% displayed noticeable lesions (score 2), and 17.54% and 0.88% had more severe lesions (scores 3 and 4), respectively. In contrast, each of the other treatment groups had over 80% of birds without any lesions (score 0; data not shown). Similarly, for the left foot, NC birds had more pronounced FPD lesions (0.86 ± 0.11) than those from the PC, PA 0.1%, PA 0.5%, PA 1%, and PA 2% (0.15 ± 0.05; 0.20 ± 0.06; 0.26 ± 0.07; 0.17 ± 0.05, and 0.18 ± 0.06, respectively; Table 4.7).

4.4 DISCUSSION

NE is a major enteric disease in poultry that has become more prevalent since the withdrawal of AGP from poultry diets (Williams, 2005). Although this disease can occur as either acute clinical or subclinical, its subclinical form is the most common and mainly responsible for the economic losses in the sector (Skinner et al., 2010). In this form, the disease is silent and sometimes only noticeable at the end of the cycle when birds fail to achieve the expected target body weight. The reduction in performance is due to the chronic damage caused by *C. perfringens* to the intestinal mucosa (focal necrosis), which hinders the digestion and absorption of nutrients (Kaldhusdal et al., 2001; Olkowski et al., 2008; Van Immerseel et al., 2009). Because of that, we were particularly interested in evaluating the effects of punicic acid in preventing subclinical NE in broiler chickens. For an accurate evaluation of this product as a potential AGP replacement, we compared diets supplemented with the test products with two control treatments (PC and NC). The PC represented a commercial diet containing a coccidiostat and a sub-therapeutic dose of an AGP. In contrast, the NC represented an antibiotic-free commercial diet (without any medications). Therefore, we expected the PC diet to prevent birds from experiencing subclinical NE and optimize their performance in the presence of the challenge. On the other hand, we expected the challenge to cause more enteric disturbances in the NC birds, reducing their performance relative to those in the PC group. However, in the present study, the challenge did not reduce the performance of NC birds compared to those fed the PC diet. It may be possible that the natural, subclinical NE challenge applied was not severe enough to decrease the performance of NC birds relative to PC birds. Similar effects were also observed in studies reported in Chapters 2 and 3, in which we applied the same challenge model. As discussed in Chapter 2, we expected the challenge applied to cause a setback in the NC birds relative to the PC birds. This would provide evidence that there

was a challenge in the experimental environment, and the inclusion of AGP in the PC diet mitigated this challenge. In the absence of reduced performance in the NC group, it is not possible to accurately evaluate the effects of potential AGP replacements. Therefore, the present study suggests again the necessity of adjustments to our challenge model. Adjustments aimed to make the natural, subclinical NE challenge model more robust would help us to accurately test the effectiveness of AGP replacements. As mentioned in Chapter 2, a way to increase the robustness of the challenge would be increasing the dosage of the coccidiosis vaccine, for example, 20 times the recommended dose on day 12. This may slightly increase the disturbance of the epithelium by allowing more *Eimeria* oocysts to invade enterocytes, creating more favorable conditions that stimulate the natural infection and proliferation of *C. perfringens*. Another way to enhance the challenge would be to use in-feed corticosterone (Zaytsoff et al., 2020) as a stress factor following the coccidia challenge. In regions with higher relative humidity, using a sanitation challenge, such as reused litter (Bortoluzzi et al., 2019), would be an option to increase the robustness of the challenge.

In the current study, the overall performance of the broilers was lower compared to the performance expected from the primary breeder guidelines (Aviagen, 2019b). Birds were 83.2 g, 318.6 g, and 460.2 g lighter than expected at the end of the starter, grower, and finisher phases, respectively. Although the NE challenge could have contributed to that decrease in performance, another possibility could be due to the total amount of oil incorporated into the diets and the limited ability of chicks to digest fats at a young age. In order to achieve the desired PA concentration in the starter diets, we included more oil than is commonly added in commercial-type diets. For a starter diet, a typical oil inclusion would be around 2% and 3.5% crude fat; however, in order to achieve the desired PA inclusion, we included 3.64% oil, increasing the crude fat to 5.2%. Young

birds have low lipase activity and low bile secretion until approximately 7-d-old (Noy and Sklan, 1995). Lipase activity in chickens increases by 20- to 100-fold from 4 to 21 d of age (Noy and Sklan, 1995). This low capability of fat digestion may have led to an increase in digesta passage rate and lowered the nutrient digestibility, causing a reduction in the overall performance of the birds. It is worth noting, however, that within each dietary phase, all diets had the same total oil inclusion (Table 4.2), thereby distributing these effects evenly across all treatment groups.

What was interesting, however, was that birds that received the highest levels of punicic acid as PSO had diminished performance compared to other treatment groups, including NC, not only during the starter phase but also in the subsequent periods. Although no detailed investigations were conducted to determine the reasons associated with this decrease in performance, we speculate that it may be related to the properties of PSO. Depending on the strain of the pomegranate, its seed oil can contain up to 80 to 90% polyunsaturated fatty acids (**PUFA**); among them, PA (18:3; n-5) is the PUFA found in greatest concentrations (Pereira de Melo et al., 2014; Aruna et al., 2016). In the current study, the PSO used was not stabilized with antioxidants and had 90.2 % PUFA, of which 85.4% was PA and 4.8% was linoleic acid (18:2; n-6). Even though PUFA are highly digestible compared to saturated fats and represent traditional fat sources in broiler diets, they are more susceptible to oxidation (Engberg et al., 1996). Fat oxidation involves the generation of fatty acid free-radicals, which react with oxygen molecules to produce peroxide free radicals and cause lipid peroxidation. This oxidative process results in the formation of undesirable products such as ketones, aldehydes, alcohols, furans, and others, leading to oxidative rancidity (Anjum et al., 2004; Pignitter and Somoza, 2012). Oxidative rancidity is a major contributor to the loss of quality in ingredients or rations, affecting flavor, aroma, color, texture, and nutritive value (Baião and Lara, 2005). Although the canola oil used as a standard fat source

in the control groups also contains PUFA, the concentration is approximately 35% (Dupont et al., 1989), which is much lower compared to the concentration in PSO. Thus, it is possible that diets containing high inclusion of PSO reduced broiler performance compared to the other diets because the PSO was more susceptible to oxidation and may have undergone peroxidation, reducing the ration palatability and nutritional value. Feeding of oxidized oil to broilers and pigs decreased their overall performance (Dibner et al., 1996; Liu et al., 2014a; Lindblom et al., 2019), impaired metabolic oxidative status (Liu et al., 2014b; Liang et al., 2015), and impaired intestinal health (Tan et al., 2019; Zhang et al., 2022). In addition to reduced performance, we observed that birds fed the highest inclusion of PA in the diet had more pronounced NE gross lesions (although still mild) in the jejunum at 22 d than those fed PC. A similar tendency of PA 2% to increase NE lesion scores was observed at 40 d ($P = 0.07$). Therefore, it is possible that PSO peroxidation not only affected the palatability and nutritive value of the ration but also increased intestinal oxidative stress, leading to increased intestinal dysbiosis (Tan et al., 2018; Zhang et al., 2022). This creates more favourable conditions for the *C. perfringens* to proliferate and cause lesions in the gut (Lee et al., 2014; Xu et al., 2023) compared to the PC.

Although not tested, there is indirect evidence that PSO oxidation may have influenced the recovery of PA from the complete diets. Initially, the inclusion level of PSO oil in the diets was calculated assuming it contained 55% PA (Pereira de Melo et al., 2016). However, the PA content of the PSO was 85% by analysis, indicating that 54.5% more PA was added to the diets vs the formulated values. However, when the complete diets were analyzed, the PA concentration in each experimental diet was relatively close to the formulated concentration for each diet (Table 4.3). This suggests that PA and potentially other fatty acids in PSO may have undergone oxidation and

were partially degraded or transformed into non-fatty acid compounds (Gardner, 1989), reducing the concentration of fatty acids detected in the gas chromatography analysis.

Although the oil oxidation could be a simple and reasonable explanation for the observed reductions in performance of birds fed PSO, studies that also observed reductions in BW in mice fed PSO suggest that the oil may induce changes in fat and glucose metabolism. When PSO was added to high-fat diets, BW, BWG and fat mass were decreased, and satiety was increased in mice (McFarlin et al., 2008; Vroegrijk et al., 2011; Mohamed and Fayed, 2020). In addition, plasma leptin and insulin were decreased, and adiponectin was increased, which the authors linked to the loss of body weight. In addition, the authors speculated that the results observed are associated with the ability of PSO to activate the PPAR-γ, a nuclear receptor that plays a crucial role in regulating lipid and glucose metabolism and hormonal signaling (Ferré, 2004; Rangwala and Lazar, 2004; Hontecillas et al., 2009). PPAR-γ is expressed in adipose tissue, lower intestine, and cells involved in immunity (Ferré, 2004). The activation of PPAR-γ by unsaturated fatty acids or lipid-derived substrates induces the differentiation of preadipocytes into mature adipocytes (which not only serve as energy storage sites but also have important endocrine functions) and stimulates triglyceride storage. The PPAR-γ activation can stimulate the secretion of insulin-sensitizing hormones by adipocytes, such as adiponectin, promoting glucose utilization and potentially reducing the need for excess energy storage in the form of fat (Ferré, 2004).

Despite the scarcity of studies on the effects of PSO and PA in poultry, a recent study also observed a linear reduction in FI and BWG of quail fed with increasing dietary levels (0, 100, and 200 mg/kg) of PSO (Sarmiento-García et al., 2023). The reasons were not further explored; however, the authors suggested that tannins present in PSO may have been responsible for the reduction in feed intake, consequently decreasing the overall quail performance (Sarmiento-García et al.,

2023). Although the actual mechanisms of action are unclear, in the present study, the PSO supplementation might have increased the fat and glucose metabolism and regulated energy intake in broilers, resulting in lower growth performance, which was more evident in treatments with high PSO inclusion.

In the current study, we observed that the NC treatment caused higher litter moisture than the PA 2% treatment. In addition, NC birds had the highest scores for FPD compared to the other treatments. FPD is characterized by inflammation and necrotic lesions, ranging from superficial to deep on the plantar surface of the footpads and toes (Greene et al., 1985; Mayne, 2005). Wet litter is the most common factor associated with FPD in broilers (Shepherd and Fairchild, 2010). Environmental and housing conditions, litter material, increased water consumption, stocking density, drinker leaks, nutrition, and certain diseases can influence litter moisture (McIlroy et al., 1987; Cengiz et al., 2011; van der Hoeven-Hangoor et al., 2013). In the current experiment, the only different factor among the treatments was the diet, which could have impacted the way birds faced the experimental NE challenge, possibly resulting in different levels of enteric disturbances or dysbiosis. Intestinal dysbiosis caused by diet or diseases can increase litter moisture and is an important risk factor associated with FPD in broilers (Teirlynck et al., 2011; Dunlop et al., 2016). Although it would make sense that NC birds had higher intestinal disturbances and higher litter moisture due to the lack of medications or supplementation of the tested product in the diet to alleviate the challenge, we observed that this group had similar intestinal NE gross lesions and performance than PC birds. In contrast, the PA 2% birds had higher NE gross lesion scores compared to PC birds, which was expected to cause lesser nutrient absorption, higher dysbiosis, and higher excretion. However, the PA 2% group resulted in lower litter moisture compared to NC and PC (pairwise comparison: $P < 0.01$ and $P = 0.01$, respectively). It is possible that the significant

reduction in feed intake by birds from this group also caused lower water intake since these two parameters are positively correlated (Manning et al., 2007; Aggrey et al., 2023), resulting in lower litter moisture.

On the other hand, the increase in litter moisture and FPD lesions in NC birds might be associated with the NE challenge. Even though we did not observe pronounced NE gross lesions in the intestines of these birds or a decrease in performance, the challenge could have caused mild intestinal dysbiosis. Some signs of enteric disturbance were observed in the checks conducted at 4-hour intervals during the 24-hour feed withdrawal at 18 d. During this period, we observed diarrhea and bloody feces, particularly in the pens of NC birds (Figure 4.1). These are some of the common signs observed in cases of enteric disturbances or diseases such as coccidiosis and NE (Williams, 2005). Intestinal lesions caused by the *Eimeria* parasites and *C. perfringens* (to a lesser extent) in this study could have caused hemorrhage of the lamina propria and submucosa, causing the appearance of blood in the feces (Songer, 1996) and reduced digestion and absorption capacity, resulting in lower passage rate and diarrhea (Williams, 2005). In addition, the fasting caused by 24-hour feed withdrawal at 18 d could have caused pronounced sloughing and excretion of the mucus layers in NC birds as an attempt to prevent the attachment and translocation of pathogens (Thompson and Applegate, 2006). The prolonged adhesion of fecal material and wet litter in the footpads can irritate and result in the deterioration of the epidermis and keratin layers over time (Shepherd and Fairchild, 2010). Therefore, a possibly greater intestinal dysbiosis in the NC birds may have led to higher litter moisture and FPD incidence (Figure 4.2) than in birds from other groups. However, it seems that this dysbiosis was mild enough that significant losses in performance were not observed. Similar results were previously observed in Chapters 2 and 3, where the same challenge model did not result in clear reductions in performance or NE intestinal

gross lesions between NC and PC groups. However, microscopically, birds from the NC group were more vulnerable to *Eimeria* infection, leading to histopathological changes in the intestinal morphology.

Although the reasons are not fully understood, this study showed that PSO was not a suitable source of PA (for doses higher than 0.5%) to be used in broiler diets. In the study conditions, increasing doses of this product did not protect the gut health or maintain performance in broilers challenged with subclinical NE. If the high susceptibility of PSO to oxidation was the main problem associated with reductions in performance, it could be solved by the addition of antioxidants in the diet or the use of antioxidants to stabilize the oil. However, if the PSO can affect the fat metabolism and hormones involved in the control of feed intake and body energy balance, it would be worth investigating if this is related to the PSO properties or PA itself. For this, it would be necessary to test other sources of PA to avoid potential confounding effects of the PSO. Moreover, the PSO, the most abundant natural source of PA, is expensive and unsuitable for large-scale feeding (Holic et al., 2018). Thus, efforts are ongoing to generate a biotechnological platform for PA production through the metabolic engineering of plants and microorganisms (Wang et al., 2021), which would facilitate the inclusion of PA in broiler diets in case the product show effectiveness to be used as an AGP replacement.

In conclusion, adjustments to the natural, subclinical challenge model used in this study are required in order to provide a more robust challenge and accurately test AGP replacements in broiler diets. PSO used to achieve PA concentrations higher than 0.5% of the diet caused reductions in broiler performance, and the highest PA inclusion was associated with pronounced NE gross lesions in the jejunum. The NC group resulted in higher litter moisture and FPD, likely due to enhanced intestinal dysbiosis caused by the challenge in birds from this group. Therefore,

in the current study conditions, PA supplemented through PSO did not protect gut health or maintain the performance of broilers challenged with a natural, subclinical NE infection model. It may be useful to explore alternative sources of PA to avoid potential confounding effects associated with PSO properties and to determine if the negative effects on performance are related to PSO or PA itself.

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4.6 TABLES

Table 4.1. Ingredient and calculated nutrient composition of the Control diets provided to birds during the starter, grower, and finisher phases.

Ingredients (%)	**Starter (Day 0-10)**	**Grower (Day 11-25)**	**Finisher (Day 26-41)**
Canola meal	7.50	10.00	12.00
Soybean meal	29.02	22.76	19.43
Wheat	53.19	59.69	60.84
Calcium carbonate	1.16	1.03	0.92
Monocalcium phosphate	1.04	0.75	0.58
NaCl	0.27	0.26	0.26
L-lysine	0.09	0.07	0.02
DL-methionine	0.30	0.25	0.23
L-threonine	0.05	0.01	---
Hy-D® premix[1]	0.05	0.05	0.05
Vitamin mineral premix[2]	0.5	0.5	0.5
Choline chloride premix[3]	0.05	0.05	0.05
Phytase[4]	0.01	0.01	0.01
Canola oil[5]	3.64	3.64	4.80
Mycotoxin binder[6]	0.15	0.15	0.15
Xylanase[7]	0.05	0.05	0.05
Sand	2.82	0.62	---
Calculated nutrient composition			
Crude protein (%)	24.88	23.50	22.69
ME (kcal/kg)	3,000	3,100	3,200
Calcium (%)	0.96	0.87	0.81
Available phosphorus (%)	0.480	0.435	0.405
Digestible lysine (%)	1.28	1.15	1.06
Digestible methionine (%)	0.61	0.55	0.52
Digestible sulfur amino acids (%)	0.95	0.87	0.83

[1]Provided 69 µg 25-hydroxycholecalciferol per kg diet (DSM Nutritional Products Inc., Parsippany, New Jersey).

