In ovo and feed application of probiotics or synbiotics and response of broiler chicks to post-hatch necrotic enteritis

By

Mallory Beth White

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Rami A. Dalloul, Chair
Samer W. El-Kadi
F. William Pierson
Eric A. Wong

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Academic Abstract

Immediately post-hatch, broiler chicks are exposed to microbes that begin colonizing the gut, including environmental pathogens. One of the costliest enteric diseases in broiler production is necrotic enteritis (NE), caused by the ubiquitous opportunistic bacteria *Clostridium perfringens* (CP). With the worldwide reduction in antibiotic growth-promoters (AGPs), there is increased interest in natural alternatives to reduce disease and improve broiler health. The overall objective of the studies described herein was to apply probiotics or synbiotics to birds by *in ovo* application or orally before they leave the hatchery, then evaluate bird performance and various intestinal responses. Data were analyzed in JMP with LS Means to separate means with significance assigned at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. The first 21-day (D) study used 480 male Cobb 500 broilers randomly divided into one of four treatments using a 2x2 factorial design: a no-additive control (**CTRL**), a one-time oral application of synbiotic at the hatchery fed a basal diet (**HS**), an oral application of water at the hatchery with dietary synbiotics (**DS**), and a hatchery synbiotic plus dietary synbiotic (**HSDS**). Performance was measured on day-of-hatch (DOH), D3, D7, D14, and D21. mRNA abundance of various intestinal markers was measured at D7 and D21, including tight junction proteins ZO-1, ZO-2, and CLD-1; nutrient transporters SGLT1 and PepT1; and immune response markers TLR2, TLR4, and IL-10. HS lowered feed intake (FI) and feed conversion ratio (FCR) without lowering body weight (BW) from D14-21. There was greater abundance of PepT1 mRNA ($P \leq 0.1$) and IL-10 mRNA ($P \leq$

0.05) on D21 in HSDS. Second, a 21-day pilot study with 480 male and female Cobb 500 broilers was conducted to determine the optimum *in ovo* dosage level of a probiotic or synbiotic (PROB or SYNB) applied at embryonic day 18 (E18) with subsequent NE challenge using seven treatments: *in ovo* application of sterile water (**CTRL**), low (**PROB-L** or **SYNB-L**: $1x10^5$ CFU), medium (**PROB-M** or **SYNB-M**: $1x10^6$ CFU), or high (**PROB-H** or **SYNB-H**: $1x10^7$ CFU) probiotic or synbiotic doses dissolved in sterile water. Performance measurements were taken on DOH, D4, D8, D14 and D21. On D8, NE lesion scores were not impacted by treatment. D8 ileal samples were taken for mRNA abundance of TLR4, IL-10, IL-1β, AvBD8, AvBD10, and AvBD13. SYNB-H had higher abundance of AvBD10 mRNA compared to CTRL ($P \leq 0.1$), and higher IL-1β mRNA compared to SYNB-L ($P \leq 0.05$). PROB-H and SYNB-H had better performance than the low and medium doses, but were not better than the CTRL. The high doses were chosen for subsequent studies. Third, a longer 42-day study using 1,630 Ross 708 male and female broilers was conducted consisting of the following six treatments. A negative control (**NC**): sterile water *in ovo* fed basal corn/soybean meal mash diet without NE challenge; antibiotic growth-promoter (**AGP+**): sterile water *in ovo* fed basal diet with virginiamycin (0.5 kg/MT) as an AGP with NE challenge; **NC+**: same as NC plus NE challenge; **SI+**: synbiotic *in ovo* fed the basal diet and NE challenged; **SD+**: sterile water *in ovo* fed basal diet supplemented with synbiotic (0.5 kg/MT feed) and NE challenged; and **SID+**: synbiotic *in ovo* fed basal diet with synbiotic (0.5 kg/MT feed) with NE challenge. Cumulatively, SID+ had lower FI and FCR than NC+, but no change in BW or BWG. The combination treatment (SID+) often had an additive effect compared to SD+ or SI+ alone on mRNA abundance and D7 cecal fatty acid profiles. SD+ and SID+ also had higher D42 lean:fat ratios compared to NC+. Last, a 42-day study was conducted using 1,630 male and female Ross 708 broilers and the *in ovo* application

of probiotics and subsequent NE challenge with five treatments. **NC**: sterile water *in ovo*, fed basal corn/soybean meal mash diet without NE challenge; **AGP**+: sterile water *in ovo*, fed basal diet with virginiamycin (0.5 kg/MT of feed) as AGP with NE challenge; **NC**+: NC treatment, with NE challenge; **PI**+: probiotic *in ovo*, fed basal diet, with NE challenge; **PD**+: sterile water *in ovo*, fed basal diet supplemented with probiotic (1.3 kg/MT of feed), with NE challenge. The use of probiotics in this study had little effect on performance, lean:fat ratios, and cecal fatty acid profiles, but PD+ increased mRNA abundance of D14 TLR2, D14 TNF-α, and D42 LEAP2 in cecal tonsils compared to controls. PI+ increased mRNA abundance of D7 and D42 MUC2, D7 LEAP2, and D42 TNF-α in the ileum. PI+ increased mRNA abundance in the cecal tonsils of D7 TLR2 and D42 TNF-α. These studies yielded interesting results about probiotics and synbiotics during a NE challenge by evaluating performance, intestinal immune responses, and fatty acid profiles in the ceca of broilers. In conclusion, the probiotic in this study did not improve broiler health during a NE challenge, but synbiotic use *in ovo* and continuation in the feed showed improvement over *in ovo* or dietary application alone. Synbiotic improved FCR over a challenged control, and altered mRNA abundance in the small intestine.

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General Audience Abstract

The poultry industry is one of the most popular animal protein sources worldwide. As with any livestock operation, industry goals include optimizing animal health and well-being, maximizing animal productivity, and producing quality products in the most cost effective manner. Improvements in genetics, nutrition, and management have increased productivity and cut costs. One important application was the low-level use of antibiotics in feed. These medications reduced the risk of disease outbreak in flocks, which led to healthier birds and improved growth rates. However, when global concern of antibiotic resistance in human medicine came to light, both the livestock industry and governing bodies implemented voluntary and mandatory reduction or elimination of antibiotics. Previously, these important antibiotics helped to control costly diseases. As they are removed, alternatives to antibiotics will be important in disease control and prevention. A major group of alternatives to antibiotics in poultry includes probiotics, prebiotics, and synbiotics. Probiotic bacteria are considered 'good bacteria' in the gut, and provide various health benefits to the host. Prebiotics are non-living substances that support the growth of healthy bacteria. A synbiotic is the combination of both probiotics and prebiotics in a single application method. The goal of this research project was to give probiotics or synbiotics to broiler chicks and evaluate their potential benefits and effects on bird performance and the immune response. Ideally, applying probiotic bacteria as early as possible might translate into early colonization of the gut with healthy bacteria. This included oral application of synbiotics at the hatchery, or by safely injecting them into part of the egg that is swallowed by

the chick embryo before hatch. This egg application, or *in ovo* application, is a safe, effective, widely-practiced method of vaccinating chicks to jumpstart their defense against disease. By vaccinating them *in ovo*, they can start to prime the immune system before they even hatch. Applying probiotics *in ovo* may improve health after early gut colonization with beneficial microbes. Numerous studies on natural alternatives to antibiotics have been conducted, with varying results. Results of this research indicate that *in ovo* application of probiotics and synbiotics is safe. Birds that received probiotics in the feed often performed similar to those that received none. However, the *in ovo* use of synbiotics combined with the continued use in the feed after hatch improved efficiency in broilers during an intestinal disease challenge and improved various aspects of gut function. Overall, as antibiotics are phased out, using probiotics and synbiotics may improve poultry health, but continued research will help understand the optimum ways to use them.

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Abbreviations

AGP = Antibiotic growth promoter
ANOVA = analysis of variance
AvBD = avian beta-defensin
BW = body weight
BWG = body weight gain
CLD-1 = claudin-1
CP = *Clostridium perfringens*
CTRL = control
D = day
DS = dietary synbiotic
DXA = dual x-ray absorptiometry
E = embryonic day
FCR = feed conversion ratio
FI = feed intake
HS = hatchery synbiotic
HSDS = hatchery synbiotic + dietary synbiotic
IL-10 = interleukin-10
LEAP2 = liver-expressed antimicrobial peptide 2
LSMeans = least square means
mRNA = messenger RNA
MT = metric ton
NC = negative control, no NE challenge
NC+ = negative control, with NE challenge
NE = necrotic enteritis
PD+ = probiotic in feed, with NE challenge
PepT1 = peptide transporter 1
PI+ = probiotic *in ovo*, with NE challenge
PROB = probiotic
qRT-PCR = quantitative real-time polymerase chain reaction
SCFA = short-chain fatty acid
SD+ = synbiotic in feed, with NE challenge
SEM = standard error means
SGLT1 = sodium glucose transporter 1
SI+ = synbiotic *in ovo*, with NE challenge
SID+ = synbiotic *in ovo* and in feed, with NE challenge
SYNB = synbiotic
TJ = tight junction
TLR2, TLR4 = Toll-like receptor 2, Toll-like receptor 4
VFA = volatile fatty acid
ZO-1, ZO-2 = zonula-occludens-1, zonula occludens-2

CHAPTER I

Introduction

Poultry is one of the most popular meats consumed worldwide, and the market continues to grow each year. Global poultry production increased 3.7% from 2018 to 2019, and the United States is one of the largest poultry producers in the world (Berkout, 2020). Increased production will also require alternative methods to address existing and future disease challenges as the use of antibiotics is phased out. Antibiotic use in livestock is becoming more difficult for various reasons. Antibiotic resistant bacteria pose a public health threat, including *Salmonella*, which continues to be isolated from poultry products (Liljebjelke et al., 2017). Concerns of antibiotic resistance and finding antibiotic residues in meat have driven both voluntary and mandatory shifts away from antibiotic growth-promoters (AGPs) in animal feed in both the European Union and United States (Castanon, 2007; Veterinary Feed Directive, 2015). Adding sub-therapeutic levels of antibiotics are effective growth promoters because they curtail levels of certain microbes in the gut that would otherwise take up some nutrients from feed for their own use and require energy to maintain, often referred to as the nutrient cost (Dibner and Richards, 2005; Yang et al., 2009). Antibiotics may also improve efficiency by decreasing the activity of intestinal inflammatory cells (Niewold, 2007). As medication restrictions increase, research into alternatives continues to draw interest. Some of these include antimicrobial plant-derived phytogenics, organic acids, enzymes to improve digestion, beneficial probiotics, prebiotics, or combinations of products (Calik and Ergün, 2015).

Although gut microbes use up some energy coming from the diet, they provide essential benefits to the host. Overall, the gut microbiota is comprised of beneficial, commensal, and pathogenic microbes found along the entirety of the gastrointestinal tract (GIT). The necessity of microbes

can be demonstrated in germ-free animals that fail to develop proper immune systems compared to animals raised in conventional systems (Berg and Savage, 1975). A healthy microbiota contributes to immune system development. Microbes produce useful fermentation byproducts like short-chain fatty acids and vitamins that can be used by the host (Broom, 2017). Beneficial probiotic bacteria can also improve host health through competitive exclusion of pathogens (Cox and Dalloul, 2015). This is achieved by the physical occupation of attachment sites in the gut, preventing pathogens from finding a place to adhere to the gut. Probiotics also compete for resources and produce bacteriocins, restricting a pathogen's ability to grow. Common probiotic genera include *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, *Streptococcus*, and *Pediococcus* (Abd El-Hack et al., 2020). Lactobacilli produce lactic acid, lowering gut pH to achieve suboptimal growing conditions for other bacteria. One way of promoting growth of probiotic bacteria is by supplementing prebiotics to stimulate growth (Gibson and Roberfroid, 1995). These non-living substances can include oligosaccharides, such as fructooligosaccharides and inulin. The combination of prebiotics and probiotics can be referred to as synbiotics, and may improve gut health and reduce disease.

A commercially important disease in poultry is necrotic enteritis (NE) caused by the ubiquitous opportunistic bacteria *Clostridium perfringens* (CP). These bacteria are considered a normal part of the chicken's gut, but proliferate and release tissue-damaging toxins when certain conditions are met. A dietary change, increased digesta viscosity, slow feed passage, increased mucus production, or existing damage to the gut can trigger a NE outbreak (Hargis, 2014). A major predisposing factor is coccidiosis, a common disease caused by parasites that infect and rupture enterocytes. There is increased mucus production to aid in flushing out the parasites, but this amplified mucogenesis aids CP proliferation and often leads to NE outbreaks (Collier et al.,

2008). The application of probiotics may be able to reduce disease severity caused by coccidiosis or NE (Dalloul et al., 2005; Lee et al., 2007; Emami et al., 2019).

Since the GIT can be colonized by environmental microbes as soon as a chick hatches, the earlier the application of probiotics, the higher chance they have of colonizing the gut and providing subsequent benefits (Broom, 2017; Yadav and Jha, 2019). *In ovo* application of probiotics or synbiotics constitutes an early, safe, effective application method. *In ovo* injections also reduce labor costs involved in post-chick handling, and allow for precise delivery (Ricks et al., 1999; Cox and Dalloul, 2014; Pender et al., 2016; Pender et al., 2017). The goals of the following experiments were to study the effects of early and *in ovo* application methods of probiotics and synbiotics with or without a NE challenge. Specific objectives were:

Chapter 3 determined 21-day broiler performance and response to early application of an oral synbiotic applied at the hatchery.

Chapter 4 assessed a 21-day pilot study conducted to determine effects of *in ovo* injection of low, medium, or high doses of probiotics or synbiotics during a NE challenge in order to select the optimum doses for larger, subsequent 42-day studies.

Chapter 5 evaluated effects of *in ovo* application of synbiotics during a post-hatch NE challenge by analyzing performance, lesion scores, volatile fatty acid concentrations in the ceca, D42 body composition, and mRNA abundance of TLR2, TLR4, CLD1, MUC2, TNF-α, LEAP2, AvBD8, AvBD10, AvBD13 and GPR43 in the ileum and cecal tonsils.

Chapter 6 measured response to a NE challenge after *in ovo* application of probiotics, including performance, lesion scores, D42 body composition, volatile fatty acid concentrations in the ceca, and mRNA abundance of the same markers evaluated in chapter 5.

References

Abd El-Hack, M.E., M.T. El-Saadony, M.E. Shafi, S.Y.A. Qattan, G.E. Batiha, A.F. Khafaga. A.E. Abdel-Moneim, and M. Alagawany. 2020. Probiotics in poultry feed: A comprehensive review. J. Ani. Physiol. Ani. Nutr. 104:1835-1850.

Berg, R.D., and D.C. Savage. 1975. Immune responses of specific pathogen-free and gnotobiotic mice to antigens of indigenous and nonindigenous microorganisms. Infect. Immun. 11:320-339.

Berkhout, N. 2020. Popularity of poultry continues globally. Poultry World. Available at https://www.poultryworld.net/Meat/Articles/2020/7/Popularity-of-poultry-continues-globally-615517E/ (verified 26 April 2021).

Broom, L.J. 2017. The sub-inhibitory theory for antibiotic growth promoters. Poult. Sci. 96:3104-108.

Calik, A., and A. Ergün. 2015. Effect of lactulose supplementation on growth performance, intestinal histomorphology, cecal microbial population, and short-chain fatty acid composition of broiler chickens. Poult. Sci. 94:2173-2182.

Castanon, J.I. 2007. History of the use of antibiotic as growth promoters in European poultry feeds. Poult. Sci. 86:2466-2471.

Collier, C.T., C.L. Hofacre, A.M. Payne, D.B. Anderson, P. Kaiser, R.I. Mackie, and H.R. Gaskins. 2008. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. Vet. Immunol. Immunopathol. 122:104-115.

Cox, C.M., and R.A. Dalloul. 2015. Immunomodulatory role of probiotics in poultry and potential *in ovo* application. Benef. Microbes 6:45-52.

Dalloul, R.A., H.S. Lillehoj, N.M. Tamim, T.A. Shellem, and J.A. Doerr. 2005. Induction of local protective immunity to *Eimeria acervulina* by a *Lactobacillus*-based probiotic. Comp. Immunol. Microbiol. Infect. Dis. 28:351-361.

Dibner, J.J., and J.D. Richards. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poult. Sci. 84:634-643.

Emami, N.K., A. Calik, M.B. White, M. Young, and R.A. Dalloul. 2019. Necrotic enteritis in broiler chickens: the role of tight junctions and mucosal immune responses in alleviating the effect of the disease. Microorganisms 7:231.

Gibson, G.R., and M.B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125:1401-1412.

Gibson, G. R.; H.M. Probert, R.A. Rastall, and M.B. Roberfroid, 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr. Res. Rev. 17:259-275.

Hargis, B.M. 2014. Overview of necrotic enteritis in poultry. Merck Veterinary Manual. https://www.merckvetmanual.com/poultry/necrotic-enteritis/overview-of-necrotic-enteritis-in-poultry.

Lee, S.H., H.S. Lillehoj, R.A. Dalloul, D.W. Park, Y.H. Hong, and J.J. Lin. 2007. Influence of *Pediococcus*-based probiotic on coccidiosis in broiler chickens. Poult. Sci. 86:63-66.

Liljebjelke, K.A., C.L. Hofacre, D.G. White, S. Ayers, M.D. Lee, and J.J. Maurer. 2017. Diversity of antimicrobial resistance phenotypes in *Salmonella* isolated from commercial poultry farms. Front. Vet. Sci. 4:96.

Niewold, T.A. 2007. The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. Poult. Sci. 86:605-609.

Pender, C.M., S. Kim, T.D. Potter, M.M. Ritzi, M. Young, and R.A. Dalloul. 2016. Effects of *in ovo* supplementation of probiotics on performance and immunocompetence of broiler chicks to an *Eimeria* challenge. Benef. Microbes 7:699-705.

Pender, C.M., S. Kim, T.D. Potter, M.M. Ritzi, M. Young, and R.A. Dalloul. 2017. *In ovo* supplementation of probiotics and its effects on performance and immune-related gene expression in broiler chicks. Poult. Sci. 96:1052-1062.

Ricks, C.A., A. Avakian, T. Bryan, R. Gildersleeve, E. Haddad, R. Ilich, S. King, L. Murray, P. Phelps, R. Poston, C. Whitfill, and C. Williams. 1999. *In ovo* vaccination technology. Adv. Vet. Med. 41:495-515.

Veterinary Feed Directive: A Rule by the Food and Drug Administration on 06/03/2015. 2015. Pages 31707-31735. Published by the Food and Drug Administration, HHS. https://www.federalregister.gov/documents/2015/06/03/2015-13393/veterinary-feed-directive.

Yadav, S., and R. Jha. 2019. Strategies to modulate the intestinal microbiota and their effects on nutrient utilization, performance, and health of poultry. J. Anim. Sci. Biotechnol. 10:2.

Yang, Y., P.A. Iji, and M. Choct. 2009. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. Worlds Poult. Sci J. 65:97-114.

CHAPTER II

Literature Review

Information in this literature review covers the avian immune system, the prevalent parasitic disease known as coccidiosis, the opportunistic bacteria causing necrotic enteritis, the benefits of feeding probiotics or synbiotics to challenged animals, and the role of the microbiome in poultry health. The subsequent studies yielded interesting results pertaining to the immune response, broiler performance, and benefits of early feeding and *in ovo* application of probiotics or synbiotics.

Avian Immune System

The avian immune system uses multiple methods to recognize, exclude, and eliminate environmental pathogens. The two branches discussed include innate and adaptive immunity. The innate system is characterized by non-specific recognition of pathogens, rapid response, but without developing memory. It involves both physical barriers, antimicrobial proteins and chemicals, and specialized innate immune cells (Juul-Madsen et al., 2014). The adaptive immune system involves pathogen recognition via highly specialized receptors, slower initial response, the development of memory, and ability to induce rapid protection upon second encounter with a pathogen. The avian immune system is capable of producing a valuable inflammatory response against pathogens, but this inflammation can often cause undesirable damage to the host (Broom and Kogut, 2018a).

Innate Immunity

The initial exclusion and elimination of pathogens falls to aspects of the innate immune system, including physical and chemical barriers, leukocytes, and cell signaling molecules. The largest physical barrier includes the tightly packed epithelial cells themselves and antimicrobial substances that cover epithelial surfaces including the skin, respiratory, and gastrointestinal tracts (Juul-Madsen et al., 2014). The innate immune response is rapid and consistent despite the number of times the antigen is encountered. An intact physical barrier is a vital first defense against the external environment, but if pathogens break past physical barriers, there are additional layers of chemical and cellular defenses in place.

Physical Barriers

Normal microbiota on the skin and in the gastrointestinal tract prevent pathogens from colonizing, a term referred to as competitive exclusion (Van Immerseel et al., 2005). Mucus in the respiratory tract traps foreign material and cilia movement takes particles away from the lungs. Low pH in the stomach and high body temperature also hinder microbial growth (Juul-Madsen et al., 2014). Antimicrobial peptides secreted by specialized epithelial cells lyse bacteria and viruses by disrupting bacterial cell walls (Cuperus et al., 2013). Saliva, serum, gut contents, and mucus contain lysozyme, which can lyse bacteria by enzymatic digestion of components of the cell wall (Al-Khalaifah and Al-Nasser, 2018). Also, lysozyme triggers other aspects of the immune system including activation of Toll-like receptors (TLRs) (Bar Shira and Friedman, 2018).

Toll-like Receptors

TLRs are innate receptors and highly conserved across many organisms, with homologues often found in both plants and animals. These receptors, collectively referred to as pattern recognition receptors (PRR), recognize highly conserved patterns belonging to broad categories of pathogens, termed microbe-associated molecular patterns (MAMPs) (Akira and Takeda, 2004). TLRs play an important role in microbe recognition by avian heterophils, the equivalent to mammalian neutrophils (Farnell et al., 2003). TLR2 is mostly associated with recognition of lipoproteins, lipoteichoic acid, peptidoglycan, glycolipids of protozoal parasites, and fungal zymosan associated with Gram-positive bacteria and yeast. TLR4 recognizes lipopolysaccharides (LPS) often associated with recognition of Gram-negative bacteria (Opitz et al., 2001; Keestra et al., 2007; Uematsu and Akira, 2008). In response to pathogenic bacteria, activated cells trigger downstream pathways that induce the production of inflammatory cytokines and chemokines to further facilitate immune responses.

TLRs can be found on cells in the spleen, cecal tonsils, liver, and especially in heterophils (Juul-Madsen et al., 2014). Avian heterophils, like mammalian neutrophils, are innate immune granulocytes capable of releasing toxic enzymes, antimicrobial peptides, and using oxidative burst to kill pathogens (Harmon, 1998; Kogut et al., 2001). Farnell et al. (2003) demonstrated that chTLR2 recognizes lipoteichoic acid from *Staphylococcus aureus* and was responsible for oxidative burst and degranulation in heterophils.

The chicken TLR4 and myeloid differentiation protein-2 (chMD-2) complex stimulated by LPS activate MyD88-dependent signaling in chicken macrophages (Keestra and van Putten, 2008). This ultimately activates the inflammatory regulatory transcription factor NF-κB, leading to

production of antimicrobial peptides, interferons (IFN), costimulatory molecules, and inflammatory cytokines such as interleukin (IL)-1β, IFN-α, and IFN-γ (Brownlie and Allan, 2010; Guriec et al., 2018). Chickens lack the mammalian equivalent of a MyD88-independent pathway that results in IFN-β secretion, which results in an animal more resistant to endotoxic shock caused by LPS (Berczi et al., 1966; Keestra and van Putten, 2008).

Chemical Barriers: Host Defense Peptides

A group of evolutionarily conserved antimicrobial peptides (AMPs) secreted by cells can be found in most animals (Hancock and Sahl, 2006). Although AMPs consist of a diverse group of molecules, they share general antimicrobial properties against some Gram-negative and Gram-positive bacteria, viruses, and yeast (Brogden, 2005). They also may bridge innate and adaptive immunity by the ability to induce chemokine and cytokine signaling, as well as activation of dendritic cells (Townes et al., 2004). AMPs include defensins, liver-expressed antimicrobial peptide-2 (LEAP2), and cathelicidins (Cuperus et al., 2013).

Of the three subtypes of defensins discovered, chickens lack α- and θ-defensins, possessing only β-defensins (Xiao et al., 2004). These important innate host defense peptides are collectively referred to as avian β-defensins (AvBDs) and demonstrate both antimicrobial and antifungal properties (Juul-Madsen et al., 2004). At least 14 chicken defensin genes have been characterized (Xiao et al., 2004; Lynn et al., 2007; Hellgren and Ekblom, 2010) and are expressed in avian heterophils, macrophages, and epithelial cells (Evans et al., 1995; Harwig et al., 1994; Zhao et al., 2001; Sugiarto and Yu, 2006; Pan and Yu, 2014). Antimicrobial activity of AvBDs is attributed to their cationic properties. Positively charged residues on the peptides interact with negatively charged components of bacterial membranes. As a result, they disrupt the membrane

of bacterial cells through permeation and pore formation to disrupt the membrane, disrupt ion balance and the ability to maintain an osmotic gradient eventually lysing the pathogen (Dathe and Wieprecht, 1999; Brogden, 2005).

Expression of these AvBDs varies across tissue type (van Dijk et al., 2008). In the skin, moderate levels of AvBD3, -9, -11, and -14 have been reported. The respiratory tract contains high levels of AvBD1, -2, -3, -9, and -13. Likewise, expression varies along the length of the gastrointestinal tract. The proximal digestive tract, consisting of the tongue, crop, and esophagus, expresses AvBD3, -5, and -9, while the small intestine and cecal tonsils express amounts of AvBD1, -2, -8, -9, -10, and -13. Although these AvBDs are often constitutively expressed, they exhibit various patterns of upregulation and downregulation depending on bird species, breed, strain, and disease type (van Dijk et al., 2008). Brumstead and Barrow (1988) demonstrated 10X higher expression of AvBD1 and -2 in *Salmonella typhimurium*-resistant line of chickens compared to a susceptible line. Su et al. (2014, 2015a, 2017) investigated expression of AvBDs in the small intestines of broilers exposed to various strains of *Eimeria* and found different patterns of upregulation and downregulation.

LEAP2 is a peptide expressed in epithelial tissues where bacterial pathogens are often encountered (Townes et al., 2004). This peptide's broad-spectrum activity includes rapid ability to associate with and permeabilize bacterial membrane, lysing cells by creating pores (Townes et al., 2009). Although Townes et al. (2004, 2009) demonstrated intestinal upregulation of LEAP2 in response to *Salmonella*, numerous studies have confirmed *Eimeria*'s ability to downregulate LEAP2 to aid in immune evasion in chickens (Casterlow et al., 2011; Paris and Wong, 2013; Fetterer et al., 2014; Su et al., 2015, 2017).

Cathelicidins (CATHs) are expressed in a variety of avian tissues including respiratory and gastrointestinal tracts, but not so much on the skin and in the crop, brain, or esophagus (Lynn et al., 2004; van Dijk et al., 2005; Achanta et al., 2012). In the gastrointestinal tract, CATH-2 expression by heterophils seems to play an important role in defense against bacterial infections with *Salmonella* or *Campylobacter jejuni* (van Dijk et al., 2009; van Dijk et al., 2012). The antimicrobial effects against both bacteria and fungi involve interactions between cathelicidins and bacterial membranes, in which they form pores by aligning themselves through the bacterial membrane, disrupting osmotic gradients, and leading to cell rupture (Porcelli et al., 2008; Saravanan and Bhattacharjya, 2011).

Avian Blood Cells

Blood cells in birds include erythrocytes, thrombocytes, and leukocytes. Erythrocytes are responsible for oxygen and carbon dioxide transport. In contrast to mammals, erythrocytes are nucleated and contain functioning mitochondria, making them capable of their own respiration (Stier et al., 2013). Thrombocytes are involved in the control of bleeding (Olah et al., 2014). Leukocyte composition includes granulocytes (heterophils, eosinophils, and basophils) and mononuclear cells (lymphocytes and monocytes) (Jones, 2015).

Cells of the innate immune system are activated by antigens and cytokines, and do not need to be activated by other cells to function. They mount a rapid initial response to pathogens. The most common leukocyte in circulation is the heterophil, a bi- or tri-nucleated granulocyte associated with inflammation. The heterophil is equivalent to the mammalian neutrophil, one of the first cells to respond to injury and inflammation, yet they lack myeloperoxidase that neutrophils possess. They instead contain other AMPs including β-defensins (Juul-Madsen et al., 2014).

Active avian macrophages are large phagocytic cells that digest foreign material, damaged host cells, and release toxic enzymes to kill bacteria by damaging cell walls. They are also considered antigen-presenting cells, and introduce antigenic material to naïve cells of the adaptive immune system (Taylor et al., 2005). Phagocytic dendritic cells are the major antigen-presenting cells involved in activation of naïve T cells (Steinman et al., 1975; Kaspers and Kaiser, 2014).

The specialized lymphocytes of the adaptive immune system are capable of mounting a more specific response, need activation by antigen-presenting cells such as dendritic cells and macrophages, and take more time to respond to antigens. Adaptive cells begin as naïve T cells and B cells. Each lymphocyte has a unique receptor capable of more specific antigen recognition than the innate immune system. Once the lymphocyte recognizes a specific antigen, it begins clonal expansion to mount a strong adaptive immune response against the specific pathogen. T cells are responsible for cell-mediated immunity. This includes the killing of infected phagocytes and host cells to reduce the spread of pathogens and activation of antibody-producing B cells. Additionally, they suppress inflammation to protect the host from the consequences of inflammation. The development of memory T cells allows the host to mount a rapid response upon reinfection with the antigen. B cells differentiate into antibody-producing plasma cells and memory B cells to circulate and reactivate upon reinfection. Plasma cells produce antigen-specific antibodies that can stop infection by marking pathogens for destruction by innate phagocytic cells, binding to pathogens to block their ability to enter host cells, and clumping the pathogens together making it harder for the pathogens to circulate and easier for the immune system to recognize (Abbas et al., 2012a,b).

Avian Cytokines

In general, a cytokine is a small signaling peptide used to communicate information between cells, particularly immune cells (Wigley and Kaiser, 2003). Cytokines are released by every cell in the body, though not every cell is capable of releasing every type of cytokine. Epithelial cells are capable of releasing inflammatory cytokines in response to tissue injury. Macrophages that clear infections also release inflammatory cytokines to recruit immune cells to the site of infection. Other immune cells release anti-inflammatory cytokines to reduce damage to host cells near the site of damage, and aid in the reduction in immune response and return to homeostasis (Wigley and Kaiser, 2003; Kaiser and Staheli, 2014).

Interferons were originally named for their ability to interfere with viral replication in host cells, but have important inflammatory functions against bacteria as well. IFN-γ is produced by numerous immune cells as an inflammatory cytokine (Quinteiro-Filho et al., 2017). It also primes macrophages to respond more strongly to bacterial LPS by stimulating nitric oxide production and oxidative burst (Schroder et al., 2004; He et al., 2011). Tumor-necrosis factor members promote inflammation, cell proliferation, and function mainly as co-stimulatory molecules (Kaiser and Staheli, 2014). TNF-α genes are induced by the transcription factor NF-κB after TLR4 binds with LPS. There is a proposed crosstalk between TNF-α and IFN-γ, as TNF-α leads to increased activation of IFN-γ, which in turn induces surface expression of TNF-α receptors on cells (Schroder et al., 2004).

The avian cytokine IL-10 has some conserved function with mammalian orthologs (Rothwell et al., 2004). It initiates anti-inflammatory responses by inhibiting pro-inflammatory cytokine production on innate and adaptive immune cells, including T and B cells, antigen presenting

cells, and natural killer cells (Ding et al., 2003; Mocellin et al., 2003). IL-10 inhibits production of inflammatory IL-1β, TNF-α, IFN-γ, and IL-12 (Fiorentino et al., 1991; Gazzinelli et al., 1992; Aste-Amezaga et al., 1998; Rothwell et al., 2004).

Gut-Associated Lymphoid Tissue (GALT)

The small intestine digests and absorbs easily digestible carbohydrates in the proximal gut, and indigestible carbohydrates by bacteria in the distal part (Hooper et al., 2002). Avian species begin to forage for food immediately after hatch, which results in rapid colonization of the gastrointestinal tract by environmental microbes (Turk, 1982; Bar-Shira and Friedman, 2006). Colonization occurs via the oral and rectal routes, and the major site of colonization, are the two paired ceca (Clench, 1999; Bar-Shira and Friedman, 2006). The gastrointestinal tract is considered one of the largest epithelial barriers and immune organs in an animal (Choct, 2009). Although the microbes it houses contribute to the overall health of an organism, some microbes increase risk of pathogens causing infection or unwanted inflammation (Peterson and Artis, 2014).

Intestinal Epithelial Cells (IEL)

The gut is composed of a single layer of epithelial cells that act as an important barrier, yet selectively absorb nutrients and water. One group of intracellular complexes that seal the space between these cells are tight junction (TJ) proteins (Tellez Jr., et al., 2017). There are approximately 50 TJ proteins, including claudin and occludin proteins, that maintain a tight seal between the paracellular space and tightly regulate permeability to select molecules and solutes. TJ will open and close for nutrients, various cellular signals, and during increased inflammation

to allow immune cells to access potential pathogens in the gut (Barekatain et al., 2019). Proper TJ protein function is important to overall gut health through maintenance of the tight barrier between intestinal cells.

The most abundant intestinal epithelial cell is the absorptive enterocyte (Stokes, 2017). Interspersed with these are specialized epithelial cells in the small intestine contributing towards the innate physical and chemical barrier that divides the host from the external lumen. Goblet cells are specialized epithelial cells that produce and secrete trefoil peptides and glycosylated mucins into the lumen of the gut, including one of the most abundant types, mucin 2 (MUC2) (Grondin et al., 2020). The mucin and trefoil populate the mucus layer and protect against bacterial pathogens and toxins (Kalabis et al., 2006; Maldonado-Contreras and McCormick, 2011). Collectively, the mucus layer itself and antimicrobial peptides within the matrix prevent microbes from penetrating and reaching the epithelial cells of the intestinal lumen (Maldonado-Contreras and McCormick, 2011). Additionally, Paneth cells secrete antimicrobial peptides as well as lysozyme, which enters the mucus layer and contributes to the barrier between the microbial communities and host cells in the gut (Bel and Hooper, 2017). Enterocytes at the base of the villi secrete immunoglobulin A (IgA) into the lumen (Mostov, 1994). IgA can be found within cells and in high concentrations in the lumen, where it deactivates LPS and neutralizes toxins (Maldonado-Contreras and McCormick, 2011). Chickens lack lymph nodes, but have areas along the gastrointestinal tract with higher lymphoid activity, including the Meckel's diverticulum, cecal tonsils, and Peyer's patches. The tissues here are rich in dendritic cells, macrophages, B cells, and T cells. Intestinal microfold (M) cells are also highly concentrated in these areas, and facilitate antigen sampling of the intestinal lumen through phagocytosis (Broom

and Kogut, 2018b). After receiving antigens from M cells or through paracellular sampling, dendritic cells travel to naïve T cells in lymphoid areas along the gut.