[2]Provided per kilogram of diet: vitamin A (retinyl acetate), 10,000 IU; cholecalciferol, 4,000 IU; vitamin E (DL-α-tocopheryl acetate), 50 IU; vitamin K, 4.0 mg; thiamine mononitrate (B_1), 4.0 mg; riboflavin (B_2), 10 mg; pyridoxine HCL (B_6), 5.0 mg; vitamin B_{12} (cobalamin), 0.02 mg; D-pantothenic acid, 15 mg; folic acid, 0.2 mg; niacin, 65 mg; biotin, 1.65 mg; iodine (ethylenediamine dihydroiodide), 1.65 mg; Mn (as $MnSO_4 \cdot H_2O$) , 120 mg; Cu, 20 mg; Zn, 100 mg, Se, 0.3 mg; Fe (as $FeSO_4 \cdot 7H_2O$), 800 mg.

[3]Provided 100 mg choline per kg of diet.

[4]Provided 500 FTU phytase/kg diet (Phyzyme XP, Danisco Animal Nutrition, Marlborough, UK).
[5]Canola oil was partially replaced by pomegranate seed oil in the diets containing 0.1, 0.25, 0.5 and 1.5% punicic acid and fully replaced in the diet containing 2% punicic acid (see Table 2 for details).
[6]Biomin II (Biomin Canada Inc., Mont-St-Hilaire, Québec, Canada).
[7]Econase XT 25 (AB Vista, Marlborough, UK) provided 80,000 BXU of endo-1, 4-beta-xylanase activity per kg diet.

Table 4.2. The percentage of pomegranate seed oil (PSO), canola oil, and total oil inclusion (%) in each of the experimental diets provided to the broilers in the starter (0-10 d), grower (11-25 d), and finisher (26-41 d) phases.

Treatments	--------- Starter (0-10 d) --------			---- Grower (11-25 d) ----------			------- Finisher (26-41 d) --------		
	PSO (%)	Canola oil (%)	Total oil inclusion (%)	PSO (%)	Canola oil (%)	Total oil inclusion (%)	PSO (%)	Canola oil (%)	Total oil inclusion (%)
Positive Control[1]	---	3.64	3.64	---	3.64	3.64	---	4.80	4.80
Negative Control[2]	---	3.64	3.64	---	3.64	3.64	---	4.80	4.80
PA 0.1%	0.18	3.46	3.64	0.18	3.46	3.64	0.18	4.62	4.80
PA 0.25%	0.45	3.19	3.64	0.45	3.19	3.64	0.45	4.35	4.80
PA 0.5%	0.91	2.73	3.64	0.91	2.73	3.64	0.91	3.89	4.80
PA 1%	1.82	1.82	3.64	1.82	1.82	3.64	1.82	2.98	4.80
PA 1.5%	2.73	0.91	3.64	2.73	0.91	3.64	2.73	2.07	4.80
PA 2%	3.64	---	3.64	3.64	---	3.64	3.64	1.16	4.80

Abbreviations: PSO = pomegranate seed oil; PA = punicic acid; 0.1, 0.25, 0.5, 1.5 and 2% represent the level of dietary punicic acid.

[1]Positive Control: Basal diet with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2]Negative Control: Basal diet without antibiotic and coccidiostat.

Table 4.3. Percentage of punicic acid recovered from the complete feed provided to the broilers in the starter, grower, and finisher phases.

Diet	PA recovery (% of the diet)		
	Starter (0-10 d)	**Grower (11-25 d)**	**Finisher (26-41 d)**
Positive Control[1]	---	---	---
Negative Control[2]	---	---	---
PA 0.1%	0.07	0.09	0.09
PA 0.25%	0.21	0.26	0.24
PA 0.5%	0.42	0.53	0.56
PA 1%	0.94	1.11	1.16
PA 1.5%	1.42	1.74	1.74
PA 2%	1.98	2.39	2.91

Abbreviations: PA = punicic acid; 0.1, 0.25, 0.5, 1.5 and 2% represent the dose of punicic acid dose included in the diet.

[1]Positive Control: Basal diet with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2]Negative Control: Basal diet without antibiotic and coccidiostat.

Table 4.4. The performance of broilers in the starter (0-10 d), grower (11-25 d), and finisher (26-41 d) phases fed varying levels of dietary punicic acid in the diet.

	Body Weight			Body Weight Gain			Feed Intake			Feed Conversion		
Age (d)	**10**	**25**	**41**	**0-10**	**11-25**	**26-41**	**0-10**	**11-25**	**26-41**	**0-10**	**11-25**	**26-41**
Treatments	**----------g----------**			**----------g/bird/d----------**						**-------g feed: g gain------**		
Positive Control[1]	223.1^{BC}	$1{,}006.3^{AB}$	2,439.2	19.1^{B}	51.2^{A}	99.8	24.6	80.2^{A}	160.8	1.29^{BC}	1.56^{CD}	1.68
Negative Control[2]	235.7^{A}	$1{,}003.4^{AB}$	2,475.1	20.4^{A}	50.3^{AB}	88.5	25.3	78.1^{AB}	161.6	1.24^{C}	1.55^{D}	1.84
PA 0.1%	234.9^{A}	$1{,}011.4^{A}$	2,475.0	20.1^{AB}	50.7^{AB}	91.1	24.8	80.3^{A}	163.3	1.24^{C}	1.58^{BCD}	1.80
PA 0.25%	226.8^{AB}	986.0^{ABC}	2,427.1	19.5^{AB}	49.7^{AB}	88.5	25.4	80.3^{A}	160.4	1.31^{BC}	1.61^{ABC}	1.82
PA 0.5%	226.1^{AB}	976.2^{BC}	2,448.2	19.1^{B}	49.1^{BC}	90.9	24.8	80.2^{A}	169.8	1.30^{BC}	1.63^{AB}	1.88
PA 1%	213.7^{C}	925.7^{D}	2,403.6	18.0^{C}	47.0^{D}	92.0	23.9	76.1^{B}	161.4	1.33^{B}	1.62^{ABC}	1.76
PA 1.5%	224.9^{B}	951.4^{CD}	2,443.4	19.3^{B}	47.4^{CD}	92.3	24.9	78.6^{AB}	163.2	1.29^{BC}	1.66^{A}	1.77
PA 2%	197.0^{D}	886.8^{E}	2,294.7	16.4^{D}	44.1^{E}	87.4	23.8	71.8^{C}	154.6	1.45^{A}	1.63^{AB}	1.77
SEM	1.88	6.64	16.19	0.19	0.36	1.38	0.19	0.46	1.36	0.01	0.01	0.02
P-value[3]	<0.01	<0.01	0.12	<0.01	<0.01	0.45	0.32	<0.01	0.31	<0.01	<0.01	0.34
P-linear	0.75	0.17	0.96	0.86	0.09	0.34	0.95	0.94	0.92	0.90	0.01	0.30
P-quadratic[4]	<0.01	<0.01	<0.01	<0.01	<0.01	0.55	0.06	<0.01	0.22	<0.01	0.01	0.26

Abbreviations: PA = punicic acid; 0.1, 0.25, 0.5, 1.5 and 2% represent the dose of punicic acid dose included in the diet.

[1]Positive Control: Basal diet with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2]Negative Control: Basal diet without antibiotic and coccidiostat.

[3]P-value for One-way ANOVA.

[A, B, C, D, E] Means with different superscripts within the same column are significantly different $P < 0.01$ (ANOVA).

[4]Quadratic response: BW at 10 d = 235.404 - 22.2692*Dose + 1.43806*Dose^2; $R^2 = 0.61$.

[4]Quadratic response: BW at 25 d= 1007.36 - 72.5024*Dose + 4.58496*Dose^2; $R^2 = 0.57$.

[4]Quadratic response: BW at 41 = 2478.71 – 99.4434*Dose + 6.47212*Dose^2; $R^2 = 0.14$

[4]Quadratic response: BWG 0-10d = 20.2473 - 2.24849*Dose + 0.145585*Dose^2; $R^2 = 0.60$.

[4]Quadratic response: BWG 11-25d = 50.7201 - 3.77692*Dose + 0.237185*Dose^2; $R^2 = 0.56$.

[4]Quadratic response: FCR 0-10d = 1.24257 + 0.116367*Dose - 0.00753789*Dose^2; $R^2 = 0.32$.

[4]Quadratic response: FCR 11-25d = 1.58793 + 0.0304494*Dose - 0.00171041*Dose^2; $R^2 = 0.13$.

[4]Quadratic response: FI 11-25d = 80.5018 - 4.62687*Dose + 0.300135*Dose^2; $R^2 = 0.47$.

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 4.5. The performance of broilers during the entire experimental period (0 to 41d) fed varying levels of dietary punicic acid in the diet.

Treatments	Body Weight Gain (g/bird/d)	Feed Intake (g/bird/d)	Feed Conversion (g feed: g gain)
Positive Control[1]	52.1	94.0[a]	1.81
Negative Control[2]	50.8	92.2[a]	1.82
PA 0.1%	51.8	93.4[a]	1.80
PA 0.25%	50.7	92.2[a]	1.82
PA 0.5%	50.7	95.7[a]	1.88
PA 1%	50.7	91.9[a]	1.81
PA 1.5%	51.0	93.4[a]	1.83
PA 2%	48.0	85.5[b]	1.78
SEM	0.41	0.63	0.01
P-value[3]	0.36	0.03	0.75
P-linear	0.94	0.69	0.83
P-quadratic	0.08	<0.01	0.63

Abbreviations: PA = punicic acid; 0.1, 0.25, 0.5, 1.5 and 2% represent the dose of punicic acid dose included in the diet; BWG = Average body weight gain per day per bird; FI = Average feed intake per day per bird; FCR = Feed conversion ratio.

[1]Positive Control: Basal diet with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2]Negative Control: Basal diet without antibiotic and coccidiostat.

[3]P-value for One-way ANOVA.

[a,b] Means with different superscripts within the same column are significantly different $P \leq 0.05$ (ANOVA).

Quadratic response: FI 0-41d = 94.1811 – 4.04764*Dose + 0.266401*Dose^2; $R^2 = 0.18$

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 4.6. Evaluation of necrotic enteritis gross lesion scores in the jejunum of broilers at 22 d and 40 d of age, fed varying levels of dietary punicic acid in the diet.

Treatments	Sample size	Necrotic enteritis gross lesion scores[4]	
		22 d	40 d
Positive control[1]	16	0.12[b]	0.93
Negative control[2]	16	0.31[ab]	1.06
PA 0.1%	16	0.37[ab]	0.94
PA 0.25%	16	0.31[ab]	1.12
PA 0.5%	16	0.50[ab]	1.06
PA 1%	16	0.25[ab]	1.12
PA 1.5%	16	0.37[ab]	1.00
PA 2%	16	0.69[a]	1.44
SEM		0.04	0.04
P-value[3]		0.04	0.07

Abbreviations: PA (punicic acid); 0.1, 0.25, 0.5, 1.5 and 2% represent the punicic acid dose of inclusion in the diet.

[1]Positive Control: Basal diet with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2]Negative Control: Basal diet without antibiotic and coccidiostat.

[3]P-value for Kruskal-Wallis One-Way Nonparametric AOV test.

[4]Scoring system used: Score 0 = No gross lesions detected; Score 1 = No ulcers, but the mucosal surface is covered with fibrin; Score 2 = Presence of an excavated ulcer with acute hemorrhage and scant crusting of fibrin around the periphery; Score 3 = Excavated ulcers of the mucosae, the periphery of which are covered by tightly-adherent layers of fibrin, necrotic tissue and inflammatory cells; Score 4 = The mucosae are covered by thick layers of fibrin, necrotic tissue, and inflammatory cells that spread widely over the intestinal mucosa.

[a,b] Means with different superscripts within the same column are significantly different $P \leq 0.05$.

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

Table 4.7. Litter moisture and footpad dermatitis scores in the right and left foot of broilers at 41 d fed varying levels of dietary punicic acid in the diet.

Treatments	Litter Moisture (%)	Footpad dermatitis score[3]	
		Right foot	Left foot
Positive Control[1]	33.87^{AB}	0.12^{B}	0.15^{B}
Negative Control[2]	41.23^{A}	0.96^{A}	0.86^{A}
PA 0.1%	37.41^{AB}	0.32^{B}	0.20^{B}
PA 0.25%	36.59^{AB}	0.30^{B}	0.29^{AB}
PA 0.5%	36.37^{AB}	0.32^{B}	0.26^{B}
PA 1%	35.66^{AB}	0.15^{B}	0.17^{B}
PA 1.5%	38.42^{AB}	0.23^{B}	0.26^{AB}
PA 2%	31.97^{B}	0.18^{B}	0.18^{B}
SEM	0.64	0.03	0.02
P-value	$0.01^{\dagger}$	$<0.01^{\ddagger}$	$<0.01^{\ddagger}$

Abbreviations: PA (punicic acid); 0.1, 0.25, 0.5, 1.5 and 2% represent the punicic acid dose of inclusion in the diet.

[1]Positive Control: Basal diet with antibiotic (Bacitracin methylene disalicylate, Zoetis Canada Inc., QC, Canada at 0.05% of the diet) and coccidiostat (Monensin, Bio Agri Mix, ON, Canada at 0.05% of the diet).

[2]Negative Control: Basal diet without antibiotic and coccidiostat.

[3]Scoring system used: Score 0 = No evidence of footpad dermatitis; Score 1 and 2 = Minimal evidence of footpad dermatitis (skin discoloration); Score 3 = Clear evidence of lesions in the entire central pad; Score 4 = There are lesions in the entire central pad and also on toes.

[†]P-value for One-Way ANOVA parametric test.

[‡]P-value for Kruskal-Wallis One-Way Nonparametric AOV test.

[A, B] Means with different superscripts within the same row are significantly different $P < 0.01$.

Note: All birds, regardless of the treatment, were challenged with a natural, subclinical necrotic enteritis model of infection (oral 15X coccidiosis vaccine dose at 12 d and a 24-hour feed removal at 18 d).

4.7 FIGURES

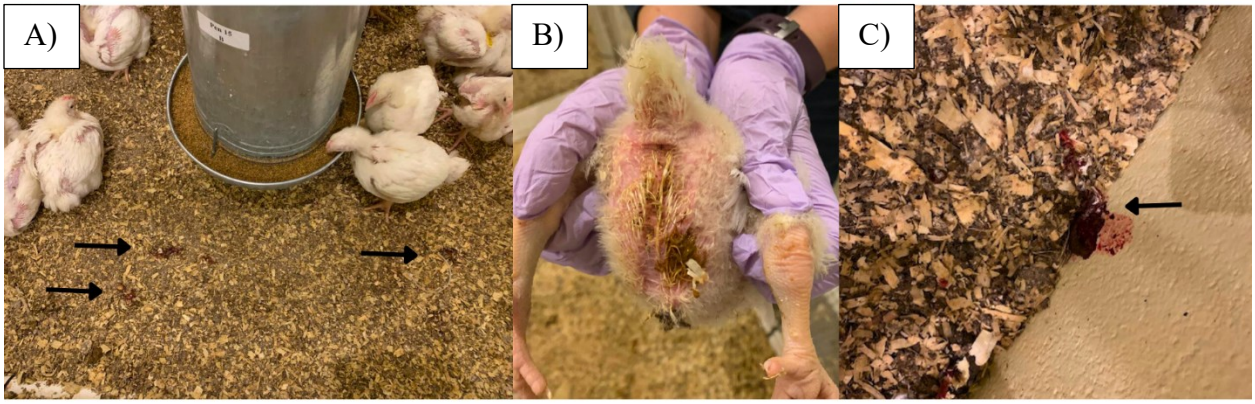

Figure 4.1. Bloody and sticky feces observed in different pens and on birds from the NC group during the 24-hour feed withdrawal at 18 d. A) bloody feces observed at 3 different points in the same pen. B) bird with sticky feces adhered to the feathers. C) presence of bloody feces.

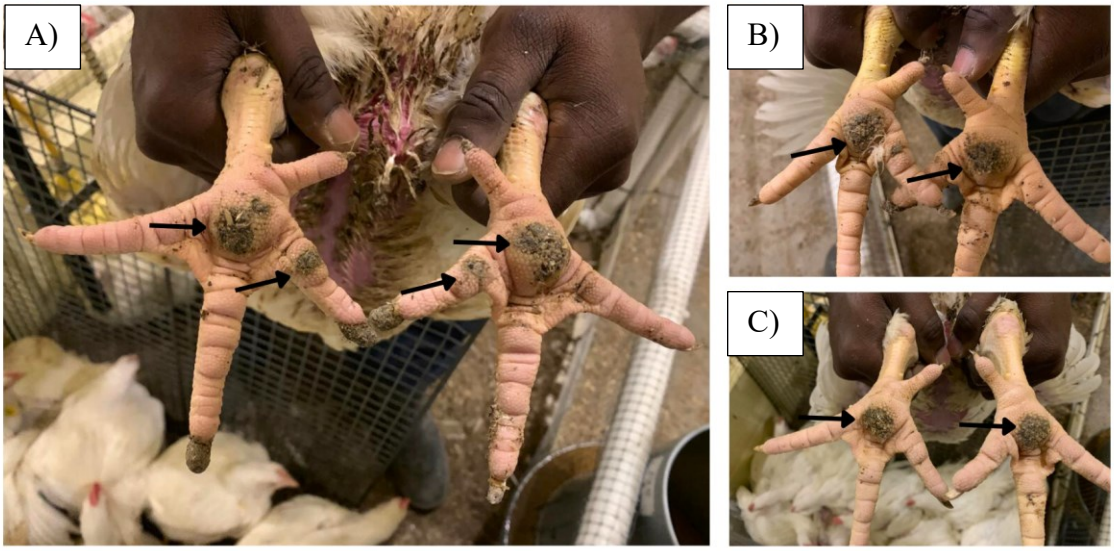

Figure 4.2. Footpad dermatitis (FPD) observed in the paws of some birds from the NC group at 41 d. Image A) represents a score of 3, in which it is possible to note lesions in the entire central and small lesions on the toes. In this image, it is also possible to observe feces stuck to the bird's feathers. B) represents a score of 3 for FPD, with clear evidence of lesions in the entire central pad, and C) represents a score of 4 for FPD, with clear evidence of deep ulcerations covering more than 50% of the central pad.

5. EFFECT OF BIOACTIVE GLUCOSAMINE-DERIVED CARAMELS ON PERFORMANCE, GAIT SCORES, BONE HEALTH AND CECAL CONCENTRATION OF SHORT-CHAIN FATTY ACIDS IN BROILERS RAISED IN WIRE-FLOOR CAGES

OVERVIEW

Bacterial chondronecrosis with osteomyelitis (**BCO**) is the most common cause of lameness in commercial broilers and can be precipitated by disruptions in intestinal integrity. Therefore, this study aimed to evaluate the potential of glucosamine-derived caramels to enhance performance and mitigate BCO in broilers. A total of 1,120 broilers were randomly assigned to 14 treatments with 8 replicate cages of 10 birds each. The treatments were a Control (commercial-type diet), Control plus glucosamine at 0.24% of the diet (**GlcN**), and the Control diet supplemented with glucosamine-derived caramels: either Light Caramel (**LC**), Brown Caramel (**BC**), LC plus caramelized fructose (**LC+F**), or BC plus caramelized fructose (**BC+F**); each product was added at 0.08, 0.16 or 0.24% of the diet. BW, feed intake (**FI**), body weight gain (**BWG**), and feed conversion ratio (**FCR**) were measured at 10, 25, and 38 d of age. Gait scoring was measured at 11, 26 and 39 d, while tibial and femoral BCO gross lesion scores were evaluated at 12, 27 and 40 d. At 10 d, birds fed LC at 0.16, 0.24% and LC+F at 0.24% were heavier and had higher BWG than those fed LC+F at 0.08%. At 25 d, birds in the LC at 0.16% group were heavier than those in the LC + F at 0.08%. At 38 d, this same group had heavier birds and higher BWG than those in the LC+F at 0.08%, BC+F and BC at 0.16% groups. In a pairwise comparison with the Control, birds in the LC at 0.24% group were heavier at 10 d and tended to have greater BWG during the starter phase. Regardless of the dietary dose, the LC group had higher FI at the starter phase than birds from the Control. Birds in the LC at 0.16% group tended to be heavier at 25 and 38 d and have higher BWG in the entire period (0 to 38 d) than the Control. Birds fed BC+F, regardless of

the inclusion dose, had lower gait defects than the Control group. Birds fed LC and LC+F at 0.08% had less severe femoral lesions than those in the LC+F, BC+F, and BC all included at 0.24%. In a pairwise comparisons, birds fed BC, BC+F, and LC, regardless of the inclusion dose, had lower BCO lesions in the tibia than those in the Control group. LC treatments showed promise in enhancing broiler performance while reducing BCO lesions possibly caused by translocated bacteria. Further research is needed to elucidate the mechanism of action of glucosamine caramel products involved in enhancing the performance and health of broilers.

Keywords: Broiler chicken, glucosamine-derived caramels, locomotor problems, performance, welfare.

5.1 INTRODUCTION

Leg disorders and lameness are among the most important issues in modern meat-type poultry production. These conditions significantly impact the well-being and performance of poultry, contributing to heightened morbidity and mortality rates in broiler flocks (Wideman et al., 2012). An important locomotor issue in broilers is bacterial chondronecrosis with osteomyelitis (**BCO**), characterized by a bacterial infection that causes the necrotic degeneration of articular bones. The most commonly affected bones are the proximal femora, proximal tibiae, and free thoracic vertebra (Wideman, 2016). The rapid growth and large body weight in modern broilers can induce excessive torque and shear stress on structurally immature bone cartilage. This stress, in turn, can result in microfractures and clefts within the epiphyseal and physeal cartilage of susceptible bones. These microfractures and clefts expose the collagenous matrix, which facilitates adhesion and infection by opportunistic bacteria. These bacteria can enter the blood via translocation from the respiratory system or gastrointestinal tract and spread hematogenously to these vulnerable sites. These sites provide limited access to immune cells but an abundance of substrate, so they are

perfect niches for bacterial proliferation (Rojas-Núñez et al., 2020). As a result, opportunistic bacteria rapidly proliferate and start to release toxins, causing abscesses and necrotic voids, ultimately leading to BCO (Wideman et al., 2012; Wideman, 2016). Among the bacteria isolated from BCO cases, the most common are *Escherichia coli*, *Staphylococcus aureus*, *S. agnetis*, *Enterococcus cecorum*, and *Salmonella enteritidis* (Wideman et al., 2012, 2015; Ekesi et al., 2021). As these bacteria are common members of the intestinal microbiota, it suggests that the translocation of bacteria from the gut to the joints due to failures in the tight junctional complexes is highly associated with the pathogenesis of BCO in broilers (Wideman et al., 2015; Rojas-Núñez et al., 2020). Therefore, maintaining the intestinal health of broilers is a key factor in preventing BCO lameness. As antibiotic growth promoters are being phased out of poultry diets, there is a growing interest in natural alternatives with bioactive properties capable of enhancing poultry gut health. Feed additives with this potential (e.g., prebiotics, probiotics, phytogenics) can potentially prevent the translocation of bacteria from the intestinal lumen to the bloodstream, which, in turn, can prevent BCO in broilers (Wideman et al., 2012, 2015; Yan et al., 2019; Alrubaye et al., 2020a; b).

Glucosamine (2-amino-2-deoxy-D-glucose) is an amino monosaccharide produced endogenously from glucose and utilized for the biosynthesis of glycoproteins and glycosaminoglycans, which are highly concentrated in connective tissues and cartilages (Salazar et al., 2014). Glucosamine can also be exogenously supplemented as sulfate, hydrochloride, *N*-acetylglucosamine, chlorohydrate salt, or as a dextrorotatory isomer (Dahmer and Schiller, 2008). Although its effectiveness is controversial, glucosamine is commonly used as a dietary supplement to prevent and relieve osteoarthritis and articular joint disease (Wu et al., 2013; Ogata et al., 2018; Conrozier and Lohse, 2022). Although in-feed supplementation of glucosamine has the potential to mitigate

locomotor problems in broilers, the application of a mild thermal process on glucosamine (i.e., caramelization) generates new pharmacological compounds (Hrynets et al., 2015, 2016) with a possibly stronger potential to alleviate joint inflammation. Among these glucosamine-derived compounds, fructosazine (**FR**) and deoxyfructosazine (**DOFR**) have gained attention due to their capacity to inhibit inflammatory mediators such as interleukins-2 and -1β and reduce damage to proteoglycans and collagen in the connective tissue of joints (Giordani et al., 2006; Zhu et al., 2007). These can, in turn, prevent pathological cartilage degradation and inflammatory diseases. Furthermore, the caramelization of glucosamine produces melanoidins, which have prebiotic properties and serve as a substrate for bifidogenic bacteria in the gut (Borrelli and Fogliano, 2005; Wu et al., 2020). These beneficial bacteria may mitigate joint inflammation indirectly by promoting gut health. To the best of our knowledge, no studies have been conducted evaluating the potential of glucosamine-derived caramels to alleviate locomotor problems in broilers. Therefore, this study aimed to evaluate the effects of glucosamine-derived caramels on growth performance, femoral and tibial BCO gross lesions, gait scores, and cecal concentrations of short-chain fatty acids (**SCFA**) in broilers raised in wire flooring cages. We hypothesized that glucosamine-derived caramels would mitigate tibial and femoral BCO lesions and enhance broiler performance.

5.2 MATERIALS AND METHODS

The animal care procedures were approved by the Animal Care and Use Committee for Livestock of the University of Alberta (AUP 00003587) and followed principles established by the Canadian Council on Animal Care (CCAC, 2009).

5.2.1 Glucosamine-derived caramels

D-glucosamine hydrochloride (≥ 99%; Sigma-Aldrich, St. Louis, MO, USA) was the starting material for generating the glucosamine-derived caramels. The glucosamine (15% w/w) was dissolved in water, and the pH adjusted to 7.0 ± 0.01 using 50% NaOH. Subsequently, the solution underwent a 12-hour incubation period at either 50°C or 90°C in the presence of oxygen and continuous stirring. After 12-hour reaction, the different caramel solutions were spray-dried using a pilot scale Anhydro spray dryer pilot 55 (SPX FLOW, Charlotte, NC, USA) with an inlet/outlet temperature of 140°C/70°C. The products derived from the incubation of glucosamine at 50°C underwent ultrafiltration with a molecular weight cut-off of 1 kDa to concentrate FR and DOFR (with molecular weights of 320 and 302 Da, respectively). This resulted in the production of decolourized caramel permeate (light caramel; **LC**). The same procedure was replicated for the glucosamine incubated at 90°C. However, in this instance, the retentate resulting from ultrafiltration was chosen due to its richness in melanoidins. This led to the creation of a brown caramel (**BC**).

Some of the treatments tested the combination of glucosamine- and fructose-derived caramels. To produce these caramel samples, all the procedures described above were the same, except for the dilution of D-fructose (1:1 molar ratio; ≥ 99%; Sigma-Aldrich, St. Louis, MO, USA) along with D-glucosamine hydrochloride in water at the beginning of the process.

The identification of FR and DOFR in the caramels was verified by mass spectrometry, and their concentration was determined by reversed-phase high-performance liquid chromatography (Hrynets et al., 2016). The melanoidins with different molecular weights present in BC were isolated and chemically characterized (Gniechwitz et al., 2008). Briefly, an ultrafiltration system using membrane cut-offs of 3, 10 and 20 kDa was applied to the BC, resulting in three fractions

of the following ranges: 3 to 10, 10 to 20 and > 20 kDa. All fractions were freeze-dried and then subjected to Sephadex LH-20 chromatography and Octyl Sepharose chromatography to isolate the melanoidins. Water and 0.5 M NaCl were used as eluents during chromatography steps. After desalting, the isolated melanoidins from the three different fractions were analyzed using Fourier Transform Infrared or Nuclear Magnetic Resonance spectroscopy to confirm the chemical structure of the compounds.

5.2.2 Animals and housing

A total of 1,120 one-day-old Ross 708 male broilers, with similar weight (38.05 ± 0.12 g), were obtained from a commercial hatchery and randomly assigned to 14 treatments with 8 replicates of 10 birds each. The animals were individually weighed and identified by neck tags. The chicks were placed in Specht pullet cages (1.2 m [length] × 0.53 m [width] × 0.43 m [height]; Specht-Ten Elsen GmbH, Sonsbeck, Germany) equipped with nipple drinkers, galvanized feeder trough, and metal wire mesh (2 × 2 cm) flooring. Initially, chick paper and flat plastic mesh (1 × 1 cm) mats covering the entire floor area of each cage were laid on top of the metal flooring to prevent chicks' feet from going through but were removed on day 10. Temperature and lighting programs followed commercial practices recommended by Ross 708 commercial management guidelines (Aviagen, 2018) and were the same as detailed in Chapters 2, 3 and 4.

5.2.3 Experimental diets

Birds were fed a wheat-soy-canola meal basal diet formulated to meet or exceed Ross 708 nutritional recommendations (Aviagen, 2019) according to each growth phase (Table 5.1). The experimental treatments consisted of a Control diet (basal commercial-type diet), Control plus glucosamine (**GlcN;** without thermal process) at 0.24% of the diet, and four glucosamine-derived caramels treatments: Control plus BC, BC plus caramelized fructose (**BC+F**), Control plus LC,

and LC plus caramelized fructose (**LC+F**), each included at 0.08%, 0.16%, and 0.24% of the diet. For each dietary phase, the basal diet was mixed as a single batch, and subdivided for addition of the respective products at the specified amounts. Diets were fed in mash form over the 40-day growth cycle; birds had free access to feed and water throughout the trial.

5.2.4 Broiler performance

Feed intake (**FI**), average BW, average BW gain (**BWG**), and feed conversion ratio (**FCR**) were measured on a pen basis at the end of the starter (10 d), grower (25 d), and finisher (38) phases, and the entire experimental period (0 to 38 d) as described in the previous chapters.

5.2.5 Gait scoring

On days 11, 26 and 39, one bird per experimental unit was randomly selected for gait score evaluation. Walking ability was evaluated using the six-point gait scoring scale as described by Kestin et al. (1992). Briefly, birds were individually removed from their cages and then placed to walk on litter inside an arena (2.5 m [length] x 1.20 m [width] x 0.50 m [height]). Two trained assessors observed the walking ability of the birds and indicated a score for each of the birds. The final score for each bird was determined by consensus between the assessors on site. A score of 0 indicated a bird with no detectable gait abnormalities; 1 indicated a detectable but unidentifiable gross abnormality; 2 indicated an identifiable gross abnormality that had little effect on overall gait function; 3 indicated an identifiable abnormality that impaired walking function; 4 indicated severe impairment of function, but the bird was still capable of moving, and 5 indicated complete lameness (complete inability to walk).

5.2.6 Bacterial chondronecrosis with osteomyelitis gross lesion scoring

On days 12, 27 and 40, the same birds used for gait scoring were euthanized by cervical dislocation, and then the proximal femoral and tibial head of each leg were analyzed for gross BCO lesions.

Gross lesion scores were evaluated according to Wideman et al. (2012), where the stages of proximal femoral head degeneration ranged from 1 to 8, and the tibial head necrosis scores ranged from 1 to 10, with a higher number indicating a more severe lesion.

For femoral head necrosis, a score of 1 was assigned for birds with a normal proximal femoral head (no detectable lesions). A score of 2 for birds with visible femoral head separation (epiphysiolysis). Scores from 3 to 5 for birds with progressive necrosis, ulceration, and fracturing of the growth plate, respectively. Scores from 6 to 8 for birds with perforation, fracturing, and necrosis/osteomyelitis of the femoral head, respectively.

For the tibia assessment, firstly, the tibia head was carefully sliced longitudinally using a boning knife until it reached the bone's central region. Excess of blood was gently removed with a paper towel, and a score was assigned to each bird. A score of 1 was given for birds with a normal proximal tibial head with struts of trabecular bone in the metaphyseal zone fully supporting the growth plate. Scores from 2 to 5 were assigned for birds with necrotic voids in the metaphyseal zone, which undermined the support of the growth plate, leading to microfractures of the growth plate. Scores from 6 to 8 for birds with macroscopic evidence of osteomyelitis (bacterial infiltration). Scores 9 to 10 for birds with necrotic voids communicating with precocious ectopic extensions of the marrow cavity.

5.2.7 Cecal content sample collection

Simultaneously with the bone evaluation, digesta contents from both ceca of each bird were collected on days 27 and 40. The samples were placed in microcentrifuge tubes in liquid nitrogen during the sample collection, and then transferred to -80 °C freezers. The SCFA analysis was conducted to measure possible shifts in bacterial fermentation in the caeca content.