Microbes and Pre- and Post-Hatch Immune Development in Broilers

As birds are immediately exposed to external microbes and intestinal colonization at hatch, the immune response must develop rapidly. Adaptive immune responses are not fully developed until 2-3 weeks post-hatch; thus, innate responses and maternal antibodies offer protection early on (Bar-Shira and Friedman, 2006). LEAP2 and all defensin genes, except AvBD11, have been detected as early as embryonic day 3 (Meade et al., 2009; Michailidis, 2010). Phagocytic macrophages have been observed as early as embryonic day 12 (E12) in the liver, and E16 in the spleen (Jeurissen and Janse, 1989). Studies using germ-free animals have shown the importance the microbiome plays in normal mucosal immune system development. Germ-free animals fail to develop normal tolerance to the diet (Hooper et al., 2012). The gut senses normal host microbiota through its PRRs and interpreting signals through microbial metabolites (Haghikia et al., 2016). Bacteria in the ceca ferment non-digestible carbohydrates into short-chain fatty acids (SCFAs), mainly butyrate, propionate, and acetate. These SCFAs can be absorbed by the host and may contribute between 5-15% of the daily maintenance energy requirements and limit pathogen growth (Annison et al., 1968; Gasaway, 1976a,b; Dunkley et al., 2007). In particular, butyrate is major energy source for enterocytes and promotes villi development in broilers (Panda et al., 2009; Donohoe et al., 2011). SCFAs can regulate various cell functions (Sun et al., 2018). Butyrate can regulate tight junction proteins between epithelial cells through increased expression of Claudin-1 (CLD-1) and Zonula Occludens-1 (ZO-1), which decreases paracellular bacterial translocation across the epithelial membrane. Decreasing bacterial and LPS

translocation inhibits macrophage activation, which decreases proinflammatory response (Wang et al., 2012; Morrison and Preston, 2016). Metabolite-sensing G-protein coupled receptors (GPR), including GPR43, sense SCFAs. In chickens, free fatty acid receptor 2 (FFAR2) is a paralog to GPR43. It is highly expressed in testis, spleen, peripheral blood mononuclear cells (PBMC), adipose tissue, intestines, and the lung (Meslin et al., 2015). SCFA production is associated with multiple benefits in the host. The binding of SCFA to GPR43 increases TJ protein expression, increases protective mucus production via activation of the gene MUC2, increases production of protective immunoglobulin IgA into the lumen, and decreases production of proinflammatory cytokines IL-1β and TNF-α (Slawinska et al., 2019; Mishra et al., 2020). These functions may contribute to overall gut health, and reduce severity of enteric diseases. Reducing infections is economically important as estimates have reported a 20-25% increase in protein usage to mount an immune response against infections (Kurpad, 2006). Up to 70% of the cost of animal production goes towards animal feed; healthy animals can achieve their genetic potential through maximum efficiency (Borda-Molina et al., 2018).

Coccidiosis

Coccidiosis caused by *Eimeria* parasites is one of the most important diseases in commercial poultry, decreasing flock productivity and causing economic losses averaging $6 billion worldwide (Dalloul et al., 2007). These species-specific parasitic protozoans are transferred via the fecal-oral route in chickens (Chapman et al., 2013). There are seven species that infect chickens. Hemorrhagic species include *E. tenella*, *E. brunetti*, and *E. necatrix* while malabsorptive species include *E. acervulina*, *E. maxima*, *E. mitis*, and *E. praecox*. Their ability to

form protective oocysts allows them to persist in the environment and resist standard cleaning methods (Blake and Tomley, 2014).

As described by Blake and Tomley (2014), the *Eimeria* life cycle begins when a bird ingests a single sporulated oocyst. This oocyst is designed to withstand the low pH in the proventriculus. Next, the oocyst actually benefits from the grinding action in the gizzard. Here, the tough oocyst wall is broken down, and each oocyst releases four sporocysts. Digestive enzymes dissolve the walls of the sporocyst, allowing sporozoites to escape, attach to and invade the epithelial wall of the intestine. Once inside the enterocyte, the sporozoite transforms into a rounded trophozoite (feeding stage) and undergoes asexual multiple fission to produce first generation merozoites. These merozoites rupture the chicken's intestinal cell and burrow into new enterocytes. Depending on the *Eimeria* species, the merozoite undergoes one, two, or more rounds of asexual reproduction and rupture of the host cell. Although each species has differing rounds of asexual reproduction, they all eventually undergo a sexual phase, where the merozoites become either large macrogametes within the host cell, or smaller, motile microgametes. A single microgamete will fertilize a large macrogamete. Then, the macrogamete develops a strong, protective wall and become an oocyst. The oocyst is shed by the host, sporulates when the right environmental temperatures are met (warmth, oxygen, moisture), and persists in the environment until it is picked up by a bird to begin the process over again. A strong cell-mediated response is more important in clearing disease and developing immunity than a strong antibody response, and birds gain immunity to specific species after subsequent infection (Soutter et al., 2020). Immunity is eventually developed, but there are specific immune reactions that occur in response to this parasite.

E. maxima infection induces high levels of IFN-γ expression, an important cytokine associated with the development of resistance to *Eimeria* infections (Yun et al., 2000; Laurent et al., 2001). Anti-inflammatory IL-10 levels counteract those of inflammatory IFN-γ, and Rothwell et al. (2004) reported equal levels of IFN-γ expression in birds susceptible and resistant to *E. maxima* infection. Interestingly, IL-10 levels were increased in the small intestines of susceptible birds, suggesting that IL-10 can inhibit protective effects of IFN-γ in birds susceptible to *E. maxima* infections. During infection with coccidiosis, macrophages infiltrate the gut and induce release of inflammatory cytokines (IL-1β, IL-6, and IL-16) and act as antigen presenting cells to stimulate adaptive immunity (Dalloul et al., 2007). It is proposed that *Eimeria* strains suppress some innate immune responses through downregulation in LEAP2 in the small intestine of broilers (Casterlow et al., 2011; Sumners et al., 2011; Fetterer et al., 2014; Su et al., 2017).

Necrotic Enteritis

Necrotic enteritis (NE) is caused by the Gram-positive spore-forming bacteria *Clostridium perfringens* (CP) (Stanley et al., 2014). This disease costs the poultry industry an average of $14.75 billion worldwide each year (Blake et al., 2020). CP is found as a commensal bacterium in poultry at low levels (Clavijo and Florez, 2018). However, various environmental factors can contribute to the pathogenicity of these opportunistic bacteria. Mycotoxins in the feed, intestinal parasites, sudden dietary changes, high levels of non-starch polysaccharides that increase viscosity of digesta and slow feed passage are all predisposing factors to NE (Williams, 2005; Clavijo et al., 2018).

Not all CP strains cause disease, and these bacteria are often found in soil, feed, sewage, and the intestines of animals. CP is classified into five groups, A-E, depending on the type of toxin they

produce: α, β, ε and ι (Shimizu et al., 2002). Its ability to cause disease has been linked with a two-component regulatory system, VirR/VirS. This region controls genes encoding for toxins and enzymes, including NetB, hyaluronidase, catalytic enzymes, and transporters, which all aid in the ability to break down tissue in live and dead birds (Ohtani and Shimizu, 2015). Sequencing of CP strains that carry the *NetB* gene continues to provide insight into disease-causing strains (Prescott et al., 2016). Many of the recently isolated genes are associated with increased fitness by *NetB* carrying strains, including sugar transporters and iron-acquisition genes (Lepp et al. 2013), and the possible presence of an additional toxin, TpeL (Chalmers et al., 2008; Coursodon et al., 2012).

Pathogenesis by NE includes different stages occurring simultaneously, but involves colonization, the ability to acquire nutrients from the damaged host, multiplication, and the ability to evade immunity (Prescott et al., 2016). Under normal circumstances, the mucus layer and bacteriocins control CP attachment and growth, but disturbances and degradation of the mucus layer seem to be a key component to disease development (Timbermont et al., 2011, 2014). Many bacterial pathogens, including disease-causing strains of NE, produce enzymes that degrade mucins, notable chitinase and glycosyl hydrolase capable of degrading glycoproteins on mucus and epithelial cell membranes (Lepp et al., 2010; Frederiksen et al., 2013). Pathogenic strains carrying the *netB* gene outcompete non-pathogenic strains. They carry a perfrin gene, encoding a protein capable of breaking down host cells and inhibiting commensal CP isolates that lack the *netB* genes (Timbermont et al., 2014; Moore, 2016). Pathogenic strains also bind more strongly to the host's extracellular matrix proteins than commensal strains (Wade et al., 2015). Breaking down mucus and host cells fuels multiplication of pathogenic CP (McGuckin et al., 2011; Ficko-Blean et al., 2012). NetB-related pore-forming toxins are responsible for

initiating intestinal damage after colonization and degradation of mucus has provided access to cells (Keyburn et al., 2008; Prescott et al., 2016). Olkowski et al. (2006, 2008) suggested that NetB-related toxins do not target the surface of the gut, but target endothelial cells. This damage results in the sloughing off of enterocytes at the surface. To aid in survival, large numbers of CP form a protective biofilm on the surface of necrotic tissue (van Hoek, 2013; Charlebois et al., 2015). After enough NetB-associated damage has exposed the extracellular matrix proteins of the intestine, CP binds via adhesins (Prescott et al., 2016). Altogether, great damage occurs to the small intestine, epithelial cells slough off, and inflammation and necrotic tissue block the animal's ability to digest and absorb nutrients.

Coinfection with *Eimeria* and *Clostridium perfringens*

In addition to anticoccidial drugs being used to prevent outbreaks of coccidiosis, vaccines containing live oocysts are routinely included as part of commercial control programs. By design, low levels of infection with live attenuated species of *Eimeria* allow the bird to develop immunity (Fatoba and Adeleke, 2018). However, the invasion, replication, and rupturing of enterocytes can trigger NE following intestinal dysbiosis caused by the coccidiosis vaccine (Yang et al., 2019).

Normally, epithelial cells of the gut are highly effective in digestive function and protection from the external environment. Enterocytes absorb nutrients and secrete mucins and antimicrobial peptides. Goblet cells secrete the majority of mucins in the gut, mostly comprised of MUC2 in the small intestine (Grondin et al., 2020). The physical and chemical barriers of the gut that are designed to inhibit enteric pathogens can be beneficially utilized by *Eimeria* and CP. *Eimeria* bind to the enterocytes on (what kind of receptors). The damage caused by coccidiosis increases

the likelihood of secondary infection by other pathogens, including *Salmonella* and CP (Blake and Tomley, 2014; Clavijo et al., 2018). *Eimeria* disrupts the physical barrier of the gut, allowing CP access to the intestinal basal layer, where it can bind to collagen and begin to colonize in early disease stages (Moore, 2016). Collier et al. (2008) demonstrated that infection with coccidiosis, specifically *E. maxima* and *E. acervulina*, increased MUC2 production by goblet cells, in an effort to flush out parasites. However, protein-rich mucus feeds the growth of CP (Moore, 2016). CP lacks certain genes needed to produce amino acids necessary for its own growth and proliferation, thus it is unable to thrive unless certain favorable conditions are met (Antonissen et al., 2016). The ruptured enterocytes release nutrient-rich serum containing growth factors, amino acids, and vitamins necessary for CP proliferation (Van Immerseel et al., 2010). As severity of NE and coccidiosis increases, enterocytes eventually slough off. Co-infection with these two pathogens is common in commercial broiler production. The use of a live vaccine containing *Eimeria* oocysts into a research facility with a known history of CP spores was the experimental method chosen to induce NE for experiments discussed in this dissertation.

Alternatives to Antibiotics in Poultry

The History and Function of Anticoccidials and Antibiotic Growth Promoters

Controlling diseases is important for animal welfare and human health, and is well-controlled by drugs (Kadykalo et al., 2018). Preventing disease is an important method of controlling enteric diseases. This includes proper sanitation of equipment, cleaning between flocks, increased biosecurity to exclude disease-carrying wildlife and humans, and good litter management (Chapman, 2018). Despite these protective measures, disease outbreaks, even at subclinical levels, can be costly. Grumbles et al. (1948) first describes the use of sulfaquinoxaline as an

anticoccidial in poultry, and in the 1970s, synthetic anticoccidials became a common method of control (Ryley and Betts, 1973). Historically, rotational (shuffle) programs in the US commercial poultry industry involve combinations of ionophores, synthetic anticoccidial drugs, and vaccination (Noack et al., 2019).

One category of anticoccidials, the ionophores, can technically be considered antibiotics, since they are fermentation products produced by *Streptomyces* spp. and *Actinomadura* spp., but they are not used as antibiotic growth promoters or important to human medicine, thus, do not contribute to drug resistant bacteria in man (Noack et al., 2019). The name 'ionophore' comes from its function – ion pore – as it functions by interfering with membranes of the parasites before they can infect the host. Monensin is an ionophore active against all species of *Eimeria* (Agtarap et al., 1967). It can form lipid-soluble complexes with both sodium and potassium ions. This increases the membrane permeability for these ions, and ruptures the parasites (Matsuoka et al., 1996). Proper dosage and adherence to species-specific use is important to reduce accidental poisonings, such as accidental deadly poisoning in horses with the ionophore Monensin (Bila et al., 2001). Another ionophore, Salinomycin, is less toxic, but binds strongly to potassium, promoting efflux of potassium ions from mitochondria and cytoplasm, again affecting membrane function of the parasites (Oehme and Pickrell, 1999; Noack et al., 2019).

The second category of anticoccidials are synthetic compounds, which have various modes of action. Some drugs target the respiratory chain of *Eimeria*, which is different from vertebrates in its use of succinate dehydrogenase rather than NADH dehydrogenase (Harder and Haberkorn, 1989). Other anticoccidial drugs inhibit the folic acid pathway, which disrupts cell replication (Lebkowska-Wieruszewska and Kowalski, 2010). One of the safest drugs, Amprolium

hydrochloride, also has poor activity against coccidiosis (Rychen et al., 2018). This drug is analogous to thiamine (vitamin B_1) but lacks any actual functionality. Since it cannot be phosphorylated, it prevents second generation schizonts of *E. tenella* from having it as a co-factor in essential metabolic reactions (James, 1980).

Virginiamycin is often used to treat outbreaks of NE. The active component is isolated from *Streptomyces virginiae* and other *Streptomyces* bacterial species, and inhibits ribosome assembly, thus prevents protein synthesis. It is used to treat infections of Gram-positive organisms (Butaye et al., 2003). Antibiotics are often included in feed at sub-therapeutic levels, and are incorporated at levels below the amount defined as bacteriocidal. Though these low levels may not be killing all bacteria, they are able to limit certain functions, including quorum sensing, toxin production, and biofilm formation (Dong et al., 2012; Broom, 2017). Low levels of antibiotics may aid in slowing infection down long enough for the host's immune system to clear infections of pathogens (Reeks et al., 2005).

As consumer concern over antibiotic resistance and potential residues in meat increases, the ability to use antibiotics has dwindled, and consumer preference for antibiotic-free alternatives has risen, further driving research into feed additives such as phytogenics, organic acids, enzymes, probiotics, and prebiotics, individually or in combination (Calik and Ergun, 2015).

Probiotics, Prebiotics, and Synbiotics

Early establishment of healthy microbiota may improve chick health and contribute to pathogen resistance. Probiotics are defined as live microorganisms able to confer a benefit to their host and often come from strains of *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Enterococcus*,

Bacteroides, *Bacillus*, *Aspergillus*, *Candida*, and *Saccharomyces* (Fuller, 1989; Parvez et al., 2006; Kabir, 2009). Probiotics are known to competitively exclude pathogens by competing for resources, occupying attachment sites in the gut, secreting lactic acid to lower gut pH, and produce bacteriocins, all of which reduce a pathogen's chances of colonizing the host (Cox and Dalloul, 2015; Clavijo and Florez, 2018).

Probiotic application in poultry has been thoroughly studied, yet conflicting results prompt continued research. Nurmi and Rantala (1973) applied gut contents isolated from healthy chickens to newly hatched chicks, and found it offered protection against *Salmonella* infection. A study using *Pediococcus acidilactici* in broilers challenged with *Eimeria* found that 0.1% incorporation into the diet resulted in improved weight gain and reduced oocyst shedding of *E. acervulina* compared to controls (Lee et al., 2007). Dalloul et al. (2005) evaluated commercial probiotic application (PrimaLac) in broilers challenged with high doses of *E. acervulina* and reported improved immune response and reduced oocyst shedding in probiotic-fed broilers. The inflammatory cytokine IFN-γ was higher 3 days post-infection in probiotic-supplemented birds. This cytokine has been associated with an improved acquired immune response and inflammatory response against coccidiosis infections. Emami et al. (2019) fed probiotics during a co-infection with coccidiosis and NE, and observed reduced lesions and mortality in birds given dietary probiotics.

In contrast to live probiotic microorganisms, prebiotics are non-living substances intended to promote the growth of probiotics (Kim et al., 2019). Gibson and Roberfroid (1995) were among the first to define these non-digestible carbohydrates that included fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), mannan-oligosaccharides (MOS), and derivatives of

such, including inulin (Ricke, 2018). A prebiotic is characterized by its ability to act as a nutrient to beneficial microbes, including *Lactobacillus* and *Bifidobacterium* species, in the distal gut without being digested by the host in the upper GIT (Ricke et al., 2020). Fermentation of prebiotics produces beneficial byproducts, including SCFAs, that provide energy to the host and contribute to pathogen inhibition. Early studies showed that *Salmonella* are capable of producing SCFAs and becoming more resistant to acidic conditions in an anaerobic environment (Kwon and Ricke, 1998). In contrast, later studies showed that the SCFA butyrate may inhibit *Salmonella* colonization by downregulating invasion genes, while propionate may decrease invasion into epithelial cells (Van Immerseel et al., 2006). The prebiotic MOS can decrease pathogen colonization by binding to the flagella of *E. coli* and *Salmonella* sp. to reduce pathogen ability to attach to host epithelial cells (Fomentini et al., 2016). The prebiotic isolated from chicory root, inulin, is a blend of fructose polymers that survives passage through the upper GIT and supports growth of beneficial *Bifidobacterium*. Inulin can increase intestinal IgA secretion and anti-inflammatory IL-10, reduce inflammatory IL-1β, and increase cell-mediated response by stimulating T cells (Madej et al., 2015).

The term synbiotics is the combined application of both prebiotics and probiotics (Roto et al., 2016). Ideally, a synbiotic would have synergistic effects; it includes both beneficial microorgansisms and the non-living nutritional substances that selectively support their growth in the GIT of the bird. Numerous studies have found a benefit of feeding probiotics, prebiotics, or synbiotics to poultry during disease challenges; however, early *in ovo* application may give additional benefit.

In ovo Applications in Poultry

History of In ovo Application and Current Uses

Historically, poultry vaccination at hatcheries was performed on the day-of-hatch by subcutaneous injection or spray cabinet, feed or drinking water application. Some of these methods can cause stress to chicks and result in non-uniform vaccine delivery. Many methods are being replaced with *in ovo* vaccination of late-stage embryos, just before they hatch (Williams, 2007). Automatic *in ovo* injection machines can rapidly create a small hole in an egg and uniformly inject vaccines and other substances in a cost-effective manner, as well as detect viable embryos before sending them to the hatcher (Jochemsen and Jeurissen, 2002; Weber and Evans, 2003). This method allows chicks to develop an earlier immune response compared to post-hatch vaccinations. Precise *in ovo* delivery and timing is essential to embryonic survival rate and vaccination success. The optimum place for delivery is in the amnion, which includes the amniotic fluid and the embryo body. A vaccine delivered in amniotic fluid will be swallowed and breathed in by the embryo before hatch. Delivery of vaccine into the embryo itself is also acceptable, but if the injection occurs too deep, it could cause trauma and reduce hatchability. The best time for *in ovo* injection is when eggs are being transferred from the incubator to the hatcher on embryonic day 17, 18, or 19 (Williams, 2007). For decades, chickens across the world have received *in ovo* vaccinations against Marek's disease virus on embryonic day 19 (Sokale et al., 2018). Vaccination against the virus began in the 1970s, and now, the majority of chickens around the world are protected by commercial use of avirulent or attenuated live virus vaccines (Witter, 2001). This has aided in rapid expansion of the poultry industry, allowing sustained, large-scale poultry production (Morrow and Fehler, 2004). This viral vaccine is well-characterized, but increasing interest in vaccinations against bacteria and parasites, including

Campylobacter jejuni and *Eimeria*, is on the rise due to restrictions on antibiotics and coccidiostats historically used to prevent these diseases.

Recent In ovo Research and Potential Applications

In commercial hatcheries, establishment of the microbiome is delayed due to the delay in access to feed. Injecting probiotics into the amnion of embryos before hatch could lead to even earlier establishment of the microbiome and promote gut health through competitive exclusion. *In ovo* injection could offer advantages over in-feed application, considering the extended time between hatch and arrival at the growout facility, a period lasting up to 72 hours (Pender et al., 2016; EFSA Panel on Animal Health and Welfare (AHAW), 2011). The earliest application of probiotics has the most benefit on immune function and subsequent microbial composition (Broom and Kogut, 2017). *In ovo* application of probiotics may be a useful method of early *in ovo* application. Previous studies have found that many probiotic strains do not negatively affect hatchability and have protective effects during post-hatch challenges. Edens et al. (1997) injected *Lactobacillus reuteri in ovo* into the air cell and amniotic fluid and found no negative effects on hatchability. After challenge with *Salmonella*, birds that received *in ovo* probiotics had reduced mortality, increased body weights, and reduced cecal loads of *Salmonella*. Pender et al. (2016) injected probiotic bacteria at embryonic day 18, and challenged birds with a mixed challenge of live *E. acervulina*, *E. maxima*, and *E. tenella* oocysts. Hatchability was not affected by application, and probiotic groups showed improved body weight and body weight gain, reduced mortality, and reduced intestinal lesions.

Although the majority of gastrointestinal development occurs post-hatch, evidence that it begins *in ovo* warrants the application of prebiotics to support the growth of beneficial probiotic species

(Ilina et al., 2016). Madej et al. (2015) applied *in ovo* prebiotics or synbiotics in Ross 308 broilers and reported no negative effects on development of immune organs such as the spleen and bursa. They found a synergistic effect of synbiotics compared to prebiotics alone in the ability to stimulate germinal centers in the spleen of birds at 21 and 35 days of age. Pruszynska-Oszmalek et al. (2015) demonstrated beneficial effects of *in ovo* prebiotics and synbiotics through increased amylase, lipase, and trypsin activity in the pancreas, which correlated with increased final body weight. A separate study investigated the effects of microbial populations on gene transcription in the cecal tonsils and ileum of adult Ross birds after *in ovo* injection with prebiotics or synbiotics (Slawinska et al., 2016). They reported a strong downregulation of immune-related genes in the cecal tonsils of adult broilers after *in ovo* GOS application, suggesting the prebiotic enhanced tolerance of the microbial populations.

This literature review summarizes the avian immune system, and some basic defenses it utilizes against pathogens, including coccidiosis and necrotic enteritis. Additionally, a background of antibiotics and their alternatives, including prebiotics, probiotics, and their synbiotic combination, is included to provide background for the subsequent studies performed with broilers and natural feed additives as reported in the chapters that follow.

References

Abbas, A.K., A.H. Lichtman, and S. Pillai. 2012a. Properties and Overview of Immune Responses. Pages 1-15 in Cellular and Molecular Immunology. Elsevier Ltd, Haryana India.

Abbas, A.K., A.H. Lichtman, and S. Pillai. 2012b. Antibodies and Antigens. Pages 89-107 in Cellular and Molecular Immunology. Elsevier Ltd, Haryana India.

Achanta, M. L.T. Sunkara, G. Dai, Y.R. Bommineni, W. Jiang, and G. Zhang. 2012. Tissue expression and developmental regulation of chicken cathelicidin antimicrobial peptides. J. Anim. Sci. Biotechnol. 3:15.

Agtarap, A., J.W. Chamberlin, M. Pinkerton, and L. Steinrauf. 1967. The structure of monensic acid, a new biologically active compound. J. Am. Chem. Soc. 89:5737-5739.

Akira, S. and K. Takeda. 2004. Toll-like receptor signaling. Nat. Rev. Immunol. 4:499-511.

Al-Khalaifah, H. and A. Al-Nasser. 2018. Cytokines as effective elements of the avian immune system. J. Microbiol. Genet. 119:19.

Annison E.F., K.J. Hill, and R. Kenworthy. 1968. Volatile fatty acids in the digestive tract of the fowl. Br. J. Nutr. 22:207-216.

Antonissen, G., V. Eeckhaut, K. Van Driessche, L. Onrust, F. Haesebrouck, R.J. Moore, and F. Van Immerseel. 2016. Microbial shifts associated with necrotic enteritis. Avian Pathol. 45:308-312.

Aste-Amezaga, M., X. Ma, A. Sartori, and G. Trinchieri. 1998. Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. J. Immunol. 160:5936.

Bar Shira, E. and A. Friedman. 2018. Innate immune functions of avian intestinal epithelial cells: Response to bacterial stimuli and localization of responding cells in the developing avian digestive tract. PLoS One 13:e0200393.

Barekatain, R., G. Nattrass, A.J. Tilbrook, K. Chousalkar, and S. Gilani. 2019. Reduced protein diet and amino acid concentration alter intestinal barrier function and performance of broiler chickens with or without synthetic glucocorticoid. Poult. Sci. 98:3662-3675.

Bel, S. and L.V. Hooper. 2017. Secretory autophagy of lysozyme in Paneth cells. Autophagy 14:719-21.

Berczi, I.L. Bertok, and T. Bereznai. 1966. Comparative studies on the toxicity of *Escherichia coli* lipopolysaccharide endotoxin in various animal species. Can. J. Microbiol. 12:1070-71.

Bila, C.G., C.L. Perreira, and E. Gruys. 2001. Accidental monensin toxicosis in horses in Mozambique. J. South African Vet. Assoc. 72:163-164.

Blake, D.P. and F.M. Tomley. 2014. Securing poultry production from the ever-present *Eimeria* challenge. Trends Parasitol. 30:12-19.

Blake, D.P., J. Knox, B. Dehaeck, B. Huntington, T. Rathinam, V. Ravipati, S. Ayoade, W. Gilbert, A.O. Adebambo, I.D. Jatau, M. Raman, D. Parker, J. Rushton, and F.M. Tomley. 2020. Re-calculating the cost of coccidiosis in chickens. Vet. Res. 51:115.

Borda-Molina, D., J. Seifert, and A. Camarinha-Silva. 2018. Current perspectives of the chicken gastrointestinal tract and its microbiome. Comput. Struct. Biotechnol. J. 16:131-139.

Brogden, K.A. 2005. Antimicrobials peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3:238-250.

Broom, L.J. 2017. The sub-inhibitory theory for antibiotic growth promoters. Poult. Sci. 96:3104-108.

Broom, L.J. and M.H. Kogut. 2018a. Inflammation: friend or foe for animal production? Poult. Sci. 97:510-514.

Broom, L.J. and M.H. Kogut. 2018b. Gut immunity: its development and reasons and opportunities for modulation in monogastric production animals. Anim. Health Res. Rev. 19:46-52.

Brownlie, R. and B. Allan. 2010. Avian toll-like receptors. Cell Tiss. Res. 343:121-130.

Bumstead, N. and P.A. Barrow. 1988. Genetics of resistance to *Salmonella typhimurium* in newly hatched chicks. Br. Poult. Sci. 29: 521-529.

Butaye, P., L.A. Devriese, and F. Haesebrouck. 2003. Antimicrobial growth promoters used in animal feed: effects of less well-known antibiotics on Gram-positive bacteria. ASM 16:175-188.

Calik, A. and A. Ergün. 2015. Effect of lactulose supplementation on growth performance, intestinal histomorphology, cecal microbial population, and short-chain fatty acid composition of broiler chickens. Poult. Sci. 94:2173-82.

Castanon, J.I. 2007. History of the use of antibiotic as growth promoters in European poultry feeds. Poult. Sci. 86:2466-2471.

Casterlow, S., H. Li, E.R. Gilbert, R.A. Dalloul, A.P. McElroy, D.A. Emmerson, and E.A. Wong. 2011. An antimicrobial peptide is downregulated in the small intestine of *Eimeria maxima*-infected chickens. Poult. Sci. 906:1212-1219.

Chalmers, G., H.L. Bruce, D.B. Hunter, V.R. Parreira, R.R. Kulkarni, Y.F. Jiang, J.F. Prescott, and P. Boerlin. 2008. Multiclocus sequence typing analysis of *Clostridium perfringens* isolates from necrotic enteritis outbreaks in broiler chicken populations. J. Clin. Microbiol. 46:3957-3964.

Chapman, D.H., J.R. Barta, D. Blake, A. Gruber, M. Jenkins, N.C. Smith, X. Suo, and F.M. Tomley. 2013. A selective review of advances in coccidiosis research. Adv. Parasitol. 83:93-171.

Chapman, H.D. 2018. Applied strategies for the control of coccidiosis in poultry. CAB Rev. 13:1-11.

Charlebois, A. M. Jacques, and M. Archambault. 2015. Comparative transcriptomic analysis of *Clostridium perfringens* biofilms and planktonic cells. In 1st International Conference on Necrotic Enteritis of Poultry. Copenhagen, Denmark. Abstract.

Choct, M. 2009. Managing gut health through nutrition. Br. Poult. Sci. 50:9-15.

Clavijo, V. and M.J.V. Florez. 2018. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. Poult. Sci. 97:1006-1021.

Clench, M. 1999. The avian cecum: update and motility review. J. Exp. Zool. 283:441-447.

Collier, C.T., C.L. Hofacre, A.M. Payne, D.B. Anderson, P. Kaiser, R.I. Mackie, and H.R. Gaskins. 2008. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. Vet. Immunol. Immunopathol. 122:104-115.

Coursodon, C.F., R.D. Glock, K.L. Moore, K.K. Cooper, and J.G. Songer. 2012. TpeL-producing strains of *Clostridium perfringens* type A are highly virulent for broiler chicks. Anaerobe 18:117-121.

Cox, C.M. and R.A. Dalloul. 2015. Immunomodulatory role of probiotics in poultry and potential *in ovo* application. Benef. Microbes 6:45-52.

Cuperus, T., M. Coorens, A. van Dijk, and H.P. Haagsman. 2013. Avian host defense peptides. Dev. Comp. Immunol. 41:352-69.

Dalloul, R.A., H.S. Lillehoj, N.M. Tamim, T.A. Shellem, and J.A. Doerr. 2005. Induction of local protective immunity to *Eimeria acervulina* by a *Lactobacillus*-based probiotic. Comp. Immunol. Microbiol. Infect. Dis. 28:351-361.

Dalloul, R.A. and H.S. Lillehoj. 2006. Poultry coccidiosis: recent advancements in control measures and vaccine development. Expert Rev. Vaccines 5:143-163.

Dalloul., R.A., T.W. Bliss, Y.-H. Hong, I. Ben-Chouikha, D.W. Park, C.L. Keeler, and H.S. Lillehoj. 2007. Unique responses of the avian macrophage to different species of *Eimeria*. Mol. Immunol. 44:558-566.

Dathe, M. and T. Wieprecht. 1999. Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. Biochem. Biophys. Acta. 1462:71-87.

Ding, Y.Z., S. Fu, D. Zamarin, and J. Bromberg. 2003. Interleukin-10. Page 603 in The Cytokine Handbook (Fourth Edition). A.W. Thomson and M.T. Lotze, eds. Academic Press, New York, US.

Dong, L., Z. Tong, D. Linghu, Y. Lin, R. Tao, J. Liu, Y. Tian, and L. Ni. Effects of sub-minimum inhibitory concentrations of antimicrobial agents on *Streptococcus mutans* biofilm formation. Int. J. Antimicrob. Agents 39:390-395.

Donohoe, D.R., N. Garge, X. Zhang, W. Sun, T.M. O'Connell, M.K. Bunger, and S.J. Bultman. 2011. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab. 13:517-526.

Dunkley, K.D., C.S. Dunkley, N.L. Njongmeta, T.R. Callaway, M.E. Hume, L.F. Kubena, D.J. Nisbet, and S.C. Ricke. 2007. Comparison of in vitro fermentation and molecular microbial profiles of high-fiber feed substrates incubated with chicken cecal inocula. Poult. Sci. 86:801-810.

Edens, F.W., C.R. Parkhurst, I.A. Casas, and W.J. Dobrogosz. 1997. Principles of ex ovo competitive exclusion and *in ovo* administration of Lactobacillus reuteri. Poult. Sci. 76:179-196.

EFSA Panel on Animal Health and Welfare (AHAW). 2011. Scientific opinion concerning the welfare of animals during transport. Chick yolk sac lasts up to 3 days post-hatch. EFSA J. 9:125 10.2903/j.efsa.2011.1966.

Emami, N.K., A. Calik, M.B. White, M. Young, and R.A. Dalloul. 2019. Necrotic enteritis in broiler chickens: the role of tight junctions and mucosal immune responses in alleviating the effect of the disease. Microorganisms 7:231.

Evans, E.W., F.G. Beach, K.M. Moore, M.W. Jackwood, J.R. Glisson, and B.G. Harmon. 1995. Antimicrobial activity of chicken and turkey heterophil peptides CHP1, CHP2, THP1, and THP3. Vet. Microbiol. 47:295-303.

Farnell, M.B., H. He, K. Genovese, and M.H. Kogut. 2003. Pharmacological analysis of signal transduction pathways required for oxidative burst in chicken heterophils stimulated by a Toll-like receptor 2 agonist. Int. Immunopharmcol. 3:1677-1684.

Fatoba, A.J. and M.A. Adeleke. 2018. Diagnosis and control of chicken coccidiosis: a recent update. J. Parasit. Dis. 42:483-493.

Fetterer, R.H., K.B. Miska, M.C. Jenkins, and E.A. Wong. 2014. Expression of nutrient transporters in duodenum, jejunum, and ileum of *Eimeria maxima*-infected broiler chickens. Parasitol. Res. 133:3891-3894.

Ficko-Blean, E., C.P. Stuart, M.D. Suits, M. Cid, M. Tessier, R.J. Woods, and A.B. Boraston. 2012. Carbohydrate recognition by an architecturally complex α-N-acetylglucosaminidase from *Clostridium perfringens*. PLoS One. 7:e33524.

Fiorentino, D.F., A. Zlotnik, T.R. Mosmann, M. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. J. Immunol. 147:3815.

Fomentini, M., D. Haese, J.L. Kill, R.P. Sobreiro, D. Del Puppo, I.R. Haddade, A.L. Lima, and A. Saraiva. 2016. Prebiotic and antimicrobials on performance, carcass characteristics, and antibody production in broilers. Cienc. Rural 46:1070-1075.

Frederiksen, R.F., D.K. Paspaliari, T. Larsen, B.G. Storgaard, M.H. Larsen, H. Ingmer, M.M. Palcic, and J.J. Leisner. 2013. Bacterial chitinases and chitin-binding proteins as virulence factors. Microbiol. 159:833-847.

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66:365-378.

Gasaway, W.C. 1976a. Seasonal variation in diet, volatile fatty acid production and size of the cecum of rock ptarmigan. Comp. Biochem. Physiol. A Physiol. 53:109-114.

Gasaway, W.C. 1976b. Volatile fatty acids and metabolizable energy derived from cecal fermentation in the willow ptarmigan. Comp. Biochem. Physiol. A Physiol. 53:115-121.

Gazzinelli, R.T., I.P. Oswald, S.L. James, and A. Sher. 1992. IL-10 inhibits parasite killing and nitric oxide production by IFN-γ-activated macrophages. J. Immunol. 148:1792.

Gibson, G.R. and M.B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125:1401-1412.

Grondin, J.A., Y.H. Kwon, P.M. Far, S. Haq, and W.I. Khan. 2020. Mucins in intestinal mucosal defense and inflammation: learning from clinical and experimental studies. Front. Immunol. 11:2054.

Grumbles, L.C., J.P. Delaplane, and T.C. Higgins. 1948. Continous feeding of low concentrations of sulfaquinoxaline for the control of coccidiosis in poultry. Poult. Sci. 27:5.