5.2.8 Short-chain fatty acids

Concentrations of SCFA were determined as previously described (Inglis et al., 2021). Briefly, cecal digesta was homogenized in phosphate buffered saline (pH 7.2) at a 1:1 ratio (w/v). One part meta-phosphoric acid (Sigma Aldrich, Oakville, ON, Canada) was added to the homogenate per four parts cecal digesta, and incubated at room temperature for 30 min. Samples were centrifuged at room temperature for 75 min at 16,100 × g, and the supernatants were collected and stored at -20°C. Concentrations of acetate, butyrate, propionate, caproate, isobutyrate, isovalerate, and valerate were quantified with a gas chromatograph (Agilent Technologies, Model 6890N with 7683 Series Injector; Agilent Technologies Canada Inc., Mississauga, ON, Canada) according to established protocols (Cottyn and Boucque, 1968; Playne, 1985). In addition, concentrations of total SCFA were determined (i.e., sum of individual SCFA).

5.2.9 Statistical analysis

Data were submitted to Bartlett's test to evaluate the homogeneity of variances, and Shapiro-Wilk test to verify the normality of residuals. After checking the normality, the performance data were analyzed by one-way ANOVA using Statistix 10 software (Analytical Software, Tallahassee, FL). Significant effects ($P < 0.05$) were further evaluated with Tukey's test for pairwise comparisons. Orthogonal contrasts were used to evaluate the performance effects of each group of compounds, regardless of the inclusion dose, in relation to the Control (i.e., Control vs BC, Control vs BC+F, Control vs LC and Control vs LC+F). In addition, each experimental treatment was also compared to the Control (i.e., Control vs BC at 0.08, 0.16 and 0.24%; Control vs BC+F at 0.08, 0.16 and 0.24%; Control vs LC at 0.08, 0.16 and 0.24% and Control vs LC+F at 0.08, 0.16 and 0.24%). As with gait scores and bone gross lesion scores data were not normally distributed, Kruskal-Wallis, a non-parametric statistical test, was used, and the statistical significance was considered at P <

0.05. Due to the non-normal distribution of gait score and bone lesion scores data, the pairwise comparison between the group of compounds and the Control, and each treatment and the Control were conducted using the Wilcoxon rank-sum test.

5.3 RESULTS

5.3.1 Growth performance

Birds fed LC at 0.16 or 0.24%, LC+F at 0.24% and BC+F at 0.24% of the diet were heavier at 10 d (228.08 ± 6.39 g, 232.82 ± 1.86 g, 227.64 ± 4.90 g and, 224.51 ± 5.90 g, respectively; $P < 0.01$) than those fed LC+F at 0.08% of the diet (195.79 ± 6.80 g; Table 5.2). However, they were not different from the other treatments. Birds fed LC at 0.16 or 0.24%, and LC+F at 0.24% had higher daily BWG (18.97 ± 0.62 g, 19.34 ± 0.20 g, 18.78 ± 0.50 g, respectively) from 0 to 10 d than those fed LC+F at 0.08% of the diet (15.78 ± 0.68 g; $P = 0.02$; Table 5.3). FCR was lower in birds that were fed LC at 0.16% or 0.24%, LC+F at 0.16% or 0.24%, BC+F at 0.24%, as well as in the GlcN and Control groups (1.34 ± 0.03 g, 1.31 ± 0.02 g, 1.35 ± 0.02 g, 1.34 ± 0.02 g, 1.33 ± 0.03 g, 1.33 ± 0.04 g, 1.35 ± 0.04 g, respectively), compared to those fed LC+F at 0.08% (1.51 ± 0.16 g; $P < 0.01$; Table 5.3).

By orthogonal contrast, birds from the LC group at 0.24% were 8% heavier ($P = 0.05$; Supplemental Table 5.1) at 10 d and tended to have higher BWG than those from the Control group ($P = 0.07$; Supplemental Table 5.2). Moreover, regardless of the inclusion level in the diet, birds fed LC had higher FI from 0 to 10 d than those from the Control group ($P = 0.04$; Table 5.3). Birds fed LC+F at 0.08% had higher FCR from 0 to 10 d than birds from the Control group ($P= 0.03$; Supplemental Table 5.2).

In the grower phase (11 to 25 d), birds fed LC at 0.16% were heavier (979.9 ± 16.7 g) than those fed LC+F at 0.08% of the diet (828.4 ± 37.3 g; Table 5.2). However, they were not different from

the other treatments. There were no treatment effects for BWG, FI or FCR during the grower phase ($P > 0.05$; Table 5.3).

No significant differences were found in the orthogonal contrast analyses during grower period. However, LC at 0.16% of the diet tended to result in heavier birds at 25 d ($P = 0.07$; Supplemental Table 5.1), higher BWG ($P = 0.09$) and FI ($P = 0.09$; Supplemental Table 5.2) than those fed the Control diet.

In the finisher phase, birds fed LC at 0.16% of the diet (2043.5 ± 45.7 g) were heavier at 38 d than those from the LC+F at 0.08% (1797.3 ± 51.8 g), LC at 0.08% (1885.1 ± 49.7 g), BC+F at 0.16% (1823.1 ± 52.1 g) and BC at 0.16% (1823.0 ± 66.7 g) groups ($P = 0.04$; Table 5.2). There were no treatment effects on BWG, FI nor FCR (Table 5.3). However, by orthogonal contrast, birds fed LC at 0.16% tended to be heavier at 38 d than those in the Control group ($P = 0.08$; Supplemental Table 5.1).

For the entire period (0 to 38 d), birds fed LC at 0.16% of the diet had a superior BWG (48.91 ± 1.1 g) to those fed LC+F at 0.08% (42.91 ± 1.2 g), LC at 0.08% (45.06 ± 1.2 g), BC+F at 0.16% (43.54 ± 1.3 g), or BC at 0.16% (43.55 ± 1.6 g; $P = 0.04$; Table 5.3). However, it was statistically similar to the other treatments. There were no treatment effects on FI ($P = 0.34$) or FCR ($P = 0.90$) during the entire experimental period.

No significant differences were found in the orthogonal contrast analyses during the entire period. However, birds fed LC at 0.16% of the diet tended to have a superior BWG ($P = 0.08$) to those in the Control group (Supplemental Table 5.2).

5.3.2 Mortality

There was no treatment effect on mortality ($P > 0.05$; data not shown). The mortality rate throughout the experiment period was 3.48% across all treatments. As a proportion of total

mortality, 72% of the animals were culled, and 28% were found dead (no specific reason detected). A total of 28 birds were culled, of which 17 were euthanized for locomotor problems, and the other 11 were culled for being underweight, weak, or failing to thrive.

5.3.3 Gait scoring

There was no treatment effect on gait scoring at 11 d (P = 0.61; Table 5.4). At 26 d of age, the treatment effect on gait score approached significance (P = 0.07). The GlcN group was responsible for the highest gait score (0.71 ± 0.26). At the same time, BC at 0.16 or 0.24, BC+F at any dose, and LC at 0.24% resulted in the lowest gait scores (all the evaluated birds received a score of 0). The contrast analysis revealed that birds fed BC+F, regardless of the dose of inclusion, had lower gait defects at 26 d than those from the Control group (P = 0.05; Table 5.4). Similarly, birds fed BC, regardless of the dose of inclusion, tended to have lower gait defects than Control birds (P = 0.09; Table 5.4).

At 39 d of age, birds fed BC at 0.24% of the diet had the highest gait scores (1.0 ± 0.5) compared to those fed the Control, GlcN, BC at 0.08 and 0.16%, BC+F at 0.16 and 0.24%, LC+F at 0.08, and LC at 0.24% of the diet (all the evaluated birds received a score of 0; Table 5.4). In a pairwise comparison between each of the experimental groups and the Control, we observed that birds from the BC at 0.24% group had higher gait scores than those in the Control group (Supplemental Table 5.3; P < 0.01).

5.3.4 Bacterial chondronecrosis with osteomyelitis gross lesion scores

There were no differences among treatment for femoral head gross lesion scores of broilers at 12 or 27 d of age (ANOVA P-value = 0.45 and 0.81, respectively; Table 5.5). However, the orthogonal contrast analysis showed that the GlcN group had more severe femoral lesions (1.75 ± 0.75) at 12 d than the Control (all birds from this group had a score of 1; P < 0.01; Table 5.5).

At 40 d of age, broilers fed BC at 0.24%, BC+F at 0.24%, or LC+F at 0.24% of the diet had higher femoral lesion scores (2.37 ± 0.50; 2.37 ± 0.37; 2.37 ± 0.46, respectively) than those fed LC+F or LC, each at 0.08% of the diet (all the evaluated birds in these two groups received a score of 1). No significant differences between the experimental groups and the Control on femoral lesions were detected by pairwise comparison ($P > 0.05$; Table 5.5 and Supplemental Table 5.4).

There were no treatment effects on tibial head gross lesion scores at 12, 27 or 40 d of age (ANOVA P-value = 0.73, 0.78, and 0.74, respectively; Table 5.5). However, when each individual treatment was compared to the Control, we observed that birds fed BC and BC+F (each at 0.08%), LC+F at 0.16%, and LC at 0.24% (scores of 2.87 ± 0.35; 3.00 ± 0.27; 3.12 ± 0.29, and 3.00 ± 0.42, respectively) had lower tibial lesion scores at 40 d than birds in the Control group (4.25 ± 0.45; Supplemental Table 5.4). Similarly, the contrast analysis revealed that birds fed BC, BC+F, and LC, regardless of the inclusion dose, had lower tibial lesions at 40 d than the Control group ($P = 0.02$, 0.03 and 0.05, respectively; Table 5.5). Additionally, birds fed LC+F, regardless of the inclusion dose, tended to have lower tibial lesions at 40 d than those fed the Control treatment ($P = 0.07$; Table 5.5).

5.3.5 Short-chain fatty acids

Dietary treatment did not affect the cecal concentration of total SCFA at 27 ($P = 0.99$) nor at 40 d of age ($P = 0.86$; Figure 5.1); similarly, there were no treatment effects at either age on the concentration of acetate, propionate, butyrate, caproate, isobutyrate, isovalerate and valerate ($P > 0.05$; data not shown).

5.4 DISCUSSION

Broilers reared on wire flooring are more prone to develop lameness than those raised on wood-shaving litter (Wideman et al., 2012). Some disorders associated with cage rearing include high

incidences of breast blisters, subclinical reductions in overall skeletal mineralization, reductions in bone strength, valgus-varus deformities, and lameness caused by slipped tendons (Wideman et al., 2012). The higher incidence of lameness is attributed to the lack of exercise coupled with the instability of footing in cage-rearing systems, which imposes additional torque and strain on the vulnerable leg joints of broilers. This can lead to osteochondrotic clefts and damaged or obstructed metaphyseal blood vessels, which are believed to create conditions conducive to infectious (BCO) and non-infectious forms of lameness (Wideman et al., 2012; Wideman and Prisby, 2013). In addition, wire flooring per se constitutes a significant stressor contributing to generalized immunosuppression and an increase in intestinal permeability. This, in turn, contributes to bacterial translocation from the lumen to the microfractures in the joints, leading to BCO (Wideman, 2016). Therefore, in the current study, broilers were reared in wire flooring cages as a model to induce lameness and, thus, create challenging conditions to test the efficacy of the glucosamine-derived caramels to enhance performance and mitigate gait defects and lesions in broiler femoral and tibial heads.

In the current study, we observed that among the glucosamine-derived caramel products tested, the LC showed promise in enhancing the performance of broilers. The LC at 0.24% tended to enhance BW at 10 d and BWG at the starter phase, while LC at 0.16% tended to enhance BW and BWG at the grower and entire experimental period (0 to 38 d) compared to the Control group. In addition, dietary supplementation of LC, regardless of the inclusion dose, also increased FI compared to birds fed the Control during the starter phase, while LC at 0.16%, specifically, tended ($P = 0.09$) to increase FI during the grower phase. These results may be related to the fact that treatments supplemented with LC were rich in FR and DOFR. These compounds belong to the non-volatile (polyhydroyalkyl)pyrazines and are produced through the self-condensation of glucosamine under

mild heat treatments (Henry et al., 2012). These pyrazines have primarily been recognized as flavouring agents and identified in roasted peanuts, caramel, and soy sauce (Henry et al., 2012). Although it has been argued that birds have a lower taste acuity than mammals due to their low taste bud numbers, recent studies based on genome sequencing showed that chickens have a well-developed taste system (Kudo et al., 2010; Roura et al., 2013; Niknafs and Roura, 2018). They can quickly adapt their feeding behaviour based on taste cues, and the ratio of the number of taste buds/oral cavity volume is higher than in most mammals (Roura et al., 2013). Thus, the increased feed intake in birds fed LC suggests that the flavour agents generated during the caramelization of glucosamine may have enhanced the taste of this diet, influencing birds to have superior feed consumption.

Besides being used as flavouring agents in the food and tobacco industries, FR and DOFR are gaining interest for their pharmaceutical properties and potential therapeutic use in humans. This includes their involvement in the potential treatment of type II diabetes, the prevention of atherosclerosis, pathological cartilage degradation and other inflammatory diseases (Zhu et al., 2007; Henry et al., 2012; Jia et al., 2014). During conditions such as osteoarthritis and articular joint disease, the concentration of the proinflammatory cytokine interleukin-1 β increases locally, causing a cascade of events that leads to cartilage damage. These include the formation of reactive oxygen species, metalloproteases, and extracellular matrix proteins absent in normal cartilage, the release of other inflammatory mediators, the inhibition of chondrocyte proliferation, and the induction of cell death (Largo et al., 2003). Although glucosamine is often used to prevent and relieve osteoarthritis and articular joint disease in humans, its effectiveness is still controversial (Block et al., 2010; Salazar et al., 2014; Conrozier and Lohse, 2022). On the other hand, FR and DOFR, products generated by the glucosamine caramelization, have been shown to inhibit inflammatory

mediators such as interleukins-2 and -1β and reduce the damage to proteoglycans and collagen in the connective tissue of joints more effectively than their parent compound (Giordani et al., 2006; Zhu et al., 2007). These can, in turn, prevent pathological cartilage degradation and inflammatory diseases. In addition, FR exerted antimicrobial action against heat-resistant *Escherichia coli* AW 1.7 by permeabilizing the cell membrane, damaging membrane integrity, and fragmenting its DNA (Bhattacherjee et al., 2016).

Therefore, the presence of FR and DOFR compounds in LC may have exerted anti-inflammatory effects on broilers. This could have mitigated the imbalanced production of reactive oxygen species induced by elevated corticosterone levels in response to physical and environmental stressors, such as the stress caused by the wire flooring cages (Wideman et al., 2012; Weimer et al., 2021). Additionally, this may reduce oxidative damage to cellular membranes, preventing unnecessary inflammation and aiding in maintaining intestinal homeostasis (Quinteiro-Filho et al., 2010; El-Senousey et al., 2018; Wang et al., 2018). Consequently, birds could direct dietary energy and nutrients toward growth instead of using them to restore intestinal homeostasis or support inflammatory processes (Broom and Kogut, 2018).

In the current study, the LC and LC+F treatments, each at 0.08% of the diet, reduced femoral head lesions at 40 d compared to some of the other caramel products. Furthermore, LC, BC and BC+F, regardless of the inclusion dose in the diet, reduced the lesions consistent with BCO in the tibia ($P \leq 0.05$) of broilers at 40 d, while LC+F tended ($P = 0.07$) to reduce the lesions compared to the Control diet. We suggest that the anti-inflammatory potential of FR and DOFR may have contributed to the reduced cartilage damage and intestinal permeability in birds from the LC groups. As a result, birds in these groups may have been more resilient to the challenge imposed and more able to maintain intestinal integrity and performance than those in the Control group. This, in turn,

aided the birds in the LC group to prevent, to some extent, the translocation of opportunistic bacteria from the lumen to the bloodstream and, consequently, mitigate potential BCO lesions (Wideman et al., 2012; Wideman, 2016). However, it is important to note that although the bone lesions observed in birds in this study are consistent with BCO, we did not attempt to isolate bacteria within the lesions to confirm this causation. Rapid growth and high body weight in broilers exert pressure on immature bone cartilage and growth plate, potentially causing microfractures or clefts (Wideman et al., 2012). This can potentially block blood supply to the growth plate and affect both epiphyseal and physical cartilage, leading to aseptic degenerative joint diseases such as osteochondrosis (Julian, 1998; Çapar Akyüz and Onbaşılar, 2020). However, osteochondrosis lesions are usually microscopic and subclinical (Çapar Akyüz and Onbaşılar, 2020). Therefore, although the lesions reported in this study are consistent with BCO (Wideman et al., 2012), the possibility of non-infectious bone lesions should also be considered since no bacterial isolation analysis was performed.