Guriec, N., F. Bussy, C. Gouin, O. Mathiaud, B. Quero, M. Le Goff, and P.N. Collen. 2018. Ulvan activates chicken heterophils and monocytes through Toll-like receptor 2 and Toll-like receptor 4. Front. Immunol. 9:2725.

Haghikia, A., S. Jorg, A. Duscha, J. Berg, A. Manzel, A. Waschbisch, A. Hammer, D.-H. Lee, C. May, N. Wilck, A. Balogh, A.I. Ostermann, N.H. Schebb, D.A. Akkad, D.A. Grohme, M. Kleinewietfeld, S. Kempa, J. Thone, S. Demir, D.N. Muller, R. Gold, and R.A. Linker. 2015. Dietary fatty acids directly impact central nervous system autoimmunity via the small intestine. Immunity 43:817-829.

Harder, A. and A. Haberkorn. 1989. Possible mode of action of toltrazuril: studies on two *Eimeria* species and mammalian and *Ascaris suum* enzymes. Parasitol. Res. 76:8-12.

Harmon, B.G. 1998. Avian heterophils in inflammation disease resistance. Poult. Sci. 77:972-977.

Harwig, S.S.L., K.M. Swiderek, V.N. Kokryakov, G.M. Aleshina, O.V. Shamova, and R.I. Lehrer. 1994. Gallinacins: cysteine-rich antimicrobial peptides of chicken leukocytes. FEBS Lett. 342:281-285.

He, H., K.J. Genovese, and M.H. Kogut. 2011. Modulation of chicken macrophage effector function by T_H1/T_H2 cytokines. Cytokine 53:363-369.

Hellgren, O. and R. Ekblom. 2010. Evolution of a cluster of innate immune genes (beta-defensins) along the ancestral lines of chicken and zebra finch. Immunome Res. 6:3.

Hooper, L.V., T. Midtvedt, and J.I. Gordon. 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu. Rev. Nutr. 22:283-307.

Hooper, L.V., D.R. Littman, and A.J. Macpherson. 2012. Interactions between the microbiota and the immune system. Science 336:1268-1273.

Ilina, L.A., E.A. Yildirim, I.N. Nikonov, V.A. Filippova, G.Y. Laptev, N.I. Novikova, A.A. Grozina, T.N. Lenkova, V.A. Manukyan, I.A. Egorov, and V.I. Fisinin. 2016. Metagenomic bacterial community profiles of chicken embryo gastrointestinal tract by using T-RFLP analysis. Dokl. Biochem. Biophys. 466:47-51.

Jeurissen, S.H., E.M. Janse, G. Koch, and G.F. De boer. 1989. Postnatal development of mucosa-associated lymphoid tissues in chickens. Cell Tissue Res. 258:119-124.

Jochemsen, P., and S.H.M. Jeurissen. 2002. The localization and uptake of *in ovo* injected soluble and particulate substances in the chicken. Poult. Sci. 81:1811-1817.

Jones, M.P. 2015. Avian Hematology. Vet. Clin. North Am. Exot. Anim. Pract. 18:51-61.

Juul-Madsen, H.R., B. Viertlboeck, A.L. Smith, and T.W.F. Göbel. 2014. Innate immune responses. Pages 121-147 in Avian Immunology (Second Edition). K.A. Schat, B. Kaspers, and P. Kaiser, eds. Elsevier Ltd., London, UK.

Kabir, S.M.L. 2009. The role of probiotics in the poultry industry. Int. J. Mol. Sci. 10:3531-3546.

Kadykalo, S., T. Roberts, M. Thompson, J. Wilson, M. Lang, and O. Espeisse. 2018. The value of anticoccidials for sustainable global poultry production. Int. J. Antimicrob. Agents. 51:304-310.

Kaiser, P. and P. Staheli. 2014. Avian cytokines and chemokines. Pages 189-204 in Avian Immunology (Second Edition). K. A. Schat, B. Kaspers, and P. Kaiser, eds. Elsevier Ltd., London, UK.

Kalabis, J., I. Rosenberg, and D.K. Podolsky. 2006. Vangl1 protein acts as a downstream effector of intestinal trefoil factor (ITF)/TFF3 signaling and regulates wound healing of intestinal epithelium. J. Biol. Chem. 281:6434-6441.

Kaspers, B. and P. Kaiser. 2014. Avian antigen-presenting cells. Pages 169-188 in Avian Immunology (Second Edition). K. A. Schat, B. Kaspers, and P. Kaiser, eds. Elsevier Ltd., London, UK.

Keestra, A.M. and J.P.M. va Putten. 2008. Unique properties of the chicken TLR4/MD-2 complex: Selective lipopolysaccharide activation of the MyD88-Dependent Pathway. J. Immunol. 181:4354-4362.

Keestra, A.M. M.R. de Zoete, L.I. Bouwman, M.M. Vaezirad, and J.P.M. van Putten. 2013. Unique features of the chicken Toll-like receptors. Dev. Comp. Immunol. 413:316-332.

Keyburn, A.L., J.D. Boyce, P. Vaz, T.L. Bannam, M.E. Ford, D. Parker, A. Di Rubbo, J.I. Rood, and R.J. Moore. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathogens. 4:e26.

Kim, S.A., M.J. Jang, S.Y. Kim, Y. Yang, H.O. Pavlidis, and S.C. Ricke. 2019. Potential for prebiotics as feed additives to limit foodborne *Campylobacter* establishment in the poultry gastrointestinal tract. Front. Microbiol. 10:91.

Kogut, M.H., K.J. Genovese, and V.K. Lowry. 2001. Differential activation of signal transduction pathways mediating phagocytosis, oxidative burst, and degranulation by chicken heterophils in response to stimulation with opsonized *Salmonella enteritidis*. Inflammation 25:7-15.

Kurpad, A.V. 2006. The requirements of protein and amino acids during acute and chronic infection. Indian J. Med. Res. 124:129-148.

Kwon, Y.M. and S.C. Ricke. 1998. Induction of acid resistance of *Salmonella* typhimurium by exposure to short-chain fatty acids. Appl. Environ. Microbiol. 64:3458-3463.

Laurent, F., R. Mancassola, S. Lacroix, R. Menezes, and M. Naciri. 2001. Analysis of chicken mucosal immune response to *Eimeria tenella* and *Eimeria maxima* infection by quantitative reverse transcription-PCR. Infect. Immun. 69:2527.

Lebkowska-Wieruszewska, B.I. and C.J. Kowalsi. 2010. Sulfachlorpyrazine residues depletion in turkey edible tissues. J. Vet. Pharmacol. Ther. 33:389-395.

Lee, S.H., H.S. Lillehoj, R.A. Dalloul, D.W. Park, Y.H. Hong, and J.J. Lin. 2007. Influence of *Pediococcus*-based probiotic on coccidiosis in broiler chickens. Poult. Sci. 86:63-66.

Lee, K.W., H.S. Lillehoj, S.H. Lee, S.I. Jang, M.S. Park, D.A. Bautista, G.D. Ritter, Y.H. Hong, G.R. Siragusa, and E.P. Lillehoj. 2012. Effect of dietary antimicrobials on immune status in broiler chickens. Asian-Australasian J. Anim. Sci. 25:382-392.

Lepp, D., J. Gong, J.G. Songer, P. Boerlin, V.R. Parreira, and J.F. Prescott. 2013. Identification of accessory genome regions in poultry *Clostridium perfringens* isolates carrying the *netB* plasmid. J. Bacteriol. 195:1152-1166.

Lepp, D., B. Roxas, V.R. Parreira, P.R. Marri, E.L. Rosey, J. Gong, J.G. Songer, G. Vedantam, and J.F. Prescott. 2010. Identification of novel pathogenicity loci in *Clostridium perfringens* strains that cause avian necrotic enteritis. PLoS One. 5:e10795.

Lynn, D.J., R. Higgs, S. Gaines, J. Tierney, T. James, A.T. Lloyd, M.A. Fares, G. Mulcahy, and C. O'Farrelly. 2004. Bioinformatic discovery and initial characterization of nine novel antimicrobial peptide genes in the chicken. Immunogenetics 56:170-177.

Madej, J.P., T. Stefaniak, and M. Bednarczyk. 2015. Effect of *in ovo*-delivered prebiotics and synbiotics on lymphoid-organs' morphology in chickens. Poult. Sci. 94:1209-1219.

Maldonado-Contreras, A.L. and B.A. McCormick. 2011. Intestinal epithelial cells and their role in innate mucosal immunity. Cell Tissue Res. 343:5-12.

Matsuoka, T., M.N. Novilla, T.D. Thomson, and A.L. Donoho. 1996. Review of monensin toxicosis in horses. J. Equine Vet. Sci. 16:8-15.

McGuckin, M.A., S.K. Linden, P. Sutton, and T.H. Florin. 2011. Mucin dynamics and enteric pathogens. Nat. Rev. Microbiol. 9:265-728.

Meade, K.G., R. Higgs, A.T. Lloyd, S. Giles, and C. O'Farrelly. 2009. Differential antimicrobial peptide gene expression patterns during early chicken embryological development. Dev. Comp. Immunol. 33:516-524.

Meslin, C., C. Desert, I. Callebaut, A. Djari, C. Klopp, F. Pitel, S. Leroux, P. Martin, P. Froment, E. Guilbert, F. Gondret, S. Lagarrigue, and P. Monget. 2015. Expanding duplication of free fatty acid receptor-2 (GPR43) genes in the chicken genome. Gen. Biol. Evol. 7:1332-1348.

Michailidis, G. 2010. Expression of chicken LEAP2 in the reproductive organs and embryos and in response to *Salmonella enterica* infection. Vet. Res. Commun. 34:459-471.

Mishra, S.P., P. Karunakar, S. Taraphder, and H. Yadev. 2020. Free fatty acid receptors 2 and 3 as microbial metabolite sensors to shape host health: pharmacophysiological view. Biomedicines 8:154.

Mocellin, S., M.C. Panelli, E. Wang, D. Nagorsen, and F.M. Marincola. 2003. The dual role of IL-10. Trends Immunol. 24:36.

Moore, R.J. 2016. Necrotic enteritis predisposing factors in broiler chickens. Avian Pathol. 45:275-281.

Morrison, D.J. and T. Preston. 2016. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 7:189-200.

Morrow, C., and F. Fehler. 2004. Marek's disease: a worldwide problem. Pages 49-61 in Marek's Disease: An Evolving Problem. F. Davisonand V. Nair, eds. San Diego, California: Elsevier Academic Press.

Mostov, K.E. 1994. Transepithelial transport of immunoglobulins. Annu. Rev. Immunol. 12:63-84.

Niewold, T.A. 2007. The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. Poult. Sci. 86:605-609.

Noack, S., H.D. Chapman, and P.M. Selzer. 2019. Anticoccidial drugs of the livestock industry. 118:2009-2026.

Nurmi, E. and M. Rantala. 1973. New aspects of *Salmonella* infection in broiler production. Nature 241:210-211.

Oehme, F.W. and J.A. Pickrell. 1999. An analysis of the chronic oral toxicity of polyether ionophores antibiotics in animals. Vet. Hum. Toxicol. 41:251-257.

Ohtani, K. and T. Shimizu. 2015. Regulation of toxin gene expression in *Clostridium perfringens*. Res. Microbiol. 166:28089.

Oláh, I., N. Nagy, and L. Vervelde. 2014. Structure of the avian lymphoid system. Pages 11-44 in Avian Immunology (Second Edition). K.A. Schat, B. Kaspers, and P. Kaiser, eds. Elsevier Ltd., London, UK.

Olkowski, A.A., C. Wojnarowicz, M. Chirino-Trejo, and M.D. Drew. 2006. Responses of broiler chickens orally challenged with Clostridium perfringens isolated from field cases of necrotic enteritis. Res. Vet. Sci. 81:99-108.

Olkowski, A.A., C. Wojnarowicz, M. Chirino-Trejo, B. Laarveld, and G. Sawicki. 2008. Sub-clinical necrotic enteritis in broiler chickens: novel etiological consideration based on ultra-structural and molecular changes in the intestinal tissue. Res. Vet. Sci. 85:543-553.

Opitz, B., N.W.J. Schroder, I. Spreitzer, K.S. Michelsen, C.J. Kirschning, W. Hallatschek, U. Zahringer, T. Hartung, U.B. Gobel, and R.R. Schumann. 2001. Toll-like receptor-2 mediates

Treponema glycolipid and lipoteichoic acid-induced NF-κB translocation. J. Biolog. Chem. 276:22041-22047.

Pan, D. and Z. Yu. 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes 5:108-119.

Panda, A.K., R.S.V. Rama, M.V.L.N. Raju, and S.G. Shyam. 2009. Effect of butyric acid on performance, gastrointestinal tract health, and carcass characteristics in broiler chickens. Asian-australian J. Anim. Sci. 22:1026-1031.

Parvez, S., K.A. Malik, S.A. Kang, and H.Y. Kim. 2006. Probiotics and their fermented food products are beneficial for health. J. Appl. Microbiol. 100:1171-1185.

Pender, C.M., S. Kim, T.D. Potter, M.M. Ritzi, M. Young, and R.A. Dalloul. 2016. Effects of *in ovo* supplementation of probiotics on performance and immunocompetence of broiler chicks to an *Eimeria* challenge. Beneficial Microbes 7:699-705.

Peterson, L.W. and D. Artis, 2014. Intestinal epithelial cells: regulators of barrier function and immune homoestasis. Nature Rev. Immunol. 14:141-153.

Porcelli, F., R. Verardi, L. Shi, K.A. Henzler-Wildman, A. Ramamoorthy, and G. Veglia. 2008. NMR structure of the cathelicidin-derived human antimicrobial peptide LL-37 in dodecylphosphocholine micelles. Biochem. 47:5565-5572.

Prescott, J.F., V.R. Parreira, I.M. Gohari, D. Lepp, and J. Gong. 2016. The pathogenesis of necrotic enteritis in chickens: what we know and what we need to know: a review. Avian Pathol. 45:288-294.

Pruszynska-Oszmalek, E., P.A. Kolodziejski, K. Stadnicka, M. Sassek, D. Chalupka, B. Kuston, L. Nogowski, P. Mackowiak, G. Maiorano, J. Jankowski, and M. Bednarczyk. 2015. *In ovo* injection of prebiotics and synbiotics affects the digestive potency of the pancreas in growing chickens. Poult. Sci. 94:1909-1916.

Reeks, B.Y., F.R. Champlin, D.B. Paulsen, D.W. Scruggs, and M.L. Lawrence. 2005. Effects of sub-minimum inhibitory concentration antibiotic levels and temperature on growth kinetics and outer membrane protein expression in Mannheimia haemolytica and Haemophilus somnus. Can. J. Vet. Res. 69:1-10.

Ricke, S.C. 2018. Impact of prebiotics on poultry production and food safety. Yale J. Biol. Med. 91:151-159.

Ricke, S.C., I.L. Sang, S.A. Kim, S.H. Park, and Z. Shi. 2020. Prebiotics and the poultry gastrointestinal tract microbiome. Poult. Sci. 99:670-677.

Rothwell, L., J.R. Young, R. Zoorob, C.A. Whittaker, P. Hesketh, A. Archer, A.L. Smith, and P. Kaiser. 2004. Cloning and characterization of chicken IL-10 and its role in the immune response to *Eimeria maxima*. J. Immunol. 173:2675-2682.

Roto, S.M., Y.M. Kwon, and S.C. Ricke. 2016. Applications of *in ovo* technique for the optimal development of the gastrointestinal tract and the potential influence on the establishment of its microbiome in poultry. Front. Vet. Sci. 3:63.

Rychen, G., G. Aquilina, G. Azimonti, V. Bampidis, M.D.L. Bastos, G. Bories, A. Chesson, P.S. Cocconcelli, G. Flachowsky, B. Kolar, M. Kouba, M. Lopez-Alonso, S.L. Puente, A. Mantovani, B. Mayo, F. Ramos, M. Saarela, R.E. Villa, R.J. Wallace, P. Wester, P. Brantom, I. Halle, P. van Beelen, O. Holczknecht, M.V. Vettori, and J. Gropp. 2018. Safety and efficacy of COXAM ® (amprolium hydrochloride) for chickens for fattening and chickens reared for laying. EFSA J. 16:e05338.

Ryley, J.F. and M.J. Betts. 1973. Chemotherapy of chicken coccidiosis. Adv. Pharmacol. Chemother. 11:221-293.

Saravanan, R. and S. Bhattacharjya. 2011. Oligomeric structure of a cathelicidin antimicrobial peptide in dodecylphosphocholine micelle determined by NMR spectroscopy. Biochim. Biophys. Acta. 1808:369-381.

Schroder, K., P.J. Hertzog, T. Ravasi, and D.A. Hume. 2003. Interferon-γ: an overview of signals, mechanisms and functions. J. Leukocyte Biol. 75:163-189.

Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi. 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. PNAS 99:966-1001.

Slawinska, A., A. Dunislawaska, A. Plowiec, M. Radomska, J. Lachmanska, M. Siwek, S. Tavaniello, and G. Maiorano. 2019. Modulation of microbial communities and mucosal gene expression in chicken intestines after galactooligosaccharides delivery *in ovo*. PLoS One 14:e0212318.

Slawinska, A., A. Plowiec, M. Siwek, M. Jaroszewski, and M. Bednarczyk. 2016. Long-term transcriptomic effects of prebiotics and synbiotics delivered *in ovo* in broiler chickens. PLoS One. 11:e0168899.

Sokale, A. O., W. Zhai, L. M. Pote, C. J. Williams, and E. D. Peebles. 2017. Effects of coccidiosis vaccination administered by *in ovo* injection on the hatchability and hatching chick quality of broilers. Poult. Sci. 96:541-547.

Stanley, D., S.-B. Wu, N. Rodgers, R.A. Swick, and R.J. Moore. 2014. Differential responses of cecal microbiota to fishmeal, *Eimeria* and *Clostridium perfringens* in a necrotic enteritis challenge model in chickens. PLoS One 9:e104739.