Similar to LC, birds fed BC and BC+F showed lower tibial lesions at 40 d, and those in the BC+F group had lower gait defects at 26 d compared to the Control birds. BC is the glucosamine caramel rich in melanoidins. Melanoidins are one of the end products of glucosamine caramelization and the end product of the Maillard reaction in some other compounds. They are responsible for the brown colour of thermally treated foods like bread and coffee (Rufián-Henares and Delgado-Andrade, 2009). The detailed structure of melanoidins remains unknown, but they seem to be composed of polysaccharides, proteins (melanoproteins) and phenolic compounds such as chlorogenic acids or catechins (Morales et al., 2012). Similar to fibre, melanoidins escape gastrointestinal digestion and are fermented by the gut microbiota (Pérez-Jiménez et al., 2014). In vitro studies showed that melanoidins increased SCFA production and favoured the growth of the beneficial genera

Bifidobacterium and *Faecalibacterium* (Pérez-Burillo et al., 2020). Moreover, when melanoidins were fed to mice exposed to both LPS and a high-fat diet, it increased SCFA production in the gut. Additionally, the abundance of beneficial bacteria such as *Lactobacillaceae* and *Akkermansiaceae* increased, while opportunistic pathogens such as *Enterobacteriaceae* decreased compared to non-supplemented mice (Wu et al., 2020). Another study showed that supplementing dietary barley malt melanoidins for mice increased the relative abundance of *Bifidobacterium* spp. and *Akkermansia* spp. It also increased SCFA concentrations in feces compared to the non-supplemented mice (Aljahdali et al., 2020).

Similar to BC, BC+F contained melanoidins but combined with caramelized fructose, which contains Di-D-fructose dianhydrides and glycosylated derivatives, also known for their prebiotic function (Peinado et al., 2013). Di-fructose dianhydride was observed to enhance performance, modulate intestinal microbiota, and enhance intestinal calcium absorption in broilers (Peinado et al., 2013; Lee and Kim, 2018). It also enhanced intestinal wound-healing processes, including migration, proliferation, and cell differentiation of in-vitro porcine epithelial cells following an LPS challenge (Lee and Kim, 2018). Thus, it is possible that BC and BC+F treatments increased the abundance of beneficial bacteria (i.e., *Lactobacilli* and *Bifidobacterium*) and improved the intestinal health of broilers through their prebiotic function. This, in turn, may have helped to prevent the establishment and translocation of pathogenic opportunistic bacteria from the lumen to the joints, mitigating lameness (Wideman et al., 2012, 2015).

It is important to note that there are limited studies investigating the metabolism and the mechanisms of action of glucosamine-derived caramels in human and animal models. To the best of our knowledge, no studies have been conducted evaluating them on poultry or specifically on the health of broilers, making it challenging to interpret our results. Some additional measurements could have

helped us have more accurate responses, such as evaluating the intestinal permeability, identifying and quantifying bacteria in the serum and bone lesions, and analyzing the presence of immune biomarkers in the serum. These are important parameters to be considered in future research in order to have more precise conclusions about the effects of glucosamine-derived caramels on preventing BCO lesions caused by translocated bacteria from the lumen. However, the current study revealed an interesting potential of light caramels, rich in FR and DOFR, to be used as AGP replacements in poultry diets, as it showed the potential to enhance the performance of broilers while reducing potential BCO lesions in the leg bones of broilers. Thus, this study can be used as a starting point for guiding future research on using glucosamine-derived caramels in poultry diets, especially for birds more susceptible to bone-related issues.

In conclusion, glucosamine-derived caramels produced at mild temperatures (50 to 90°C) to concentrate FR, DOFR, or melanoidins and included in up to 0.24% of the diet are safe for broilers. Glucosamine-derived caramels containing FR and DOFR demonstrated the potential to improve broiler performance while mitigating femoral and tibial lesions potentially caused by opportunistic bacterial translocation. Further research is necessary to understand the mechanisms of action involved in order to use glucosamine-derived caramels effectively to improve gut and bone health in poultry.

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5.6 TABLES

Table 5.1. Ingredient and calculated nutrient composition of experimental diets for broilers during starter, grower, and finisher phases.

Ingredients (%)	Starter (Day 1-10)	Grower (Day 11-25)	Finisher (Day 26-42)
Canola meal	10.00	12.00	15.00
Soybean meal	26.90	21.81	17.36
Wheat	56.65	59.03	59.50
Calcium carbonate	1.06	0.91	0.77
Monocalcium phosphate	0.99	0.73	0.49
NaCl	0.26	0.26	0.26
L-lysine	0.09	0.06	-
DL-methionine	0.29	0.25	0.19
L-threonine	0.040	0.006	-
Hy-D® premix[1]	0.05	0.05	0.05
Vitamin mineral premix[2]	0.5	0.5	0.5
Choline chloride premix[3]	0.05	0.05	0.05
Phytase[4]	0.01	0.01	0.01
Canola oil	2.45	3.67	5.15
Mycotoxin binder[5]	0.15	0.15	0.15
Xylanase[6]	0.05	0.05	0.05
Calculated nutrient composition			
Crude protein (%)	25.27	23.72	22.61
ME (kcal/kg)	3000	3100	3200
Calcium (%)	0.96	0.87	0.79
Available phosphorus (%)	0.480	0.435	0.395

[1]Provided 69 µg 25-hydroxycholecalciferol per kg diet (DSM Nutritional Products Inc., New Jersey, United States).

[2]Provided per kilogram of diet: vitamin A (retinyl acetate), 10,000 IU; cholecalciferol, 4,000 IU; vitamin E (DL-α-tocopheryl acetate), 50 IU; vitamin K, 4.0 mg; thiamine mononitrate (B_1), 4.0 mg; riboflavin (B_2), 10 mg; pyridoxine HCL (B_6), 5.0 mg; vitamin B_{12} (cobalamin), 0.02 mg; D-pantothenic acid, 15 mg; folic acid, 0.2 mg; niacin, 65 mg; biotin, 1.65 mg; iodine (ethylenediamine dihydroiodide), 1.65 mg; Mn ($MnSO_4 \cdot H_2O$) , 120 mg; Cu, 20 mg; Zn, 100 mg, Se, 0.3 mg; Fe ($FeSO_4 \cdot 7H_2O$), 800 mg.

[3]Provided 100 mg choline per kg of diet.

[4]Provided 500 FTU phytase/kg diet (Phyzyme XP, Danisco Animal Nutrition, Marlborough, UK).

[5]Biomin II (Biomin Canada Inc., Mont-St-Hilaire, Québec, Canada).

[6]Econase XT 25 (AB Vista, Marlborough, UK) provided 80,000 BXU of endo-1, 4-beta-xylanase activity per kg diet.

Table 5.2. The average body weight (g) per bird of broilers at 0, 10, 25 and 38 days of age fed with or without glucosamine-derived caramels in the diet.

Treatments	Dietary inclusion (%)	BW (g) per bird at 0 d	BW (g) per bird at 10 d	BW (g) per bird at 25 d	BW (g) per bird at 38 d
Control Diet[1]	---	37.60	215.97^{AB}	906.14^{ab}	1,910.30^{abcd}
Control+GlcN[2]	0.24	38.34	216.82^{AB}	914.19^{ab}	1,981.50^{ab}
BC[3]	0.08	38.15	216.61^{AB}	933.46^{ab}	1,976.30^{abc}
	0.16	37.39	218.07^{AB}	833.81^{ab}	1,823.00^{cd}
	0.24	37.60	218.26^{AB}	902.27^{ab}	1,996.10^{ab}
BC + F[4]	0.08	37.17	216.44^{AB}	918.55^{ab}	1,913.50^{abcd}
	0.16	38.00	205.75^{AB}	846.84^{ab}	1,823.10^{cd}
	0.24	38.38	224.51^{A}	934.95^{ab}	1,985.00^{ab}
LC[5]	0.08	37.78	218.97^{AB}	881.53^{ab}	1,885.10^{bcd}
	0.16	38.38	228.08^{A}	979.90^{a}	2,043.50^{a}
	0.24	38.67	232.82^{A}	955.42^{ab}	2,006.70^{ab}
LC + F[6]	0.08	37.96	195.79^{B}	828.37^{b}	1,797.30^{d}
	0.16	38.40	222.76^{AB}	913.27^{ab}	1,961.00^{abc}
	0.24	38.80	227.64^{A}	892.75^{ab}	1,938.60^{abcd}
SEM	---	0.12	5.86	30.87	55.20
P-value[7]	---	0.38	<0.01	0.02	0.04
Orthogonal contrast			P-value[8]		
Control[1] vs GlcN[2]	---	---	0.92	0.86	0.39
Control[1] vs BC[3]	---	---	0.82	0.66	0.75
Control[1] vs BC+F[4]	---	---	0.96	0.87	0.96
Control[1] vs LC[5]	---	---	0.15	0.38	0.31
Control[1] vs LC+F[6]	---	---	0.94	0.46	0.86

[1] Control diet = A basal, commercial-type diet.

[2] Control+GlcN = Control diet plus glucosamine at 0.24% of the diet.

[3] BC = Brown caramel produced at 90°C, and rich in melanoidins.

[4] BC+F = Brown Caramel plus caramelized fructose.

[5] LC = Light Caramel produced at 50°C, and rich in fructosazine and deoxyfructosazine.

[6] LC+F = Light Caramel plus caramelized fructose.

[a,b,c,d] Means with different superscripts within the same column are significantly different $P \leq 0.05$.

[A, B] Means with different superscripts within the same row are significantly different $P < 0.01$.

[8] P-value for the orthogonal contrast analysis of the Control vs each group of compounds.

Table 5.3. The performance of broilers in the starter (0-10 d), grower (11-25 d), finisher (26-38 d), and entire (0-38 d) phases fed with or without glucosamine-derived caramels in the diet.

Treatments	Dietary inclusion (%)	----------Starter----------			----------Grower---------			--------Finisher----------			------Entire period------		
		BWG 0-10d (g/day)	FI 0-10d (g/day)	FCR 0-10d (g/g)	BWG 11-25d (g/day)	FI 11-25d (g/day)	FCR 11-25d (g/g)	BWG 26-38d (g/day)	FI 26-38d (g/day)	FCR 26-38d (g/g)	BWG 0-38 d (g/day)	FI 0-38d (g/day)	FCR 0-38d (g/g)
Control Diet[1]	---	17.78^{ab}	23.91	1.35^{B}	45.34	72.05	1.59	63.59	118.68	1.90	45.67^{abcd}	71.53	1.57
Control+GlcN[2]	0.24	18.13^{ab}	23.88	1.33^{B}	46.07	72.44	1.58	59.33	115.79	2.01	47.40^{ab}	70.54	1.49
BC[3]	0.08	17.83^{ab}	24.90	1.40^{AB}	47.65	74.01	1.55	59.72	117.65	1.98	47.27^{abc}	72.47	1.53
	0.16	17.92^{ab}	24.86	1.39^{AB}	41.26	69.50	1.76	60.58	115.56	1.92	43.55^{cd}	69.43	1.60
	0.24	18.06^{ab}	25.06	1.39^{AB}	45.76	72.76	1.59	62.64	115.68	1.86	47.77^{ab}	71.45	1.49
BC + F[4]	0.08	17.87^{ab}	24.29	1.36^{AB}	45.60	71.29	1.57	63.22	116.07	1.84	45.76^{abcd}	70.89	1.55
	0.16	16.77^{ab}	24.19	1.45^{AB}	42.02	67.08	1.60	62.55	109.05	1.76	43.54^{cd}	66.75	1.54
	0.24	18.61^{ab}	24.66	1.33^{B}	46.06	73.62	1.60	60.69	120.10	1.98	47.48^{ab}	72.64	1.53
LC[5]	0.08	17.85^{ab}	25.06	1.41^{AB}	44.98	74.32	1.65	61.24	110.22	1.81	45.06^{bcd}	70.21	1.56
	0.16	18.97^{a}	25.38	1.34^{B}	49.32	77.37	1.57	63.73	120.94	1.90	48.91^{a}	74.45	1.52
	0.24	19.34^{a}	25.36	1.31^{B}	47.76	76.43	1.61	66.20	119.31	1.81	48.00^{ab}	73.60	1.53
LC + F[6]	0.08	15.78^{b}	23.73	1.52^{A}	40.26	64.59	1.63	59.32	109.30	1.86	42.91^{d}	65.51	1.53
	0.16	18.35^{ab}	24.74	1.35^{B}	45.73	72.83	1.60	58.17	116.31	2.04	46.89^{abc}	71.42	1.52
	0.24	18.78^{a}	25.16	1.34^{B}	43.99	72.38	1.67	64.02	117.32	1.85	46.33^{abcd}	71.90	1.55
SEM	---	0.61	0.58	0.03	2.20	2.66	0.04	0.89	1.39	0.02	1.34	2.28	0.03
P-value	---	0.02	0.52	<0.01	0.21	0.09	0.32	0.95	0.92	0.37	0.04	0.34	0.90
Orthogonal Contrasts							P-value[7]						
Control[1] vs GlcN[2]		0.77	0.97	0.64	0.80	0.91	0.77	0.50	0.70	0.44	0.36	0.76	0.25
Control[1] vs BC[3]		0.84	0.12	0.34	0.86	0.98	0.49	0.50	0.69	0.74	0.75	0.87	0.54
Control[1] vs BC+F[4]		0.97	0.48	0.53	0.76	0.65	0.94	0.71	0.56	0.78	0.96	0.59	0.54
Control[1] vs LC[5]		0.21	0.04	0.99	0.44	0.20	0.80	0.97	0.76	0.60	0.32	0.64	0.49
Control[1] vs LC+F[6]		0.85	0.33	0.23	0.44	0.50	0.50	0.42	0.48	0.77	0.85	0.47	0.45

Abbreviations: BWG = body weight gain per day/bird (g); FI = feed intake per day/bird (g); FCR = feed conversion ration (g/g).

[1] Control diet = A basal, commercial-type diet.
[2] Control+GlcN = Control diet plus the supplementation of glucosamine at 0.24% of the diet.
[3] BC = Brown caramel produced at 90°C, and rich in melanoidins.
[4] BC+F = Brown Caramel plus caramelized fructose.
[5] LC = Light Caramel produced at 50°C, and rich in fructosazine and deoxyfructosazine.
[6] LC+F = Light Caramel plus caramelized fructose.
[a,b,c,d] Means with different superscripts within the same column are significantly different $P \leq 0.05$.
[A, B] Means with different superscripts within the same row are significantly different $P < 0.01$.
[8] P-value for the orthogonal contrast analysis of the Control vs each group of compounds.

Table 5.4. Gait score of broilers at 11, 26 and 39 days of fed with or without glucosamine-derived self-condensation products in the diet.

Treatments	**Dietary inclusion (%)**	**Gait Score[1] at 11 d**	**Gait Score[1] at 26 d**	**Gait Score[1] at 39 d**
Control Diet[2]	---	0	0.62	0^{B}
Control+GlcN[3]	0.24	0.12	0.71	0^{B}
BC[4]	0.08	0	0.22	0^{B}
	0.16	0	0	0^{B}
	0.24	0	0	1.0^{A}
BC + F[5]	0.08	0	0	0.37^{AB}
	0.16	0	0	0^{B}
	0.24	0	0	0^{B}
LC[6]	0.08	0	0.12	0.12^{B}
	0.16	0.12	0.28	0.29^{AB}
	0.24	0	0	0^{B}
LC + F[7]	0.08	0.12	0.50	0^{B}
	0.16	0	0.10	0.5^{AB}
	0.24	0	0.70	0.12^{B}
SEM	---	0.01	0.07	0.05
P-value[8]	---	0.61	0.07	<0.01
Orthogonal contrast			P-value[9]	
Control[2] vs GlcN[3]	---	0.13	0.83	1.00
Control[2] vs BC[4]	---	1.00	0.09	0.13
Control[2] vs BC+F[5]	---	1.00	0.05	0.57
Control[2] vs LC[6]	---	0.53	0.12	0.54
Control[2] vs LC+F[7]	---	0.53	0.52	0.35

[1]Gait scoring system: Score 0 = no detectable abnormality; Score 1 = detectable but unidentifiable abnormality; Score 2 = Identifiable abnormality that has little effect on overall function; Score 3 = Identifiable abnormality that impairs function; Score 4 = Severe impairment of function but still capable of walking; Score 5 = complete lameness.

[2] Control diet = A basal, commercial-type diet.

[3] Control+GlcN = Control diet plus the supplementation of glucosamine at 0.24% of the diet.

[4] BC = Brown caramel produced at 90°C, and rich in melanoidins.

[5] BC+F = Brown Caramel plus caramelized fructose.

[6] LC = Light Caramel produced at 50°C, and rich in fructosazine and deoxyfructosazine.

[7] LC+F = Light Caramel plus caramelized fructose.

[8] P-value for Kruskal-Wallis One-Way Nonparametric AOV test.

[a,b] Means with different superscripts within the same column are significantly different $P \leq 0.05$.

[9] P-value for the orthogonal contrast analysis of the control vs each group of compounds.

Table 5.5. Evaluation of femoral (from 1 to 8) and tibial head (from 1 to 10) lesion scores of broilers at 11, 26, and 42 d fed with or without glucosamine-derived self-condensation products in the diet.

Treatments	Dietary inclusion (%)	Femoral lesion scores[1]			Tibial lesion scores[2]		
		12 d	27 d	40 d	12 d	27 d	40 d
Control Diet[3]	---	1.00	1.00	1.62^{ab}	2.50	2.50	4.25
Control+GlcN[4]	0.24	1.75	1.25	1.37^{ab}	2.62	3.00	3.50
BC[5]	0.08	1.00	1.37	1.62^{ab}	2.37	3.12	2.87
	0.16	1.00	1.37	2.25^{ab}	1.75	3.00	3.37
	0.24	1.00	1.00	2.37^{a}	3.00	3.00	3.25
BC + F[6]	0.08	1.00	1.12	2.00^{ab}	1.87	3.00	3.00
	0.16	1.00	1.00	1.87^{ab}	2.12	2.87	3.37
	0.24	1.00	1.00	2.37^{a}	2.25	2.75	3.37
LC[7]	0.08	1.00	1.12	1.00^{b}	2.12	3.12	3.62
	0.16	1.00	1.00	1.50^{ab}	2.00	2.25	3.50
	0.24	1.00	1.12	1.37^{ab}	2.37	2.62	3.00
LC + F[8]	0.08	1.00	1.00	1.00^{b}	3.00	2.75	3.37
	0.16	1.00	1.25	1.62^{ab}	2.25	2.87	3.12
	0.24	1.00	1.12	2.37^{a}	1.87	3.50	3.75
SEM	---	0.05	0.04	0.09	0.12	0.10	0.11
P-value[9]	---	0.45	0.81	0.03	0.73	0.78	0.74
Orthogonal contrast				P-value[10]			
Control[3] vs GlcN[4]	---	<0.01	0.29	0.62	0.84	0.34	0.17
Control[3] vs BC[5]	---	1.00	0.20	0.27	0.80	0.20	0.02
Control[3] vs BC+F[6]	---	1.00	0.27	0.27	0.41	0.37	0.03
Control[3] vs LC[7]	---	1.00	0.67	0.42	0.51	0.69	0.05
Control[3] vs LC+F[8]	---	1.00	0.52	0.92	0.81	0.20	0.07

[1]Femoral head necrosis scoring system: Score 1 = no lesions; Score 2 = femoral head separation (epiphyseolysis); Scores 3 to 5 = progressive necrosis, ulceration, erosion, and fracturing of the growth plate; Scores 6 to 8 = perforation, fracturing, and necrosis/osteomyelitis of the femoral head.

[2]Tibial head necrosis scoring system: Score 1 = no lesions or abnormalities; Scores 2 to 5 = necrotic voids in the metaphyseal zone undermine the support of the growth plate, leading to microfractures of the growth plate; Scores 6 to 8 = macroscopic evidence of osteomyelitis (bacterial infiltration); Scores 9 to 10 = necrotic voids communicating with precocious ectopic extensions of the marrow cavity.

[3] Control diet = A basal, commercial-type diet.

[4] Control+GlcN = Control diet plus the supplementation of glucosamine.

[5] BC = Brown caramel produced at 90°C, and rich in melanoidins.

[6] BC+F = Brown Caramel plus caramelized fructose.

[7] LC = Light Caramel produced at 50°C, and rich in fructosazine and deoxyfructosazine.

[8] LC+F = Light Caramel plus caramelized fructose.

[9] P-value for Kruskal-Wallis One-Way Nonparametric AOV test.

[a,b] Means with different superscripts within the same column are significantly different $P \leq 0.05$.

[10] P-value for the orthogonal contrast analysis of the control vs each group of compounds.

5.7 FIGURES

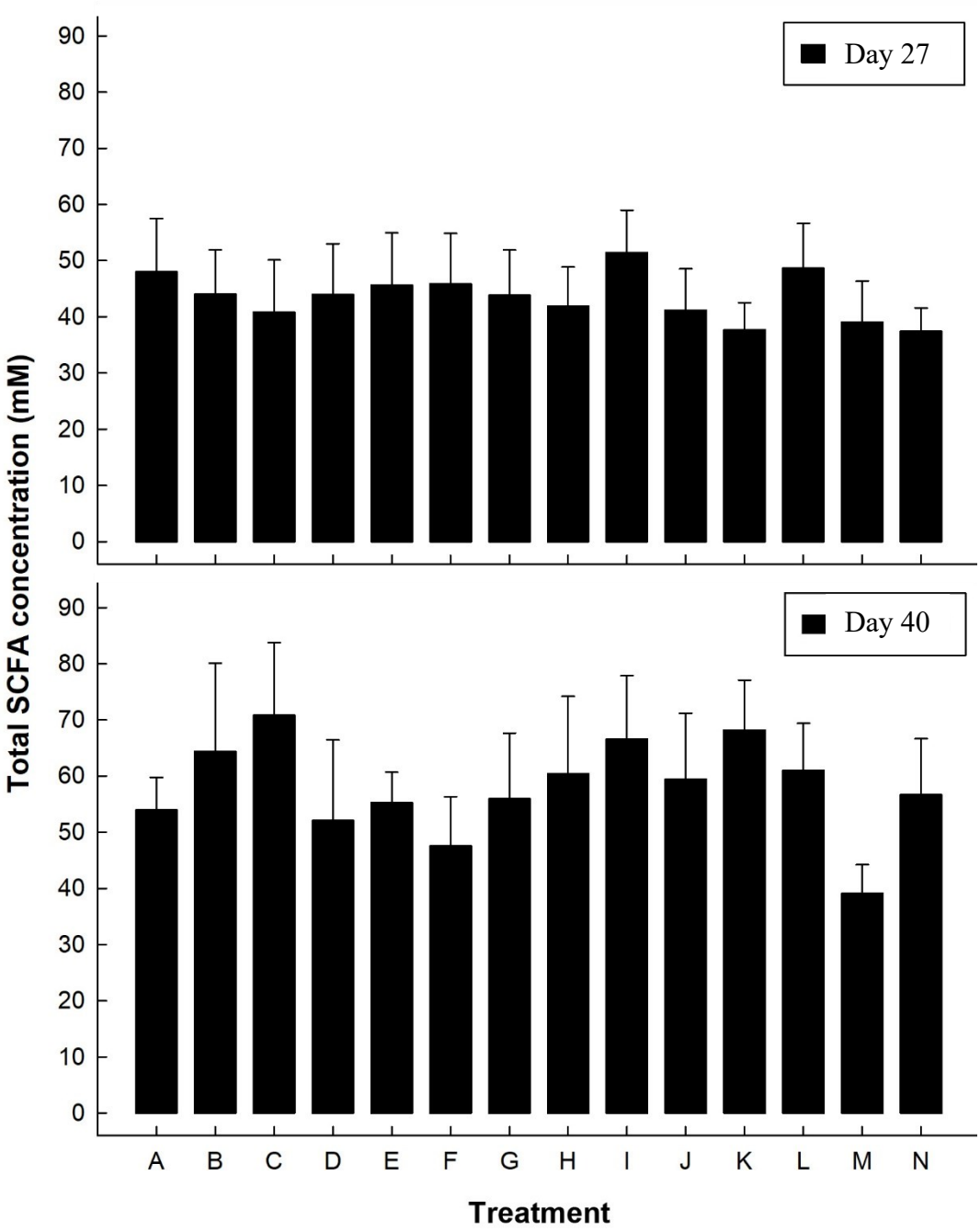

Figure 5.1. Total concentration of short-chain fatty acid (SCFA) in the cecal content of broilers at 27 and 40 d fed with or without glucosamine-derived caramels in the diet.
A = Control diet; B = Control + glucosamine included at 0.24% of the diet; C = Brown caramel included at 0.08% of the diet; D = Brown caramel included at 0.16% of the diet; E = Brown caramel included at 0.24% of the diet; F = Brown + caramelized fructose included at 0.08% of the diet; G = Brown + caramelized fructose included at 0.16% of the diet; H = Brown + caramelized fructose

included at 0.24% of the diet; I = Light caramel + caramelized fructose included at 0.08%; J = Light caramel + caramelized fructose included at 0.16% of the diet; K = Light caramel + caramelized fructose included at 0.24% of the diet; L = Light caramel included at 0.08% of the diet; M = Light caramel included at 0.16% and N = Light caramel included at 0.24% of the diet.

6. RESEARCH SYNTHESIS

6.1 OVERVIEW

Antibiotic growth promoters (**AGP**) have been used to enhance growth rates and protect broiler chickens from the adverse effects of pathogenic and non-pathogenic enteric microorganisms since the 1940s (Bedford, 2000; Castanon, 2007). However, due to the emergence of bacterial resistance to antibiotics used to treat animal and human infections, many countries have banned or increased the veterinary oversight of AGP use in animal diets (Castanon, 2007; Wallinga et al., 2022; Da Silva et al., 2023). The phasing out of AGP from broiler diets has increased the exposure of birds to potentially harmful pathogens, resulting in a recurring activation of the innate immune system and increasing the risks of clinical and subclinical enteric diseases (Broom and Kogut, 2018). Enteric diseases pose an important concern in the poultry industry as they cause reduced productivity, increased mortality, and an elevated risk of contaminated poultry products for human consumption (Dahiya et al., 2006; Grass et al., 2013; van der Klein et al., 2023). Necrotic enteritis (**NE**) is a major enteric disease in poultry that has become more prevalent since the withdrawal of AGP, resulting in estimated losses of $6 billion per year worldwide (Wade and Keyburn, 2015). This disease develops due to infection with pathogenic strains of *Clostridium perfringens* and the presence of predisposing factors (Annett et al., 2002; Keyburn et al., 2008; Wu et al., 2014; Bortoluzzi et al., 2019). At high levels in the gut, *C. perfringens* can activate a series of virulence factors that enable them to outcompete commensal bacteria, form localized microcolonies on the mucosal surface, and produce necrotic toxins that cause damage to the intestinal tissue of the host (Prescott et al., 2016). This damage can, in turn, lead to intestinal inflammation, impair digestion and absorption of nutrients, and increase intestinal permeability, allowing toxins, microorganisms, and other compounds to reach the bloodstream, potentially opening the gate for other diseases. NE

may present as either a clinical or subclinical disease. The clinical form is characterized by a sudden increase in flock mortality, while subclinical NE leads to performance losses (Van Immerseel et al., 2009).

Another issue associated with enteric disturbances and disease is the development of bacterial chondronecrosis with osteomyelitis (**BCO**). BCO is the leading cause of broiler lameness and is responsible for significant losses in poultry production (Ekesi et al., 2021). This condition occurs when opportunistic bacteria enter the blood via translocation from the respiratory system or gastrointestinal tract and spread hematogenous to vulnerable sites, such as microfractures and clefts in the joint cartilages, especially from the femur and tibiotarsus, caused by the rapid growth of broilers (Wideman et al., 2012). The overgrowth and the release of lytic substances by opportunistic bacteria in these vulnerable sites cause gross abscesses and necrotic voids, ultimately leading to BCO (Wideman et al., 2012; Wideman, 2016). The most common bacteria isolated from BCO cases are *Escherichia coli*, *Staphylococcus aureus*, *S. agnetis*, *Enterococcus cecorum*, and *Salmonella enteritidis,* common inhabitants of the intestinal microbiota. This suggests that the translocation of bacteria from the gut to the joints due to failures in the tight junctional complexes is highly associated with the pathogenesis of BCO in broilers (Wideman et al., 2015; Rojas-Núñez et al., 2020).

Given the global shift towards antibiotic-free production and the significant impact of enteric and associated diseases on the poultry industry, there is an urgent need to develop AGP alternatives in broiler production (M'Sadeq et al., 2015; Pham et al., 2020; Emami et al., 2021). Although over the past two decades, extensive research has focused on the development of AGP alternatives, there is still a lack of consistency in the results and limited knowledge about their mechanisms of action. In addition, none of the current alternatives appear to be as effective, cost-effective,

reliable, and easy to apply as AGP (Moore, 2023). Hence, it is important to understand the mode of action of the products used as AGP replacements in order to use them effectively in the industry. In the case of AGP replacements aimed to improve gut health, it is important to consider their efficacy and mechanism of action in broilers under enteric challenge conditions that closely simulate field conditions, such as the simulation of in-field NE development. Therefore, the main objective of this Ph.D. thesis was to investigate the potential of three novel products to replace AGP and enhance the gut health of broilers under challenging conditions: chitosan oligosaccharides (**COS**), punicic acid (**PA**), and glucosamine-derived caramels.

COS are functional oligosaccharides, natural alkaline polymers of glucosamine obtained by chemical and enzymatic hydrolysis of chitosan or chitin (Zou et al., 2016). COS can exert a broad range of biological properties, including antimicrobial (Kim et al., 2003), anti-inflammatory (Ma et al., 2011; Moine et al., 2021), and antioxidant activities (Je et al., 2004; Li et al., 2017). However, no single type of COS displays all the observed biological activities. The versatile functions of COS are closely related to the compound characteristics, such as molecular weight (**Mw**) and degree of deacetylation (Zou et al., 2016). To the best of our knowledge, no study has evaluated the ability of different COS Mw to prevent NE in broilers. Therefore, dietary shellfish COS were assessed as potential AGP replacement in broilers challenged with a natural, subclinical NE infection model. Two studies were conducted to address this objective. The objective of the first study was to determine the optimal COS Mw and levels of inclusion in the diet of broilers on performance, NE intestinal lesion scores, and BW uniformity. Eight COS products ranging from low to high molecular weight (14, 17, 25, 30, 95, 110, 180, 220 kDa) included at three levels in the diet (0.2, 2, and 5 g/kg) were tested, and compared with a Positive Control (commercial-type diet with antibiotic and coccidiostat) and Negative Control diet (commercial-type diet without any

medication; Chapter 2, Study 1). From this experiment, the most promising treatments to recover performance and mitigate NE gross lesions in the jejunum were then further evaluated and compared to PC and NC groups in a larger-scale experiment (a greater number of animals and replicates). In this study, COS 180 kDa included at 0.2, 2, and 5 g/kg, COS 110 kDa at 5 g/kg, and COS 95 kDa included at 0.2 and 5 g/kg were evaluated (Chapter 2, Study 2). Among them, COS 95 kDa, included at 0.2 or 5 g/kg, showed a higher potential to enhance performance under the subclinical NE challenge. Thus, samples from these specific treatments were selected for further investigation of their effects on performance, cecal microbial abundance, cecal quantification of *C. perfringens* and concentration of short-chain fatty acid, immune modulation, intestinal morphology, and *Eimeria* oocysts count in the intestine of broilers challenged with a natural, subclinical NE infection model (Chapter 3).

The second screened product was PA, an unusual polyunsaturated fatty acid (C18:3-9 cis, 11 trans, 13 cis) that is mainly derived from pomegranate seed oil (**PSO**) (Pereira de Melo et al., 2014). Studies evaluating PA have gained attention, especially in humans, due to its range of beneficial pharmacological properties, including anti-diabetes, anti-obesity, antioxidant, and anti-inflammatory properties (Saha and Ghosh, 2009; Hontecillas et al., 2009; Bassaganya-Riera et al., 2011; Yuan et al., 2021). To the best of our knowledge, no studies have evaluated the potential of dietary PA to mitigate NE in broilers. Therefore, the objective of the PA study was to assess the effects of PA provided through the supplementation of PSO in the diet as a potential AGP replacement to mitigate NE on the performance, intestinal NE gross lesion, litter quality, and footpad dermatitis of broilers challenged with a natural, subclinical NE infection model. In order to evaluate the optimal PA level of inclusion to mitigate NE, PSO was added to a final PA concentration of 0.1, 0.25, 0.5, 1, 1.5, or 2% of the feed. Birds in these experimental treatments

were compared to those fed PC (diet with antibiotic and coccidiostat) or NC (diet without any medication).

The last set of products evaluated in this thesis were the glucosamine-derived caramels, which are compounds originating from the self-condensation of glucosamine under mild temperatures (Hrynets et al., 2015, 2016). Among these compounds, we were particularly interested in the pyrazines, known as fructosazine (**FR**) and deoxyfructosazine (**DOFR**), and in the melanoidins. Light caramels (colorless) were generated by the caramelization of the glucosamine at 50 °C and were rich in FR and DOFR. These pyrazines seem to have stronger anti-inflammatory properties than the parent compound (Giordani et al., 2006; Zhu et al., 2007; Hrynets et al., 2015). When a slightly higher temperature of 90 °C was applied, brown caramels rich in melanoidins were generated. Melanoidins are compounds that seem to exert prebiotic properties and are commonly formed during the heat processing of foods like coffee, bread, and malt (Borrelli and Fogliano, 2005; Nunes and Coimbra, 2010). To the best of our knowledge, no studies evaluating the potential of glucosamine-derived caramels to alleviate BCO in broilers have been conducted. Therefore, the objective of the glucosamine-derived caramels study was to evaluate them on growth performance, femoral and tibial BCO gross lesions, gait scores, and cecal concentrations of short-chain fatty acids in broilers raised in wire flooring cages as a model to induce lameness.