Steinman, R.M., J.C. Adams, and Z.A. Cohn. 1975. Identificatin of a novel cell type in peripheral lymphoid organs of mice. IV. Identification and distribution in mouse spleen. J. Exp. Med. 141:804-820.

Stier, A., P. Bize, Q. Schull, J. Zoll, F. Singh, B. Geny, F. Gros, C. Royer, S. Massemin, and F. Criscuolo. 2013. Avian erythrocytes have functional mitochondria, opening novel perspectives for birds as animal models in the study of ageing. Front. Zool. 10:33.

Stokes, C.R. 2017. The development and role of microbial-host interactions in gut mucosal immune development. J. Anim. Sci. Biotech. 8:12.

Su, S., K.B. Miska, R.H. Fetterer, M.C. Jenkins, and E.A. Wong. 2015. Expression of digestive enzymes and nutrient transporters in *Eimeria*-challenged broilers. Exp. Parasitol. 150:13-21.

Su, S., D.M. Dwyer, K.B. Miska, R.H. Fetterer, M.C. Jenkins, and E.A. Wong. 2017. Expression of host defense peptides in the intestine of *Eimeria*-challenged broilers. Poult. Sci. 96:2421-2427.

Sun, M., W. Wu, L. Chen, W. Yang, X. Huang, C. Ma, F. Chen, Y. Xiao, Y. Zhao, C. Ma, S. Yao, V.H. Carpio, S.M. Dann, Q. Zhao, Z. Liu, and Y. Cong. 2018. Microbiota-derived short-chain fatty acids promote Th1 cell IL-10 production to maintain homeostasis. Nat. Comm. 9:3555.

Sugiarto, H. and P.-L. Yu. 2006. Identification of three novel ostricacins: an update on the phylogenetic perspective of β-defensins. Int. J. Antimicrob. Agents 27:229-35.

Sumners, L.H., K.B. Miska, M.C. Jenkins, R.H. Fetterer, C.M. Cox, S. Kim, and R.A. Dalloul. 2011. Expression of Toll-like receptors and antimicrobial peptides during *Eimeria praecox* infection in chickens. Exp. Parasitol. 1273:714-18.

Tellez Jr., G., G. Tellez-Isaias, and S. Dridi. 2017. Heat stress and gut health in broilers: role of tight junction proteins. Adv. Food Technol. Nutr. Sci. Open J. 3:e1-e4.

Timbermont, L., L. De Smet, F. Van Nieuwerbergh, V.R. Parreira, G. Van Driessche, F. Haesebrouck, R. Ducatelle, J. Prescott, D. Deforce, B. Devreese, and F. Van Immerseel. 2014. Perfrin, a novel bacteriocin associated with netB-positive *Clostridium perfringens* strains from broiler chickens with necrotic enteritis. Vet. Res. 45:40.

Timbermont, L., F. Haesebrouck, R. Ducatelle, and F. Van Immerseel. 2011. Necrotic enteritis in broilers: an updated review on the pathogenesis. Avian Path. 40:341-347.

Townes, C.L., G. Michailidis, C.J. Nile, and J. Hall. 2004. Induction of cationic chicken liver-expressed antimicrobial peptide 2 in response to *Salmonella enterica* infection. Infect. 72:6987-6993.

Turk, D.E. 1982. The anatomy of the avian digestive tract as related to feed utilization. Poult. Sci. 61:1225-1244.

Uematsu, S. and S. Akira. 2008. Toll-like receptors (TLRs) and their ligands. Handb. Exp. Pharmacol. 1-20.

van Dijk, A., E.J.A. Veldhuizen, and H.P. Haagsman. 2008. Avian defensins. Vet. Immunol. Immunopathol. 124:1-18.

van Dijk, A., M.H. Tersteeg-Zijderveld, J.L. Tjeerdsma-van Bokhoven, A.J. Jansman, E.J. Veldhuizen, and H.P. Haagsman. 2009. Chicken heterophils are recruited to the site of *Salmonella* infection and release antibacterial mature Cathelicidin-2 upon stimulation with LPS. Mol. Immunol. 46:1517-1526.

van Dijk, A., M. Herrebout, M.H. Tersteeg-Zijderveld, J.L. Tjeerdsma-van Bokhoven, N. Bleumink-Pluym, A.J. Jansman, E.J. Veldhuizen, and H.P. Haagsman. 2012. *Campylobacter jejuni* is highly susceptible to killing by chicken host defense peptide cathelicidin-2 and suppresses intestinal cathelicidin-2 expression in young broilers. Vet. Microbiol. 160:347-354.

van Immerseel, F., J. De Buck, F. Pasmans, G. Huyghebaert, F. Haesebrouck, and R. Ducatelle. 2004. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian Pathol. 33:537-549.

van Immerseel, F., U. Methner, I. Rychlik, B. Nagy, P. Velge, G. Martin, N. Foster, R. Ducatelle, and P.A. Barrow. 2005. Vaccination and early protection against non-host-specific Salmonella serotypes in poultry: exploitation of innate immunity and microbial activity. Epidemiol. Infect. 133:959-978.

van Immerseel, F., J.B. Russell, M.D. Flythe, I. Gantois, L. Timbermont, F. Pasmans, F. Haesebrouck, and R. Ducatelle. 2006. The use of organic acids to combat *Salmonella* in poultry: a mechanistic explanation of the efficacy. Avian Pathol. 35:182-188.

Veterinary Feed Directive: A Rule by the Food and Drug Administration on 06/03/2015. 2015. Pages 31707-31735. Published by the Food and Drug Administration, HHS. https://www.federalregister.gov/documents/2015/06/03/2015-13393/veterinary-feed-directive.

Wang, H.B., P.Y. Wang, X. Wang, Y.L. Wan, and Y.C. Liu. 2012. Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein Claudin-1 transcription. Dig. Dis. Sci. 57:3126-3135.

Weber, F.H., and N.A. Evans. 2003. Immunization of broiler chicks by *in ovo* injection of *Eimeria tenella* sporozoites, sporocysts, or oocysts. Poult. Sci. 82:1701-1707.

Wigley, P. and P. Kaiser. 2003. Avian cytokines in health and disease. Braz. J. Poult. Sci. 5:1-14.

Williams, R.B. 2005. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Av. Pathol. 34:159-180.

Williams, C. 2007. *In ovo* vaccination for disease prevention. Int. Poult. Prod. 15:7-8.

Witter, R.L., G.H. Burgoyne, and B. R. Burmester. 1968. Survivial of Marek's disease agent in litter and droppings. Avian Dis. 12:522-530.

Xiao, Y., A.L. Hughes, J. Ando, Y. Matsuda, J.F. Cheng, D. Skinner-Noble, and G. Zhang. 2004. A genome-wide screen identifies a single beta-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. BMC Genomics 5:56.

Yang, W.Y., Y. Lee, H. Lu, C.H. Chou, and C. Wang. 2019. Analysis of gut microbiota and the effect of lauric acid against necrotic enteritis in *Clostridium perfringens* and *Eimeria* side-by-side challenge model. PLoS One 14:e0205784.

Yun, C.H., H.S. Lillehoj, J. Zhu, and W. Min. 2000. Kinetic differences in intestinal and systemic interferon-γ and antigen-specific antibodies in chickens experimentally infected with *Eimeria maxima*. Avian Dis. 44:305-312.

Zhao, C. T. Nguyen, L. Liu, R.E. Sacco, K.A. Brogden, and R.I. Lehrer. 2001. Gallinacin-3, an inducible epithelial beta-defensin in the chicken. Infect. Immun. 69:2684-2691.

CHAPTER III

Hatchery application of synbiotics in broilers: performance and mRNA abundance of ileum tight junction proteins, nutrient transporters, and immune response markers

Abstract

Once they hatch, broiler chicks typically do not receive feed and water until they reach a growout facility, and rely on yolk reserves from the time of hatch until first meal. Immediately after hatching, environmental microbes begin to colonize the gut. By providing beneficial synbiotics immediately post-hatch, establishment of a healthy gut could start earlier than any in-feed supplementation not received until they reach the growout farm. This study investigated the effects of oral synbiotics given to day-old broilers at the hatchery prior to transport, and the effects of continuing the synbiotic in the feed for a 21-day (D) period. A total of 480 Cobb male broilers were randomly divided into one of four treatments using a 2x2 factorial design: a no-additive control (**CTRL**), a one-time oral application of synbiotic at the hatchery fed a basal diet (**HS**), an oral application of water at the hatchery with dietary synbiotics (**DS**), and a hatchery synbiotic plus dietary synbiotic (**HSDS**). Feed intake and performance were measured at DOH, D3, D7, D14, and D21 on a per-pen basis. Ileal samples were collected (8 birds/treatment) at D7 and D14 to assess mRNA abundance of tight junction proteins (ZO-1, ZO-2, CLD-1), nutrient transporters (PepT1, SGLT1), and immune response markers (IL-10, TLR2, TLR4). Data were analyzed in JMP with LS Means to separate means with significance assigned at $P \leq 0.05$ and trends assigned at $0.05 \leq P \leq 0.10$. While no statistical differences were observed in BW and mortality, FCR was significantly reduced in HS birds from D14 – D21. The main effect of diet

significantly lowered FI from D3 – D7 ($P \leq 0.05$). The main effect of oral synbiotics at the hatchery lowered BW at D3 and D7, and FI from D7-14, and temporarily raised FCR from D3 – D7 while lowering FCR from D7 – D14. On D21, there was a trend ($P \leq 0.1$) where HSDS had higher PepT1 level compared to other treatments. On D7, IL-10 expression was lower and TLR4 expression was higher in birds given the synbiotic feed additive. By D21, HSDS had higher IL-10 expression compared to HS or DS alone. Overall, these results indicate an early effect of synbiotic application that slightly reduced early performance, yet there were no negative effects observed by the end of the study. HSDS also had higher anti-inflammatory IL-10 mRNA expression on D21, a potential benefit for birds facing a stress challenge. Future research involving extended growout studies with a disease challenge would expand the implications of early-application of probiotics.

Keywords: synbiotics, hatchery, broiler, immune response genes, nutrient transporter genes

Introduction

Reduced antibiotic use in food animal production does not eliminate the need to curtail disease and promote animal health (Cervantes, 2015). Although antibiotic resistance in bacteria can often be attributed to improper human use (Phillips et al., 2004; Phillips, 2007), increased public pressure on agricultural use has led to more regulation and voluntary reduction of antibiotic use by many companies (Davies and Davies, 2010). Ultimately, the decline in using antibiotics has led to interest in alternatives, including probiotics and prebiotics. In this study, a combination of probiotics and prebiotics, commonly referred to as synbiotics, were given at the hatchery prior to transportation. After arrival, some birds continued to receive synbiotics in the feed to further evaluate potential benefits in performance and gut development.

An early establishment of a healthy gut microbiota may improve growth and development in broilers, and prime early immunity. In the commercial poultry industry, it is standard practice to transport chicks from hatcheries to growout operations without feed or water because chicks have a built-in nutrient supply, the yolk sac, which provides nutrients for up to 72 hrs post-hatch (EFSA Panel on Animal Health and Welfare (AHAW), 2011). Although chicks have this natural system to sustain them, negative consequences have been documented in chicks that experienced prolonged periods in transit without feed or water. Chicks are temporarily affected by prolonged transport due to dehydration (Fairchild et al., 2006); in contrast, there are benefits to providing early access to feed and water. Exogenous nutrients like synbiotics, immediately available post-hatch, may improve development of the gastrointestinal tract, which ultimately helps broilers reach their full genetic potential and maximize growth performance.

According to Fuller (1989), probiotics are defined as living microbes applied as a feed supplement to confer a benefit to the host by affecting enteric microbial balance. Numerous probiotics are known to stimulate gastrointestinal development and immunological activity in young chicks. Common probiotic strains include *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, and *Streptococcus* (Yang et al., 2009). One way, probiotics promote gut health is through competitive exclusion of pathogens and enhancing mucosal immunity (Pender et al., 2016). Prebiotics are substances that survive enzymatic digestion in the upper gastrointestinal tract (GIT) and promote growth of beneficial bacteria in the lower part of the GIT (Gibson et al., 2004; Abd El-Hack et al., 2020). They are often products of fermentation, and include fructooligosaccharides (FOS) like inulin to promote growth of probiotic bacteria like *Bifidobacterium* and *Lactobacillus* (Yang et al., 2009; Samanta et al., 2012; Teng and Kim, 2018). Inulin is a common way by which plants store carbohydrates in their roots, and it is not

digestible in monogastric organisms but can pass to the large intestine and act as an energy source for beneficial bacteria (Miremadi and Shah, 2012).

Most digestion and absorption occur in the small intestine (Awad et al., 2007). The broiler gut prepares for exogenous feed prior to hatch by increasing expression of nutrient transporters (Gilbert et al., 2007). Glucose is mainly transported into enterocytes by sodium-dependent glucose transporter protein 1 (SGLT1), while peptide transporter 1 (PepT1) transfers di- and tripeptide products of amino acids across the lumen (Shimizu et al., 2017; Miska et al., 2015). The gut maintains a strict barrier to ensure important nutrients are absorbed properly while undesirable microbes and material are kept out. Tight junction proteins including zonula occludens (ZO)-1, ZO-2, and claudin (CLD)-1 maintain a tight barrier by shutting the paracellular space between enterocytes (Emami et al., 2019).

The GIT is constantly exposed to the external environment and has an active immune system to defend against pathogens. To gauge how early application or in-feed synbiotics influence the intestinal immune system, the three immune response genes measured in this study were Toll-like receptor (TLR) 2, TLR4, and IL-10. TLRs are highly conserved receptors found in many animal cells, functioning to recognize bacterial components that are distinct from the host cell (Keestra et al., 2013). The ligands for TLR2 are di- and tri-acetylated lipopeptides found on cell walls of Gram-positive bacteria (Oliveira-Nascimento et al., 2012). TLR4 surveys for Gram-negative bacteria by detecting lipopolysaccharides (LPS), a common component in their cell wall (Ateya et al., 2019). Although an active immune system is important for host defense, an excess of inflammation in the gut damages host tissue, reduces nutrient absorption, and slows bird growth (Awad et al., 2017). A key anti-inflammatory cytokine, interleukin (IL)-10, balances the immune response, suppressing release of pro-inflammatory cytokines (Dokka et al., 2001).

This study applied an oral synbiotic to day-old broiler chicks before transportation from the hatchery to a growout facility. The objective was to gauge the effects of early synbiotic application by measuring weekly performance and immune response.

Materials and Methods

Birds and Experimental Treatments

This 21-day study using male Cobb 500 male broilers was conducted under the approval of IACUC # 18-037-APSC at the Virginia Tech animal research facilities. The 2x2 factorial design (Table 3.1) consisted of diet (basal with or without synbiotic) and hatchery application (oral gel application with or without synbiotic) for a total of 4 treatments (8 replicate pens/treatment; 15 birds/replicate; 120 birds/treatment). The basal diet formulation is presented in Table 3.2. The control (**CTRL**) treatment received an oral gel only application without synbiotics on day-of-hatch (DOH) at the hatchery, and a non-medicated basal corn/soybean starter mash diet throughout the trial (D0 - D21). The second treatment, hatchery synbiotic (**HS**), received an oral synbiotic application at the hatchery (16 g soluble synbiotic/1 L gel) and the basal diet. The third treatment group, dietary synbiotic (**DS**), received a gel only hatchery application and the basal diet plus synbiotics in the feed (0.5 kg/MT) throughout the trial. Treatment 4 (**HSDS**) received synbiotics at the hatchery and in the basal diet (0.5 kg/MT) for the duration of the experiment. The synbiotic consisted of dehydrated probiotic bacterial species (minimum 10^8 CFU/g) *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, *Lactobacillus reuteri*, and the prebiotic inulin (fructooligosaccharides).

Bird Management

In total, 480 day-old male broilers were obtained from a commercial hatchery, randomly divided in half and received either an individual oral dose of 0.5 mL gel without synbiotics (gel only) or 0.5 mL gel containing soluble synbiotics. Chicks were then transported for 6 hours in hatch trays to mimic industry transportation methods. Upon arrival at the animal research facility, each group of birds was randomly divided in half and assigned to either basal dietary treatment or the basal diet plus in-feed synbiotics within each group to yield the four treatment groups.

Housing

Each replicate consisted of a 1.2 x 1.2 meter floor pen using clean pine shavings on top of concrete flooring. Water was supplied by adjustable nipple waterers, and each pen had a bucket feeder with adjustable height. Water and feed were provided ad libitum. Temperature and humidity were recorded daily and maintained at industry standards. Temperature began at 36℃ at DOH and was lowered gradually by 1℃ each day until reaching 24℃ by D21. The light schedule was set at 24 hours of light for the first week, and gradually adjusted to 20 hours of light and 4 hours of dark between D7-D14.

Performance Measurements

Daily observations and mortality were recorded. Performance measurements were adjusted for mortality and reported on an average bird per pen basis. All the birds from one pen were weighed together as a single replicate. Bird weight (per pen basis) and feed amounts were recorded on DOH, D3, D7, D14, and D21 to calculate average body weight (BW), body weight gain (BWG), total feed intake (FI), and feed conversion ratio (FCR).

mRNA Abundance in Ileal Tissues

On D7 and D21, one bird per pen was euthanized, the ileum was immediately removed, rinsed with 10% ice-cold PBS, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. When considering which areas to investigate potential effects of synbiotics, the ileum was chosen because of its proximity to the distal GIT and ceca, where many probiotic microbes colonize. Tissue samples were homogenized using a TissueLyser (Qiagen) and RNA was extracted following standard manufacturer's protocol (Qiagen RNeasy Mini Kit). Optical density (OD) was used to assess RNA quality (OD 260/280) and concentration (OD 260) as measured by a NanoDrop-1000. The high-capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to make cDNA for qRT-PCR). Reactions (20 µL/well) were run in triplicate using Fast SYBR™ Green Master Mix (Applied Biosystems) on a 7500 FAST Real-Time PCR Machine (Applied Biosystems) with melt curves to determine product specificity. Target genes included tight junction proteins ZO-1, ZO-2, and CLD-1; nutrient transporters PepT1 and SGLT1; and immune response markers IL-10, TLR2 and TLR4. Primer details are provided in Table 3.3. Changes in mRNA abundance of each target gene were calculated using glyceraldehye-3-phosphate (GAPDH) as the endogenous control and the CTRL treatment as the calibrator. Results for each treatment are reported as average mRNA abundance relative to GAPDH using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Analysis of data was performed using JMP Pro 15 using two-way ANOVA between diet and hatchery application using Fisher's least significant difference (LSD) method. The results report the interaction between diet and application, the main effect of diet, and the main effect of hatchery application. Significance ($P \leq 0.05$) or a trend ($0.05 \leq P \leq 0.10$) between treatments was

determined using a student's *t*-test. Results are reported as least square means (LS Means) with standard error means (SEM).