6.1.1 Review of thesis hypotheses

1) The natural, subclinical NE infection model would be a suitable model to mimic field conditions in which NE takes place and effectively test the efficacy of the tested products in replacing AGP in broiler diets. This hypothesis was partially accepted and discussed in Chapters 2, 3, and 4.

2) COS at a specific Mw and level of inclusion in the diet would partially restore the growth performance decreased by subclinical NE, compared to the AGP, through the modulation of gut

health and immune responses. This hypothesis was partially accepted and is discussed in Chapters 2 and 3.

3) Supplementation of PA through PSO would partially restore the performance decreased by subclinical NE, compared to the AGP, and mitigate NE intestinal lesions in broilers. The results of this study would serve as a guide for developing new biotechnological sources of PA (i.e., genetically modified yeast enriched with PA). This hypothesis was rejected and is discussed in Chapter 4.

4) Glucosamine-derived caramels would enhance broiler performance and mitigate BCO lesions potentially caused by translocated bacteria from the lumen to the joints. This hypothesis was partially accepted and is discussed in Chapter 5.

6.2 FINDINGS AND ANALYSES

In this thesis, the effects of the COS and PA on enhancing gut health and mitigating NE were tested in broilers subjected to a natural, subclinical NE challenge model. This model is a novel approach developed by our research group, and involves exposing the birds to predisposing factors that disturb gut homeostasis, leading to a natural proliferation of pathogenic *C. perfringens* in the gut, ultimately causing subclinical NE (He et al., 2022). As part of the model, birds are challenged with 15x the recommended dose of a coccidiosis vaccine containing live *Eimeria* oocysts by gavage at 12 d and 24-hour feed withdrawal at 18 d. Coccidiosis was used as a predisposing factor because it is the most important risk factor associated with NE based on the strong correlation between the prevalence of both diseases in the field (Dierick et al., 2021). As *Eimeria* spp. are enteric parasites, they invade the host's enterocytes to complete their maturation, and upon exiting, they cause damage to the epithelium, which includes disruption in the extracellular matrix and plasma protein leakage, leading to an increase in mucus production (López-Osorio et al., 2020).

These changes create favorable conditions (nutrient substrate and adhesion sites) for the proliferation and establishment of *C. perfringens* in the gut (Collier et al., 2008; Wade et al., 2015). The 24-hour feed withdrawal introduced on day 18 was important for enhancing microbiota disturbance, favoring the conditions for *C. perfringens* to thrive, and facilitating the timely onset of a subclinical disease outbreak (He et al., 2022). As *C. perfringens* is an opportunistic pathogen, it gains a selective survival advantage over other bacteria during the feed withdrawal, using the host's mucus and tissues as a substrate for growth (Shimizu et al., 2002). Thus, these predisposing factors were used to facilitate the natural infection and proliferation of *C. perfringens* in the gut and simulate the progression of the subclinical NE development in broilers. Before the experiments were conducted, samples from the litter, digesta of birds from a previous flock, and a swab from different points of the barn were collected to confirm the presence of pathogenic strains of *C. perfringens* in the experimental facility. We confirmed the presence of *C. perfringens* with the following toxin genes: *cpa*, *netB, cpb2*, *tpeL*, *pfoA*, and *cnaA*, which include toxin genes associated with the development of poultry NE (Keyburn et al., 2008; Praveen Kumar et al., 2019; Kiu et al., 2019). In the COS study reported in Chapter 3, *C. perfringens* was detected in the cecal digesta of all the evaluated birds regardless of the time point of assessment. In contrast, *C. perfringens* pathogenic *netB*-positive strains were detected in 87.5% of the sampled birds following the challenge and in 78.1% of the evaluated birds at 38 d. Nevertheless, the natural, subclinical NE challenge model used to induce the disease in broilers only caused a reduction in performance in birds from the NC compared to those in PC in the COS study 1, specifically for BW gain during the grower phase. For COS study 2 and PA (Chapter 2, Study 2, and Chapter 4, respectively), the challenge did not reduce performance in birds from the NC compared to those in the PC group. Since the main effect of subclinical NE is a reduction in broiler performance (Timbermont et al.,

2011), we expected to see a consistent decrease in the performance of birds fed NC relative to those fed the PC, especially following the challenge. In the absence of reduced performance in the NC group, it was not possible to accurately evaluate the effects of potential AGP replacements (Korver, 2020). Despite that, the results of the intestinal morphology of COS study 2 (Chapter 3) clearly show that the challenge applied caused histopathological changes in the gut structure, affecting especially birds from the NC group. Birds fed NC were the most vulnerable to *Eimeria* infection, which was clear not only from the highest numbers of oocysts found in the villi of these birds but also due to the lowest villi height to crypt depth ratio, thickest epithelium, lamina propria, and deepest crypts compared to all the other treatments. These histopathological changes suggest that the challenge caused mild gut dysbiosis and local intestinal inflammation, especially in the NC birds. Although the natural, subclinical NE challenge model used disturbed the intestinal morphology architecture of the gut and caused mild gut dysbiosis in birds, these changes were not severe enough to have a discernable impact on the growth of birds fed NC compared to those fed PC. Therefore, slight adjustments of the natural, subclinical challenge model used in this study are required in order to capture a more substantial decrease in the performance of birds fed the NC relative to the PC. This would help us to test the potential of COS and PA products more accurately as AGP replacements to prevent or mitigate subclinical NE in broilers.

In the COS study 1 presented in Chapter 2, we observed that supplementation of dietary shellfish COS with Mw up to 220 kDa and inclusion levels up to 5 g/kg in the diet are safe for broilers. Among the evaluated products, COS at medium to high Mw (95 to 220 kDa) exhibited a greater capacity to partially restore performance following the NE challenge compared to COS at low Mw (30 to 14 kDa). In the two experiments described in Chapter 2, COS 95 kDa demonstrated some potential to enhance performance and mitigate NE-associated damage in the jejunum of broilers.

When we further investigated the effects of COS 95 kDa on the gut health of broilers challenged with the natural, subclinical NE model (Chapter 3), we observed that this product increased the relative abundance of *Lactobacillus* species in the ceca compared to NC, enhanced the intestinal morphology integrity of broilers, and mitigated *Eimeria* infection at the same level as the AGP (a diet with antibiotic and coccidiostat). COS 95 kDa also seemed to contribute to a vigorous local immune response, increasing the serum levels of the pro-inflammatory chemokine MIP-3α and cytokine IL-16 in order to restore homeostasis following the challenge. Although the reasons are not fully elucidated, we speculate that the effects observed in this study might be related to the prebiotic and immunomodulatory/stimulant properties of COS. This is because COS can serve as a substrate for bacteria that can digest these polymers and as an immune adjuvant when adhered to the intestinal mucosa and recognized by the immune system (Lee et al., 2002; Moine et al., 2021).

In the PA study presented in Chapter 4, the supplementation of PSO to achieve PA concentrations higher than 0.5% of the diet caused significant reductions in the feed intake, which negatively affected BW and BW gain of the broilers, especially during the starter and grower phases compared to birds fed the Control diets. Although we did not further investigate the cause of the reduced performance associated with increasing levels of PSO in the diet, we suspect that the reason is related to the properties of PSO. For this study, we utilized virgin PSO, which contained 91% polyunsaturated fatty acids (**PUFA**), a higher concentration than in canola oil, which contains approximately 35% PUFA (Dupont et al., 1989). Canola oil was used to equalize the total oil inclusion in the experimental diets, and in the PC and NC diets to represent the common source of fat used in poultry diets in Western Canada. Even though PUFA are highly digestible compared to saturated fats and represent traditional fat sources in broiler diets (e.g., canola oil, soy oil), they

are more susceptible to oxidative processes (Engberg et al., 1996). Thus, we speculate that the high concentration of PUFA in the PSO-supplemented diets may have led to oxidative rancidity. Oxidative rancidity is a major contributor to the loss of quality in ingredients or rations, affecting flavor, aroma, color, texture, and nutritive value (Baião and Lara, 2005). Thus, it is possible that diets containing high inclusion of PSO reduced broiler performance compared to the other diets because the PSO was more susceptible to peroxidation, reducing the ration palatability and nutritional value. In addition to reduced performance, we observed that birds fed the highest inclusion of PA in the diet had more pronounced NE gross lesions (although still mild) in the jejunum at 22 d than those fed PC. A similar tendency of PA 2% to increase NE lesion scores was observed at 40 d ($P = 0.07$). Therefore, it is possible that PSO peroxidation has not only affected the palatability and nutritive value of the ration but also increased intestinal oxidative stress, leading to increased intestinal dysbiosis (Tan et al., 2018; Zhang et al., 2022). This creates more favourable conditions for the *C. perfringens* to proliferate and cause lesions in the gut (Lee et al., 2014; Xu et al., 2023) compared to the PC.

Although the oil oxidation could be a simple and reasonable explanation for the observed reductions in performance of birds fed PSO, studies that also observed reductions in BW in mice fed PSO suggest that the oil may induce changes in fat and glucose metabolism. When PSO was added to high-fat diets, BW, BWG and fat mass were decreased, and satiety was increased in mice (McFarlin et al., 2008; Vroegrijk et al., 2011; Mohamed and Fayed, 2020). The authors speculated that these results are associated with the ability of PSO to activate the peroxisome proliferator-activated receptors gamma (PPAR-γ), a nuclear receptor that plays a crucial role in regulating lipid and glucose metabolism and hormonal signaling (Ferré, 2004; Rangwala and Lazar, 2004). Therefore, it is possible that PSO supplementation led to increased fat and glucose metabolism and

reduced energy intake in broilers, contributing to reduced growth performance, especially noticeable in broilers fed higher PSO levels. Although the actual reason remains unclear, this study clearly demonstrated that PSO supplementation above 0.5% in the diet reduced performance and did not mitigate NE in broilers.

For the glucosamine-derived caramels study (Chapter 5), broilers were reared in wire-floored cages as a model to induce lameness and, thus, create challenging conditions to test the efficacy of the tested products to prevent bone lesions caused by the potential translocation of bacteria from the gut to susceptible joints (Wideman et al., 2012, 2015). According to these authors, broilers raised on wire flooring are more prone to lameness compared to those on wood-shaving litter because the movement of the floors in cages causes instability and additional stress on immature and vulnerable leg joints. The increased shear stress in the joints leads to osteochondrosis in the cartilage and blockage of blood vessels around the joints, creating conditions for bacterial colonization and resulting in lameness associated with BCO. Furthermore, wire flooring acts as a stressor, contributing to immunosuppression and affecting gut health and permeability, thus contributing to bacterial proliferation and increasing the risks of BCO (Wideman et al., 2012; Wideman, 2016).

In this study, we observed that glucosamine-derived caramels produced at mild temperatures (50 to 90°C) to concentrate the melanoidins, FR and DOFR and included at up to 0.24% of the diet are safe for broilers. Light caramels (**LC**) rich in FR and DOFR and included at 0.24% resulted in heavier birds at 10 d, while birds in the LC at 0.16% group tended to be heavier at 25 and 38 d and have higher BWG in the entire period (0 to 38 d) than the Control ($P \leq 0.09$). Regardless of the dose included in the diet, the LC group had higher FI at the starter phase than birds from the Control. In addition, birds fed brown caramel produced at 90 °C rich in melanoidins (**BC**), BC

plus caramelized fructose, and LC had lower lesions consistent with BCO in the tibia than those in the Control group. The LC, rich in FR and DOFR, compounds that seem to have anti-inflammatory properties, demonstrated a potential to improve broiler performance while mitigating bone lesions compared to the Control birds. Previous experiments conducted in vitro observed that FR and DOFR have stronger inhibitory activity against interleukins (IL-1β and IL-2) than glucosamine (Giordani et al., 2006; Zhu et al., 2007). Although immune responses were not evaluated in this study, we suspect that FR and DOFR present in LC exerted immunomodulatory activities and helped to prevent pathological cartilage degradation in broilers. None of the products tested in this thesis were as effective as AGP for growth promotion and disease prevention. However, COS 95 kDa and glucosamine-derived caramels rich in FR and DORF products might be used as part of the strategy to replace in-feed AGP in broiler diets. As the studies conducted in this thesis are novel, further exploration of the mechanisms of action of the tested products under more challenging conditions should be considered in order to make more accurate conclusions and potentially make recommendations for their use in the industry.

6.3 STUDY LIMITATIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

The natural, subclinical challenge model used to test the efficacy of COS and PA (Chapter 2 Study 2 and Chapter 4) to prevent or mitigate subclinical NE in broilers did not cause reductions in performance in birds fed an NC diet (commercial-type diet without any medication) compared to those fed PC (commercial-type diet with AGP and coccidiostat). Therefore, it was not possible to accurately test the efficacy of the tested products in preventing NE in broilers. Reduction in performance is the hallmark of subclinical NE in broilers (Van Immerseel et al., 2009; Timbermont et al., 2011). Hence, conditions in which reductions in performance between the NC and PC birds are observed would be important to confirm the presence of the challenge in the experimental

facility and that the challenge was especially affecting birds from the NC group. Since the PC diet was supplemented with coccidiostat and AGP, we expected that in the presence of the challenge, the coccidiostat would reduce *Eimeria* infection and prevent coccidiosis from being a risk factor for NE development. Likewise, in the presence of the challenge, we expected the AGP present in the PC diet would prevent the proliferation of *C. perfringens* by inhibiting their potential virulence factors and preventing inflammatory processes. This would ultimately help birds to get closer to their maximum potential for growth. Therefore, the challenge used was not strong enough to cause discernible reductions in performance between NC and PC birds. This suggests that refinements to the challenge model to yield a more potent, yet still subclinical NE episode may be necessary to understand more clearly the effects of the tested products in preventing NE in broilers. One way to increase the robustness of the challenge model in future research would be to increase the coccidiosis vaccine dosage (e.g. 20x recommended dose) on day 12. This might increase the epithelium disturbance by providing more oocysts invading enterocytes, creating more favorable conditions for *C. perfringens* proliferation. In addition, the relative low humidity in Alberta, Canada (~ 67% average in 2022; Alberta Climate Information Service) contributes to dry weather and dry litter, hindering the sporulation of *Eimeria* oocysts (Venkateswara Rao et al., 2015). To address this, spraying water over the litter could help maintain the right conditions for *Eimeria* re-cycling and bird reinfection. Another way to enhance the challenge would be to use in-feed corticosterone (Zaytsoff et al., 2020) as a stress factor following the coccidia challenge. In regions with higher relative humidity, using a sanitation challenge, such as reused litter (Bortoluzzi et al., 2019), would be an option to increase the robustness of the challenge.

Although the potential of COS products to prevent losses in performance could not be properly evaluated due to the limitations of the model, COS 95 kDa demonstrated promising results in both

experiments (Chapters 2 and 3). While COS 95 kDa alone is unlikely to replace AGP, it may have the potential to be used as a part of a non-AGP strategy to prevent subclinical NE in broilers. However, further exploration of its mechanism of action under a more potent subclinical NE challenge model should be considered, as well as investigating potential synergistic relationships with other proposed AGP replacements (e.g., probiotics, enzymes, organic acids). In addition, when we further investigated the effects of COS on immunomodulation (Chapter 3), we observed that COS 95 kDa increased the pro-inflammatory cytokine IL-16 and the MIP-3α chemokine, both from the acquired immune system in the serum of broilers following the challenge. On the other hand, COS reduced the concentration of IFN-α, a cytokine from the innate immune system. It may suggest that in the study conditions, COS 95 kDa stimulated a vigorous response from the immune system and a more efficient transition from innate to adaptive immune responses compared to the NC group. However, we can only speculate that since the immune biomarkers in the serum were measured only at 22 d of age. Therefore, we suggest the collection of blood samples prior to the coccidiosis vaccine application and at different time points following the coccidia and feed withdrawal challenge. This would help us to investigate the COS effects in the transition from innate to acquired immunity and identify which immune biomarkers are involved in this process. Similarly, for future studies would be interesting to look at some additional intestinal health biomarkers, such as the detection of host proteins in gut contents (e.g., fibronectin, myeloid protein-1, or hemoglobin subunit beta) that are indicative of gut barrier damage, as well as the presence of acute phase proteins involved in intestinal inflammation (e.g., ovotransferrin, alpha-1 antitrypsin) in the blood (De Meyer et al., 2019; Bindari and Gerber, 2022; Ducatelle et al., 2023). For the PA study, the supplementation of high levels of PA through PSO probably caused some confounding effects. The addition of increasing dietary doses of PSO caused a reduction in broiler

feed intake and BW compared to both PC and NC birds. It was unclear whether the reduction in performance was due to the properties of PSO or PA itself. However, we suspect that the PSO properties were the main responsible for the reduction in the feed intake, which affected the overall performance of the birds fed higher inclusions of PSO in the diet. The PSO used in this study was virgin, and its high content of PUFA may have increased its susceptibility to oxidation, thereby possibly reducing the quality of the ration. Another possible reason might be related to the ability of PSO to increase glucose and fat metabolism and regulate energy intake through the activation of PPAR-γ (McFarlin et al., 2008). This could lead to increased fat metabolism and reduced energy intake in broilers, contributing to reduced growth performance, especially noticeable in diets with higher PSO levels. Therefore, we still believe that it is worth investigating the effects of PA eliminating the oil as a possible confounding effect. For this, we are collaborating with a research team to develop a baker's yeast (*Saccharomyces cerevisiae*) genetically modified to contain PA to be tested in future experiments and serve as an alternative PA source to be supplemented in the diet of broilers. Although the use of baker's yeast can also provide confounding effects in the determination of the PA effects due to its potential to work as a prebiotic (contains α-mannans and β-glucans and other potential growth-enhancing factors in its cell wall) (Ahiwe et al., 2021), we expect to observe an additive effect. Since the production of pure PA is severely limited by the fact that its natural source, the PSO, is expensive and not readily available on a large scale, the use of enriched baker's yeast with PA might be an alternative (Wang et al., 2021) worth exploring in future studies.

For the glucosamine-derived caramels study (Chapter 5), the tested products rich in FR and DOFR demonstrated some potential to enhance performance and mitigate BCO in broilers. BCO is closely linked to increased intestinal permeability, allowing bacteria to translocate from the lumen to the

joints, ultimately leading to bone necrosis (Wideman et al., 2012; Ekesi et al., 2021). However, it is important to note that in our study, we did not assess the host intestinal integrity or isolate bacteria from the bone lesions to confirm whether the lesions were caused by bacteria commonly found in the gastrointestinal tract of birds. Therefore, for future studies, we suggest the addition of analysis that allows better characterization of bacterial translocation. In addition, since no specific enteric challenge was used to test the potential of the caramel products to enhance gut health and indirectly prevent lameness in broilers, it would be interesting to test these products in broilers under enteric challenge conditions. Our research group has recently received funding for the continuation of this project, and we intend to further evaluate the most promising treatments found in this study (Chapter 5) in a new experiment with broilers challenged with the adjusted natural, subclinical NE challenge model. In addition to the aforementioned analysis to be included in further research, additional analysis of bone histology, serum immune biomarkers related to inflammation, and the measurement of keratan sulfate as a marker for cartilage degradation in broilers will also be included.

Except for the COS study 1 (Chapter 2, Study 1), all the other experiments described in this thesis were carried out amidst the challenges posed by the COVID-19 pandemic. The pandemic significantly constrained the availability of volunteers and staff, particularly on sample collection days, and temporarily restricted access to the campus and laboratories. Additionally, adherence to social distancing measures compelled us to revise our sampling logistics, necessitating careful prioritization of the most important samples to be collected while excluding others. The pandemic has not affected the overall conduction of the experiments but prevented a more in-depth investigation of the effects of the tested products.

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APPENDICES

Supplemental Table 2.1. Body weight uniformity (CV%) of broilers at 10, 25 and 38 d fed with or without chitosan oligosaccharide (COS) in the diet in Experiment 1.

Treatment	COS Inclusion	BW uniformity (CV%)		
		10 d	25 d	38 d
PC[1]	----	15.33	21.22	15.97
NC[2]	----	16.54	15.36	16.82
COS 220 kDa	0.2 g/kg	11.63	13.94	10.99
	2.0 g/kg	20.64	18.60	15.52
	5.0 g/kg	11.33	18.89	17.22
COS 180 kDa	0.2 g/kg	17.14	13.08	11.47
	2.0 g/kg	13.70	15.39	16.11
	5.0 g/kg	19.87	19.35	12.24
COS 110 kDa	0.2 g/kg	16.84	20.17	14.96
	2.0 g/kg	17.47	14.88	11.43
	5.0 g/kg	15.21	19.09	17.06
COS 95 kDa	0.2 g/kg	14.92	17.36	16.80
	2.0 g/kg	15.83	19.35	14.15
	5.0 g/kg	14.68	18.60	17.29
COS 30 kDa	0.2 g/kg	16.96	18.34	20.96
	2.0 g/kg	18.40	19.57	17.85
	5.0 g/kg	16.49	24.65	30.40
COS 25 kDa	0.2 g/kg	19.24	24.22	22.83
	2.0 g/kg	20.29	21.97	18.64
	5.0 g/kg	17.39	22.23	22.82
COS 17 kDa	0.2 g/kg	18.82	23.05	18.88
	2.0 g/kg	17.17	19.56	14.31
	5.0 g/kg	17.57	19.37	16.52
COS 14 kDa	0.2 g/kg	17.83	17.40	15.87
	2.0 g/kg	18.42	24.41	23.08
	5.0 g/kg	18.72	19.34	20.42
SEM		0.51	0.69	0.83
P-value[3]		0.76	0.80	0.39

[1] PC: Positive Control (basal diet with antibiotic and coccidiostat).
[2] NC: Negative Control (basal diet without antibiotic and coccidiostat).
[3] P-value for One-way ANOVA parametric test.

Supplemental Table 2.2. Body weight uniformity (CV%) of broilers at 10, 17, 25 and 36 d fed with or without chitosan oligosaccharide (COS) in the diet in Experiment 2.

Treatment	COS Inclusion	Body weight uniformity (CV%)			
		10 d	17 d	25 d	36 d
PC[1]	----	13.21	16.83	15.17	15.00
NC[2]	----	14.95	16.97	16.02	15.98
COS 95 kDa	0.2 g/kg	15.41	17.21	16.80	17.10
COS 95 kDa	5.0 g/kg	14.68	15.90	14.47	14.15
COS 110 kDa	5.0 g/kg	14.59	16.48	15.58	16.20
COS 180 kDa	0.2 g/kg	14.50	17.00	17.69	17.39
COS 180 kDa	2.0 g/kg	13.58	15.54	14.03	14.82
COS 180 kDa	5.0 g/kg	15.75	17.98	16.30	14.92
SEM		0.94	1.07	1.15	1.04
P-value[4]		0.57	0.82	0.38	0.29

[1] PC: Positive Control (basal diet with antibiotic and coccidiostat).
[2] NC: Negative Control (basal diet without antibiotic and coccidiostat).
[3] P-value for One-way ANOVA parametric test.

Supplemental Table 2.3. Evaluation of necrotic enteritis (NE) gross lesion scores in the jejunum of broilers at 22 d and 38 d of age, fed with or without chitosan oligosaccharides (COS) in the diet in Experiment 2.

Treatment	COS Inclusion	Sample size	---------- NE gross lesion scores[4] ----------	
			22 d	38 d
PC[1]	----	16	0.94	1.13
NC[2]	----	16	0.69	0.94
COS 95 kDa	0.2 g/kg	16	0.56	1.00
COS 95 kDa	5.0 g/kg	16	0.69	1.25
COS 110 kDa	5.0 g/kg	16	0.63	1.25
COS 180 kDa	0.2 g/kg	16	0.69	0.94
COS 180 kDa	2.0 g/kg	16	0.94	0.88
COS 180 kDa	5.0 g/kg	16	0.94	1.25
SEM			0.05	0.04
P-value[3]			0.46	0.14

[1] PC: Positive Control (Basal diet with antibiotic and coccidiostat).
[2] NC: Negative Control (Basal diet without antibiotic and coccidiostat).
[3] P-value for Kruskal-Wallis One-Way Nonparametric AOV test.
[4] The lesion scoring system used ranged from 0 (no detected lesions) to 4 (extensive presence of fibrin, necrotic tissue, and inflammation covering mucosae).

Supplemental Table 5.1. P-values for orthogonal contrasts between the experimental groups and the Control for the average body weight (BW) of broilers at 10, 25 and 38 d of age.

Treatments	---------- Orthogonal Contrast P-value ----------		
	Average BW/bird at 10 d	**Average BW at 25 d/bird**	**Average BW at 38 d/bird**
Control vs GlcN	0.92	0.86	0.39
Control vs BC 0.08%	0.95	0.58	0.41
Control vs BC 0.16%	0.82	0.21	0.33
Control vs BC 0.24%	0.81	0.93	0.32
Control vs BC+F 0.08%	0.96	0.79	0.97
Control vs BC+F 0.16%	0.34	0.21	0.27
Control vs BC+F 0.24%	0.40	0.51	0.26
Control vs LC 0.08%	0.76	0.15	0.73
Control vs LC 0.16%	0.25	0.07	0.08
Control vs LC 0.24%	0.05	0.30	0.21
Control vs LC+F 0.08%	0.07	0.15	0.16
Control vs LC+F 0.16%	0.48	0.87	0.53
Control vs LC+F 0.24%	0.23	0.81	0.72

Control = A basal, commercial-type diet.
GlcN = Control diet plus the supplementation of glucosamine at 0.24% of the diet.
BC = Brown caramel produced at 90°C and rich in melanoidins, included at 0.08, 0.16 or 0.24% of the diet.
BC+F = Brown Caramel plus caramelized fructose, included at 0.08, 0.16 or 0.24% of the diet.
LC = Light Caramel produced at 50°C, and rich in fructosazine and deoxyfructosazine.
LC+F = Light Caramel plus caramelized fructose, included at 0.08, 0.16 or 0.24% of the diet.

Supplemental Table 5.2. P-values for orthogonal contrasts between the experimental groups and the Control for the performance parameters of broilers at starter (0 to 10 d), grower (11 to 25 d), finisher (26 to 38 d), and entire period (0 to 38 d).

Treatments	Orthogonal Contrast P-value											
	BWG 0-10 d	FI 0-10 d	FCR 0-10 d	BWG 11-25 d	FI 11-25 d	FCR 11-25 d	BWG 26-38 d	FI 26-38 d	FCR 26-38 d	BWG 0-38 d	FI 0-38 d	FCR 0-38 d
Control vs GlcN	0.77	0.97	0.64	0.80	0.91	0.77	0.50	0.70	0.44	0.36	0.76	0.25
Control vs BC 0.08%	0.96	0.31	0.34	0.43	0.65	0.36	0.52	0.91	0.29	0.42	0.82	0.58
Control vs BC 0.16%	0.88	0.29	0.48	0.33	0.55	0.26	0.56	0.69	0.78	0.33	0.50	0.58
Control vs BC 0.24%	0.76	0.19	0.45	0.88	0.84	0.98	0.86	0.71	0.69	0.31	0.98	0.07
Control vs BC+F 0.08%	0.92	0.59	0.85	0.93	0.86	0.39	0.95	0.78	0.60	0.97	0.86	0.82
Control vs BC+F 0.16%	0.34	0.75	0.06	0.26	0.20	0.84	0.85	0.13	0.22	0.26	0.06	0.59
Control vs BC+F 0.24%	0.40	0.33	0.62	0.79	0.67	0.83	0.54	0.82	0.23	0.27	0.67	0.39
Control vs LC 0.08%	0.95	0.17	0.31	0.21	0.12	0.55	0.67	0.29	0.39	0.74	0.69	0.87
Control vs LC 0.16%	0.25	0.07	0.80	0.09	0.09	0.36	0.98	0.77	0.87	0.08	0.29	0.24
Control vs LC 0.24%	0.07	0.08	0.31	0.41	0.24	0.75	0.58	0.92	0.41	0.21	0.46	0.40
Control vs LC+F 0.08%	0.07	0.85	0.03	0.21	0.12	0.55	0.43	0.23	0.74	0.15	0.08	0.48
Control vs LC+F 0.16%	0.54	0.28	0.93	0.88	0.84	0.98	0.30	0.74	0.36	0.53	0.97	0.33
Control vs LC+F 0.24%	0.29	0.08	0.79	0.69	0.93	0.29	0.94	0.86	0.73	0.73	0.90	0.76

BWG = body weight gain; FI = feed intake; FCR = feed conversion ration.

Control = A basal, commercial-type diet.

GlcN = Control diet plus the supplementation of glucosamine at 0.24% of the diet.

BC = Brown caramel produced at 90°C and rich in melanoidins, included at 0.08, 0.16 or 0.24% of the diet.

BC+F = Brown Caramel plus caramelized fructose, included at 0.08, 0.16 or 0.24% of the diet.

LC = Light Caramel produced at 50°C, and rich in fructosazine and deoxyfructosazine.

LC+F = Light Caramel plus caramelized fructose, included at 0.08, 0.16 or 0.24% of the diet.

Supplemental Table 5.3. P-values for the effects of the pairwise comparison between the experimental treatments and the Control group on gait scores at 11, 26 and 39 days of age of broilers.

Treatments	---------- Orthogonal Contrast P-value ----------		
	---------- Gait score ----------		
	11 d	**26 d**	**39 d**
Control vs GlcN	0.13	0.83	1.00
Control vs BC at 0.08%	1.00	0.30	1.00
Control vs BC at 0.16%	1.00	0.12	1.00
Control vs BC at 0.24%	1.00	0.12	<0.01
Control vs BC+F at 0.08%	1.00	0.12	0.13
Control vs BC+F at 0.16%	1.00	0.12	1.00
Control vs BC+F at 0.24%	1.00	0.12	1.00
Control vs LC at 0.08%	1.00	0.21	0.61
Control vs LC at 0.16%	0.13	0.41	0.26
Control vs LC at 0.24%	1.00	0.12	1.00
Control vs LC+F at 0.08%	0.13	0.75	1.00
Control vs LC+F at 0.16%	1.00	0.17	0.04
Control vs LC+F at 0.24%	1.00	0.84	0.61

Control = A basal, commercial-type diet.
GlcN = Control diet plus the supplementation of glucosamine at 0.24% of the diet.
BC = Brown caramel produced at 90°C and rich in melanoidins, included at 0.08, 0.16 or 0.24% of the diet.
BC+F = Brown Caramel plus caramelized fructose, included at 0.08, 0.16 or 0.24% of the diet.
LC = Light Caramel produced at 50°C, and rich in fructosazine and deoxyfructosazine.
LC+F = Light Caramel plus caramelized fructose, included at 0.08, 0.16 or 0.24% of the diet.

Supplemental Table 5.4. P-values for the effects of the pairwise comparison between the experimental treatments and the Control group on femoral and tibial head gross lesion scores at 12, 27 and 40 days of age of broilers.

Treatments	---------- Orthogonal Contrast P-value ----------					
	--- Femoral lesion scores ---			---- Tibial lesion scores ----		
	12 d	27 d	40 d	12 d	27 d	40 d
Control vs GlcN	<0.01	0.29	0.62	0.84	0.34	0.17
Control vs BC at 0.08%	1.00	0.12	1.00	0.84	0.23	0.02
Control vs BC at 0.16%	1.00	0.12	0.21	0.23	0.34	0.12
Control vs BC at 0.24%	1.00	1.00	0.14	0.42	0.34	0.08
Control vs BC+F at 0.08%	1.00	0.60	0.45	0.32	0.34	0.03
Control vs BC+F at 0.16%	1.00	1.00	0.62	0.55	0.48	0.12
Control vs BC+F at 0.24%	1.00	1.00	0.14	0.69	0.64	0.12
Control vs LC at 0.08%	1.00	0.60	0.21	0.55	0.24	0.27
Control vs LC at 0.16%	1.00	1.00	0.80	0.42	0.63	0.18
Control vs LC at 0.24%	1.00	0.60	0.61	0.84	0.81	0.03
Control vs LC+F at 0.08%	1.00	1.00	0.21	0.42	0.64	0.12
Control vs LC+F at 0.16%	1.00	0.30	1.00	0.69	0.48	0.05
Control vs LC+F at 0.24%	1.00	0.60	0.14	0.31	0.06	0.37

Control = A basal, commercial-type diet.

GlcN = Control diet plus the supplementation of glucosamine at 0.24% of the diet.

BC = Brown caramel produced at 90°C and rich in melanoidins, included at 0.08, 0.16 or 0.24% of the diet.

BC+F = Brown Caramel plus caramelized fructose, included at 0.08, 0.16 or 0.24% of the diet.

LC = Light Caramel produced at 50°C, and rich in fructosazine and deoxyfructosazine.

LC+F = Light Caramel plus caramelized fructose, included at 0.08, 0.16 or 0.24% of the diet.