Results

Performance Measurements

Mortality. There were no significant differences in the interaction between diet and hatchery application on mortality. However, from DOH-D3, there was a trend ($P \leq 0.1$) in which basal-fed birds had lower mortality compared to synbiotic-fed birds, yet from D3-D7 there was a trend ($P \leq 0.1$) where the basal-fed birds had higher mortality compared to the synbiotics-fed birds (Table 3.4). There were no other significant effects on mortality due to treatment type.

Body Weight. There was a trend ($P \leq 0.1$) in D14 BW per bird (Table 3.5) where CTRL had higher BW compared to HS and HSDS. DS had a similar BW to all treatments. There were no effects of diet*hatchery application from DOH-D21. There was a trend in main effect of application at D3 ($P \leq 0.1$) and significant difference at D7 ($P \leq 0.05$) in which birds that received gel only had higher BW compared to birds that received gel with synbiotics. There were no differences seen by D14 and D21.

Body Weight Gain. Table 3.6 shows there were no significant differences between diet *hatchery application on BWG, yet there was a trend ($P \leq 0.1$) from D7-D14 in which CTRL had higher BWG compared to HS. There was a difference due solely to the main effect of hatchery application during the first two weeks. The birds given gel only tended to have higher BWG compared to the birds given gel plus synbiotics from DOH-D3 ($P \leq 0.1$) The main effect of gel led to significantly higher BWG from D3-D7 ($P \leq 0.05$) compared to gel plus synbiotic

application. The main effect of gel led to significantly higher BWG from D3-D7 ($P \leq 0.05$) compared to gel plus synbiotic application.

Feed Intake. There was no significant difference observed during the first week of the trial (Table 3.7). There was a trend ($P \leq 0.1$) from D7-D14 where the CTRL had higher FI compared to the other three treatments. Similarly, there was a significant difference due to the interaction of diet*hatchery application ($P \leq 0.05$) from D14-D21 in which CTRL had a higher FI compared to HS and DS, yet HSDS had a similar FI compared to all other treatments. Cumulatively, from DOH-D21, there was a significant interaction between diet* hatchery application ($P \leq 0.05$). CTRL had a higher overall FI compared to HS and DS, while HSDS had a FI similar to all of the treatments. For the main effect of diet, the birds fed the basal diet had a significantly higher FI compared to birds fed the synbiotic during the period from D3-D7 ($P \leq 0.05$).

Feed Conversion Ratio. There was a significant interaction ($P \leq 0.05$) between diet* hatchery application from D14-D21 (Table 3.8) where the HS treatment had a significantly lower FCR compared to the CTRL. DS and HSDS had a similar FCR to all treatments. When looking at the main effect of hatchery application, there was a trend ($P \leq 0.1$) from D3-D7 where birds given the gel only had a lower FCR compared to birds given the gel with a synbiotic. Overall, there were no significant differences in FCR between DOH-D21.

mRNA Abundance

Tight Junction Proteins. Results of diet and hatchery application on ileum mRNA abundance of ZO-1, ZO-2, and CLD-1 are given in Table 3.9. There were no significant differences or trends observed during the experiment at D7 or D21.

Nutrient Transporters. Table 3.10 reports results of ileum mRNA abundance of PepT1 and SGLT1. No significant differences were observed in expression of SGLT1. However, on D21,

there was a trend ($P \leq 0.1$) in greater PepT1 abundance in HSDS compared to other treatments due to diet*hatchery application.

Immune Response Markers. mRNA abundance of IL-10, TLR2, and TLR4 are shown in Table 3.11. There was a main effect of diet on D7 IL-10 expression in which synbiotics in the feed led to significantly lower IL-10 expression compared to the control diet ($P \leq 0.05$). By D21, there was a significant interaction between diet*hatchery application on IL-10 expression ($P \leq 0.05$) in which HSDS had significantly greater IL-10 expression compared to HS and DS. There were no significant interactions between diet* hatchery application on TLR2 or TLR4. A significant difference ($P \leq 0.05$) was observed due to the main effect of diet on D7 TLR4 expression, in which birds fed synbiotics had lower TLR4 expression, although the effect did not carry over to D21.

Discussion

During chick transportation from the hatchery to the growout facility, it is common practice to keep chicks in the dark to reduce their activity, and withhold feed and water in transit (Dam and Fitzgerald, 2017). The time from hatch to growout facility, including transportation, can be up to 48 hours, and chicks that hatch early spend additional time in the hatchery without feed and water access (Moran and Reinhart, 1980). Since broilers reach slaughter age within a short period of time, it may be beneficial to have application of probiotics and prebiotics (synbiotics) at the hatchery to jumpstart gut health as early as possible. This study sought to investigate the effects of using a single-time oral dose of synbiotic before a 6-hour transportation time, and continuing synbiotic in the feed in some treatments.

Most chick mortality occurs within the first week of life at the growout facility due to many factors: poor chick quality, stress from the hatchery, transportation, disease, and other environmental factors (Heier et al., 2002; Uni and Ferket, 2004). From D3-D7 of this study, there was a trend towards reduced mortality in chicks fed synbiotics in the diet (0% mortality vs 1.25%). Although this cannot be attributed to early application of synbiotics at the hatchery, it could be a result of synbiotics in the feed promoting bird health.

Improving body weight with feed additives is an ultimate goal that results in increased profit and product. Although many studies show that birds given synbiotics have little difference in performance measurements by the time they reach slaughter age, this early feeding may be able to help when it comes to exposure to disease or stress during the first few weeks of life. Bergoug et al. (2013) found that transportation times of 0 hr, 4 hr, and 10 hr all resulted in no effect on feed intake, FCR, mortality, or increased BW past D21. In the present study, there was no significant change in end body weight due to the interactions of diet and hatchery application, yet CTRL birds tended to have higher BW on D14 compared to HS and HSDS. This could be due to the higher feed intake in CTRL birds from D7-14 compared to other treatments. The CTRL birds continued to have higher FI than HS and DS from D14-21, and cumulatively consumed more feed than their counterparts in other groups, yet resulted in similar final body weights. The birds that received oral synbiotics on DOH had lower BW until compensatory growth took effect by D21. Similarly, Sarangi et al. (2016) saw no difference in BW or BWG on D21. In the present study, synbiotic application seemed to lower BW and BWG for the first two weeks. A developing gut microbiome in birds receiving synbiotics may have required more energy compared to CTRL birds growing on clean shavings. Previous studies have demonstrated slower growth in conventional birds compared to germ-free animals, perhaps due to energy use of the

gut microbiome for the first few weeks (Klasing, 1987; Dibner and Richards, 2005; Lan et al., 2005). One attribute of synbiotics is their ability to produce SCFAs, which may contribute energy towards the animal's needs. Birds typically eat to meet their energy requirements, which may explain why HS and DS birds consumed less than the CTRL treatment.

The trend in higher D3-7 FCR in birds that received synbiotics at the hatchery did not last past the first week, perhaps because early establishment of a gut microbiome uses energy and resources, but ultimately improves bird performance (Yang et al., 2009). This was observed as a trend in lower D14-21 FCR in birds that got hatchery synbiotics and a significant reduction in FCR in HS birds during the final week, even though final BWG and BW remained the same in all treatments. Other studies also found that synbiotics lowered overall FCR (Awad et al. 2009; Sarangi et al., 2016). Cumulatively, this lower FCR could possibly indicate that birds fed synbiotics at the hatchery or in the diet were converting feed more efficiently towards the end of the experiment.

In this study, there was a trend for higher PepT1 mRNA levels on D21 in birds given synbiotics, indicative of the ability for increased amino acid absorption in the gut (Shimizu et al., 2017). The higher PepT1 could contribute to better FCR in the same groups. In this study, synbiotics did not have an effect on the abundance of SGLT1, important in carbohydrate absorption in the small intestine (Sklan et al., 2003).

Intercellular tight junction (TJ) proteins maintain a tight epithelial barrier in the gut, functioning to prevent harmful microbes and toxins from entering the body (Di Pierro et al., 2001). Enteric pathogens can degrade TJ proteins, compromise gut integrity, and lead to a condition called 'leaky gut' which negatively impacts performance (Fasano and Nataro, 2004). This study found no significant change in mRNA abundance in TJ proteins ZO-1, ZO-2, or CLD-1 due to

synbiotic application. Some studies have found supplementation with oligosaccharides and strains of *Lactobacilli* improved barrier function by increasing TJ relocation in cells (Arnhe and Hagslett, 2011; Song et al., 2013).

Feeding synbiotics would stimulate an immune response as the gut microbiome is established early in life (Givisiez et al., 2020). In this experiment, mRNA abundance of IL-10 was measured to determine if synbiotics may lower inflammation in the gut and TLR2 and TLR4 were measured to determine the gut's innate ability to detect microbes. Although there were no differences in TLR2 expression in this study, there were differences in TLR4 and IL-10 mRNA. Interestingly, D7 IL-10 and TLR4 mRNA were lower in birds fed synbiotics, suggesting a temporary downregulation of immune response. However, by D21, the HSDS had significantly greater levels of IL-10 than HS or DS, suggesting the combination of early synbiotic application and continued feed application could have a stronger anti-inflammatory protection than either application method alone.

This study observed effects of early application of synbiotics and the continuous application in the feed for 21 days. Overall, broilers fed synbiotics exhibited lower mRNA abundance of anti-inflammatory IL-10 and TLR4. This also correlates with the lower BW and BWG observed at the beginning of the experiment in birds receiving synbiotics, which may have the ability to downregulate the immune response. Initially, the establishment of synbiotics in the gut may take up energy, thus, the reduced BW and BWG during the first two weeks. However, the reduction was short-lived, as there were no significant differences in final BW and BWG, FI was lower in HS and DS birds, and FCR was improved in HS birds. Correlating with the improved FI and FCR was a trend for greater mRNA abundance of PepT1 in synbiotic-fed birds and greater IL-10 levels in HSDS compared to HS or DS alone. It is important to remember that measuring mRNA

abundance of a particular gene has limitations, and does not necessarily translate to protein abundance or indicate a specific area where genes are being expressed. In conclusion, early application of synbiotics and continuation in the feed could help improve some aspects of broiler performance. Further studies should include longer trials and the addition of an enteric challenge, to test if such feed additives containing synbiotics can be more effective during disease conditions.

References

Abd El-Hack, M.E., M.T. El-Saadony, M.E. Shafi, S.Y.A. Qattan, G.E. Batiha, A.F. Khafaga. A.E. Abdel-Moneim, and M. Alagawany. 2020. Probiotics in poultry feed: A comprehensive review. J. Ani. Physiol. Ani. Nutr. 104:1835-50.

Ahmed, I.A., N. Arafat, R.M. Saleh, H.M. Ghanem, D. Naguib, H.A. Radwan, and Y.Y. Elseady. 2019. Intestinal gene expressions in broiler chickens infected with *Escherichia coli* and dietary supplemented with probiotic, acidifier and synbiotic. Vet. Res. Commun. 43:131-42.

Ahrne, S. and M.L. Hagslatt. 2011. Effect of lactobacilli on paracellular permeability in the gut. Nutrients 3:104-17.

Awad, W.A., C. Hess, and M. Hess. 2017. Enteric pathogens and their toxin-induced disruption of the intestinal barrier through alteration of tight junctions in chickens. Toxins 9:60.

Awad, W.A., K. Ghareeb, S. Abdel-Raheem, and J. Bohm. 2009. Effects of dietary inclusion of prebiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poult. Sci. 88:49-56.

Bergoug, H., M. Guinebretière, Q. Tong, N. Roulston, C.E.B. Romanini, V. Exadaktylos, D. Berckmans, P. Garain, T.G.M. Demmers, I.M. McGonnell, C. Bahr, C. Burel, N. Eterradossi, and V. Michel. 2013. Effect of transportation duration of 1-day-old chicks on postplacement production performances and pododermatitis of broilers up to slaughter age. Poult. Sci. 92:3300-09.

Cervantes, H. 2015. Antibiotic-free poultry production: Is it sustainable? J. Appl. Poult. Res. 24:91-7.

Dam, A., and S. Fitzgerald. 2017. Poultry Handling and Transportation Manual. Poultry Service Association.

Davies, J. and D. Davies. 2010. Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. Rev. 74:417-33.

Di Pierro, M., R. Lu, S. Uzzau, W. Wang, K. Margaretten, C. Pazzani, F. Maimone, and A. Fasano. 2001. Zonula occludens toxin structure-function analysis. Identification of the fragment biologically active on tight junctions and of the zonulin receptor binding domain. J. Biol. Chem. 276:19160-65.

Dibner, J.J. and J.D. Richards. 2005. Antibiotic growth promoters in agriculture: History and mode of action. Poult. Sci. 84:634-43.

EFSA Panel on Animal Health and Welfare (AHAW). 2011. Scientific opinion concerning the welfare of animals during transport. Chick yolk sac lasts up to 3 days post-hatch. EFSA J. 9:125 10.2903/j.efsa.2011.1966.

Emami, N.K., A. Calik, M.B. White, M. Young, and R.A. Dalloul. 2019. Necrotic enteritis in broiler chickens: the role of tight junctions and mucosal immune responses in alleviating the effect of the disease. Microorganisms 7:231.

Fairchild, B.D., J.K. Northcutt, J.M. Mauldin, R.J. Buhr, L.J. Richardson, and N.A. Cox. 2006. Influence of water provision to chicks before placement and effects on performance and incidence of unabsorbed yolk sacs. J. Appl. Poult. Res. 15:538-43.

Fasano, A. and J.P. Nataro. 2004. Intestinal epithelial tight junctions as targets for enteric bacteria-derived toxins. Adv. Drug Deliv. Rev. 56:795-807.

Fuller, R. 1989. Probiotics in man and animals: a review. J. Appl. Bacteriol. 66:365-78.

Gibson, G. R.; H.M. Probert, R.A. Rastall, and M.B. Roberfroid, 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr. Res. Rev. 17:259-75.

Gilbert, E.R., H. Li, D.A. Emmerson, K.E. Webb, Jr., and E.A. Wong. 2007. Developmental Regulation of Nutrient Transporter and Enzyme mRNA Abundance in the Small Intestine of Broilers. Poult. Sci. 86:1739-53.

Givisiez, P., A. Moreira-Filho, M. Santos, H.B. Oliveira, P.R. Ferket, C. Oliveira, and R.D. Malheiros. 2020. Chicken embryo development: metabolic and morphological basis for *in ovo* feeding technology. Poult. Sci. 99:6774–82.

Heier, B, H. Høgåsen, and J. Jarp. 2002. Factors associated with mortality in Norwegian broiler flocks. Prev. Vet. Med. 53:147-58.

Jacobs, L., E. Delezie, L. Duchateau, K. Goethals, B. Ampe, E. Lambrecht, X. Gellynck, and F.A.M. Tuyttens. 2016. Effect of post-hatch transportation duration and parental age on broiler chicken quality, welfare, and productivity. Poult. Sci. 95:1973-79.

Kabir, S.M.L., M.M. Rahman, M.B. Rahman, M.M. Rahman, and S.U. Ahmed. 2004. The dynamics of probiotics on growth performance and immune response in broilers Int. J. Poult. Sci. 3:361-64.

Kidd, M.T., J.W. Taylor, C.M. Page, B.D. Lott, and T.N. Chamblee. 2007. Hatchery feeding of starter diets to broiler chicks. J. Appl. Poult. Res. 16:234-39.

Klasing, K.C., D.E. Laurin, R.K. Peng, and M. Fry. 1987. Immunologically mediated growth depression in chicks: influence of feed intake, corticosterone and interleukin-1. J. Nutr. 117:1629-37.

Lan, Y., M.W. Verstegen, S. Tamminga, and B.A. Williams. 2005. The role of the commensal gut microbial community in broiler chickens. Worlds Poult. Sci. J. 61:95-104.

Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2- ΔΔCt method. Methods 25:402-8.

Miremadi, F. and N.P. Shah. 2012. Applications of inulin and probiotics in health and nutrition. Int. Food Res. J. 19:1337-50.

Miska, K.B., R.H. Fetterer, and E.A. Wong. 2015. mRNA expression of amino acid transporters, aminopeptidase, and the di- and tri-peptide transporter PepT1 in the intestine and liver of posthatch broiler chicks. Poult. Sci. 96:1323-32.

Negi, S., D.K. Das, S. Pahari, S. Nadeem, and J.N. Agrewala. 2019. Potential role of gut microbiota in induction and regulation of innate immune memory. Front. Immunol. 10:2441.

Noy, Y. and D. Sklan. 1999. Different types of early feeding and performance in chicks and poults. J. Appl. Poult. Res. 8:16-24.

Oliveira-Nascimento, L., P. Massari, and L.M. Watzler. 2012. The role of TLR2 in infection and immunity. Front. Immunol. 3:79.

Philips, Ian. 2007. Withdrawal of growth-promoting antibiotics in Europe and its effects in relation to human health. Int. J. Antimicrob. Agents 30:101-7.

Phillips, I., M. Casewell, T. Cox, B. DeGroot, C. Friis, R. Jones, C. Nightingale, R. Preston, and J. Waddell. 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J. Antimicrob. Chemother. 53:28-52.

Samanta, A. K., S. Senani, A.P. Kolte, M. Sridhar, R. Bhatta, and N. Jayapal. 2012. Effect of prebiotic on digestibility of total mixed ration. Indian Vet. J. 89:41-2.

Sarangi, N.R., L.K. Babu, A. Kumar, C.R. Pradhan, P.K. Pati, and J.P. Mishra. 2016. Effect of dietary supplementation of prebiotic, probiotic, and synbiotic on growth performance and carcass characteristics of broiler chickens. Vet. World 9:313-19.

Sklan D., Geyra A., Tako E., Gal-Gerber O., and Z. Uni. 2003. Ontogeny of brush border carbohydrate digestion and uptake in the chick. Br. J. Nutr. 89:747-753.

Shimizu, K., Y. Komaki, N. Fukano, and T. Bungo. 2017. Transporter gene expression and transference of fructose in broiler chick intestine. J. Poult. Sci. 55:137-41.

Song, J., L.F. Jiao, K. Xiao, Z.S. Luan, C.H. Hu, B. Shi, and X.A. Zhan. 2013. Cello-oligosaccharide ameliorates heat stress-induced impairment of intestinal microflora, morphology and barrier integrity in broilers. Anim. Feed Sci. Technol. 185:175-81.

Su, S., D.M. Dwyer, K.B. Miska, R.H. Fetterer, M.C. Jenkins, and E.A. Wong. 2017. Expression of host defense peptides in the intestine of Eimeria-challenged chickens. Poult. Sci. 96:2421-27.

Sujatha D., S. Xianglin, S. Leonard, L. Wang, V. Castranova, and Y. Rojanasakul. 2001. Interleukin-10-mediated inhibition of free radical generation in macrophages. Am. J. Physiol. 280:L1196-L1202.

Teng, P.Y. and W.K. Kim. 2018. Review: roles of prebiotics in intestinal ecosystem of broilers. Front. Vet. Sci. 5:245.

Uni, Z. and R.P. Ferket. 2004. Methods for early nutrition and their potential. Worlds Poult. Sci. J. 60:101-11.

Xin, H. and S.R. Rieger. 1995. Physical conditions and mortalities associated with international air transport of young chicks. Am. Soc. Ag. Eng. 38:1863-67.

Yang, Y., P.A. Iji, and M. Choct. 2009. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. Worlds Poult. Sci. J. 65:97-114.

Table 3.1. Treatment Groups.

Trt	Hatchery Application	Dietary Trt	Replicates	Animals/Rep	Animals/Trt
1. CTRL	Gel only	Basal	8	15	120
2. HS	Gel+Synbiotic	Basal	8	15	120
3. DS	Gel only	Basal + Synbiotic	8	15	120
4. HSDS	Gel+Synbiotic	Basal + Synbiotic	8	15	120

CTRL = Control. HS = Hatchery Synbiotic. DS = Dietary Synbiotic.

Table 3.2. Feed Composition of broiler mash starter diet (D0-21).

Ingredient, g/kg	0-21 d
Corn	59.73
Soybean meal, CP 48%	33.20
Vegetable oil	3.00
Limestone	0.68
Dicalcium phosphate	2.20
DL-Methionine (98%)	0.20
L-Lysine HCl (78%)	0.24
L-Threonine	0.09
Salt	0.30
Vitamin-Mineral Premix (NB3000)*	0.36
Total	100.00
Chemical composition (Calculated)	
Dry Matter, %	87.75
Crude Protein, %	21.00
AME_n, kcal/kg	3060
Lysine, %	1.32
Dig. Lysine, %	1.18
Methionine + cysteine, %	0.88
Dig. Methionine + cysteine, %	0.78
Threonine, %	0.90
Dig. Threonine, %	0.77
Calcium, %	0.90
Available phosphorus, %	0.45

*Vitamins per kg diet: retinol 3.33 mg, cholecalciferol 0.1 mg, α-tocopherol acetate 23.4 mg, vitamin K3 1.2 mg, vitamin B1 1.6 mg, vitamin b2 9.5 mg, niacin 40 mg, pantothenic acid 9.5 mg, vitamin B6 2 mg, folic acid 1 mg, vitamin B12 0.016 mg, biotin 0.05 mg, choline 556 mg. Minerals per kg diet: Mn 144 mg, Fe 72 mg, Zn 144 mg, Cu 16.2 mg, I 2.1 mg, Se 0.22 mg.

Table 3.3. Primers used for qRT-PCR in mRNA abundance. Forward primer sequence is listed before reverse primer sequence.

Gene[a]	Primer Sequence (5' – 3')	NCBI Accession Number
GAPDH	CCTAGGATACACAGAGGACCAGGTT GGTGGAGGAATGGCTGTCA	NM_204305
Zonula occluden-1	GGAGTACGAGCAGTCAACATAC GAGGCGCACGATCTTCATAA	XM_413773
Zonula occluden-2	GCGTCCCATCCTGAGAAATAC CTTGTTCACTCCCTTCCTCTTC	NM_205149.1
Claudin-1	GTGTTCAGAGGCATCAGGTATC CTCAGGTCAAACAGAGGTACAA	NM_001013611.2
PepT1	CCCCTGAGGAGGATCACTGTT CAAAAGAGCAGCAGCAACGA	NM_204365
SGLT1	GCCATGGCCAGGGCTTA CAATAACCTGATCTGTGCACCAGTA	XM_415247
TLR2	GCGAGCCCCCACGAA GGAGTCGTTCTCACTGTAGGAGACA	NM_204278
TLR4	CCACACACCTGCCTACATGAA GGATGGCAAGAGGACATATCAAA	NM_001030693
IL-10	CGCTGTCACCGCTTCTTCA CGTCTCCTTGATCTGCTTGATG	NM_001004414

[a]GAPDH = Glyceraldehyde 3-phosphate dehydrogenase. PepT1 = Peptide Transporter 1. SGLT1 = Sodium-dependent glucose transporter 1. TLR = Toll-like receptor. IL = Interleukin.

Table 3.4. Effect of synbiotic application method on mortality (%). Includes interaction between diet*hatchery application, main effect of diet, and main effect of hatchery application method.

Interaction between Diet * Hatchery Application (*n* = 120 birds/trt)					
Trt[1]	DOH-3 (%)	D3-7 (%)	D7-14 (%)	D14-21 (%)	DOH-21 (%)
1. CTRL	0	1.67	2.73	0	4.17
2. HS	0	0.83	0.90	0	1.67
3. DS	1.67	0	0.91	0	2.50
4. HSDS	0.83	0	0.90	0	1.67
	P > 0.10	*P > 0.10*	*P > 0.10*	-	*P > 0.10*
	SEM = 0.10	*SEM = 0.12*	*SEM = 0.17*	-	*SEM = 0.22*
Main Effect of Diet (*n* = 240 birds/trt)					
Diet	**DOH-3 (%)**	**D3-7 (%)**	D7-14 (%)	D14-21 (%)	DOH-21 (%)
1. Basal	**0**	**1.25**	1.81	0	2.92
2. Synbiotic	**1.25**	**0**	0.90	0	2.08
	P ≤ 0.10	***P ≤ 0.10***	*P > 0.10*	-	*P > 0.10*
	SEM = 0.07	***SEM = 0.07***	*SEM = 0.12*	-	*SEM = 0.15*
Main Effect of Hatchery Application (*n* = 240 birds/trt)					
Application	DOH-3 (%)	D3-7 (%)	D7-14 (%)	D14-21 (%)	DOH-21 (%)
1. Gel Only	0.83	0.84	1.82	0	3.33
2. Synbiotic	0.42	0.42	0.90	0	1.67
	P > 0.10	*P > 0.10*	*P > 0.10*	-	*P > 0.10*
	SEM = 0.07	*SEM = 0.07*	*SEM = 0.12*	-	*SEM = 0.15*

[1]Treatments: CTRL = basal diet and gel application only; HS = hatchery synbiotic; DS = dietary synbiotic; HSDS = hatchery synbiotic and dietary synbiotic. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. SEM: standard error means.

Table 3.5. Effect of synbiotic application method on average body weight (BW) per bird. Includes interaction between diet*hatchery application, main effect of diet, and main effect of hatchery application method.

Interaction between Diet * Hatchery Application (*n* = 120 birds/trt)					
Trt[1]	DOH (g)	D3 (g)	D7 (g)	D14 (g)	D21 (g)
1. CTRL	43	78	162	**470**[a]	932
2. HS	43	75	151	**430**[b]	876
3. DS	43	76	154	**441**[ab]	903
4. HSDS	43	75	150	**438**[b]	921
	P > 0.10	*P > 0.10*	*P > 0.10*	***P ≤ 0.10***	*P > 0.10*
	[2]*SEM = 0.31*	*SEM = 1.0*	*SEM = 2.6*	***SEM = 11.0***	*SEM = 30.0*
Main Effect of Diet (*n* = 240 birds/trt)					
Diet	DOH (g)	D3 (g)	D7 (g)	D14 (g)	D21 (g)
1. Basal	-	76	156	451	904
2. Synbiotic	-	75	152	440	912
		P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*
		SEM = 0.71	*SEM = 1.8*	*SEM = 7.6*	*SEM = 20.0*
Main Effect of Hatchery Application (*n* = 240 birds/trt)					
Application	DOH (g)	D3 (g)	D7 (g)	D14 (g)	D21 (g)
1. Gel Only	-	**77**[a]	**158**[a]	456[a]	917
2. Synbiotic	-	**75**[b]	**151**[b]	435[b]	899
		P ≤ 0.10	***P ≤ 0.05***	*P ≤ 0.10*	*P > 0.10*
		SEM = 0.71	***SEM = 1.8***	*SEM = 7.6*	*SEM = 20.0*

[1]Treatments: CTRL = basal diet and gel application only; HS = hatchery synbiotic; DS = dietary synbiotic; HSDS = hatchery synbiotic and dietary synbiotic. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 3.6. Effect of synbiotic application method on average body weight gain (BWG) per bird (kg). Includes interaction between diet*hatchery application, main effect of diet, and main effect of hatchery application method.

Interaction between Diet * Hatchery Application (*n* = 120 birds/trt)					
Trt[1]	DOH-3 (g)	D3-7 (g)	D7-14 (g)	D14-21 (g)	DOH-21 (g)
1. CTRL	34	84	**308[a]**	506	933
2. HS	32	76	**277[b]**	473	872
3. DS	33	78	**288[ab]**	487	886
4. HSDS	32	75	**288[ab]**	504	900
	P > 0.10	*P > 0.10*	***P ≤ 0.10***	*P > 0.10*	*P > 0.10*
	SEM = 0.8	*SEM = 2.3*	***SEM = 8.7***	*SEM = 12*	*SEM = 21*
Main Effect of Diet (*n* = 240 birds/trt)					
Diet	DOH-3 (g)	D3-7 (g)	D7-14 (g)	D14-21 (g)	DOH-21 (g)
1. Basal	34	80	293	490	904
2. Synbiotic	33	77	288	496	893
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 0.59	*SEM = 1.6*	*SEM = 6.3*	*SEM = 8.8*	*SEM = 15*
Main Effect of Hatchery Application (*n* = 240 birds/trt)					
Application	DOH-3 (g)	D3-7 (g)	D7-14 (g)	D14-21 (g)	DOH-21 (g)
1. Gel Only	**34[a]**	**81[a]**	298[a]	497	909
2. Synbiotic	**32[b]**	**76[b]**	283[b]	489	887
	P ≤ 0.10	***P ≤ 0.05***	*P > 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 0.59	***SEM = 1.6***	*SEM = 6.3*	*SEM = 8.8*	*SEM = 15*

[1]Treatments: CTRL = basal diet and gel application only; HS = hatchery synbiotic; DS = dietary synbiotic; HSDS = hatchery synbiotic and dietary synbiotic. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. SEM: standard error means.

Table 3.7. Effect of synbiotic application method on average feed intake (FI) per bird (kg). Includes interaction between diet*hatchery application, main effect of diet, and main effect of hatchery application method.

Interaction between Diet * Hatchery Application (*n* = 120 birds/trt)					
Trt[1]	DOH-3 (g)	D3-7 (g)	**D7-14 (g)**	**D14-21 (g)**	**DOH-21 (g)**
1. CTRL	54	108	**400[a]**	**732[a]**	**1294[a]**
2. HS	56	114	**369[b]**	**647[b]**	**1187[b]**
3. DS	54	100	**376[b]**	**664[b]**	**1195[b]**
4. HSDS	53	99	**376[b]**	**695[ab]**	**1223[ab]**
	P > 0.10	*P > 0.10*	***P ≤ 0.10***	***P ≤ 0.05***	***P ≤ 0.05***
	SEM = 3.4	*SEM = 5.3*	***SEM = 7.7***	***SEM = 17.2***	***SEM = 25.0***
Main Effect of Diet (*n* = 240 birds/trt)					
Diet	DOH-3 (g)	**D3-7 (g)**	D7-14 (g)	D14-21 (g)	DOH-21 (g)
1. Basal	55	**111[a]**	384	690	1240
2. Synbiotic	53	**100[b]**	376	679	1209
	P > 0.10	***P ≤ 0.05***	*P > 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 0.24	***SEM = 3.7***	*SEM = 5.4*	*SEM = 12.0*	*SEM = 18.0*
Main Effect of Hatchery Application (*n* = 240 birds/trt)					
Application	DOH-3 (g)	D3-7 (g)	D7-14 (g)	D14-21 (g)	DOH-21 (g)
1. Gel Only	54	104	388[a]	698	1244
2. Synbiotic	55	106	373[b]	671	1205
	P > 0.10	*P > 0.10*	*P ≤ 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 2.4	*SEM = 3.7*	*SEM = 5.4*	*SEM = 12.2*	*SEM = 18.0*

[1]Treatments: CTRL = basal diet and gel application only; HS = hatchery synbiotic; DS = dietary synbiotic; HSDS = hatchery synbiotic and dietary synbiotic. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. SEM: standard error means.

Table 3.8. Effect of synbiotic application method on average feed conversion ratio (FCR) per bird. Includes interaction between diet*hatchery application, main effect of diet, and main effect of hatchery application method.

Interaction between Diet * Hatchery Application (*n* = 120 birds/trt)					
Trt[1]	DOH-3	D3-7	D7-14	**D14-21**	DOH-21
1. CTRL	1.54	1.28	1.30	**1.42**[a]	1.39
2. HS	1.75	1.53	1.34	**1.34**[b]	1.36
3. DS	1.64	1.28	1.31	**1.37**[ab]	1.35
4. HSDS	1.64	1.31	1.31	**1.38**[ab]	1.36
	P > 0.10	*P > 0.10*	*P > 0.10*	***P ≤ 0.05***	*P > 0.10*
	SEM = 0.10	*SEM = 0.08*	*SEM = 0.03*	***SEM = 0.02***	*SEM = 0.02*
Main Effect of Diet (*n* = 240 birds/trt)					
Diet	DOH-3	D3-7	D7-14	D14-21	DOH-21
1. Basal	1.65	1.41	1.32	1.38	1.38
2. Synbiotic	1.64	1.30	1.31	1.37	1.36
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 0.07	*SEM = 0.05*	*SEM = 0.02*	*SEM = 0.01*	*SEM = 0.01*
Main Effect of Hatchery Application (*n* = 240 birds/trt)					
Application	DOH-3	**D3-7**	D7-14	D14-21	DOH-21
1. Gel Only	1.59	**1.28**[b]	1.31	1.39[a]	1.37
2. Synbiotic	1.69	**1.42**[a]	1.33	1.36[b]	1.36
	P > 0.10	***P ≤ 0.10***	*P > 0.10*	*P ≤ 0.10*	*P > 0.10*
	SEM = 0.07	***SEM = 0.05***	*SEM = 0.02*	*SEM = 0.01*	*SEM = 0.01*

[1]Treatments: CTRL = basal diet and gel application only; HS = hatchery synbiotic; DS = dietary synbiotic; HSDS = hatchery synbiotic and dietary synbiotic. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. SEM: standard error means.

Table 3.9. Effect of synbiotic application method on fold change of D7 and D21 ileum gene expression of tight junction proteins (ZO-1, ZO-2, CLD-1). Includes interaction between diet*hatchery application, main effect of diet, and main effect of hatchery application method.

Interaction between Diet * Hatchery Application (*n* = 8 birds/trt)						
Trt[1]	D7 ZO-1	D21 ZO-1	D7 ZO-2	D21 ZO-2	D7 CLD-1	D21 CLD-1
1. CTRL	1.04	1.10	1.12	1.03	0.00082	0.0025
2. HS	0.85	1.94	1.22	1.12	0.00108	0.0022
3. DS	0.89	0.87	0.89	0.90	0.00061	0.0021
4. HSDS	0.75	1.05	0.90	0.96	0.00094	0.0030
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 0.15	*SEM = 0.16*	*SEM = 0.17*	*SEM = 0.16*	*SEM = 0.0003*	*SEM = 0.0008*
Main Effect of Diet (*n* = 16 birds/trt)						
Diet	D7 ZO-1	D21 ZO-1	D7 ZO-2	D21 ZO-2	D7 CLD-1	D21 CLD-1
1. Basal	0.95	1.02	1.17	1.08	0.00095	0.0024
2. Synbiotic	0.82	0.96	0.90	0.93	0.00074	0.0026
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 0.10	*SEM = 0.11*	*SEM = 0.12*	*SEM = 0.37*	*SEM = 0.0002*	*SEM = 0.0006*
Main Effect of Hatchery Application (*n* = 16 birds/trt)						
Application	D7 ZO-1	D21 ZO-1	D7 ZO-2	D21 ZO-2	D7 CLD-1	D21 CLD-1
1. Gel Only	0.97	0.99	1.00	0.97	0.00071	0.0023
2. Synbiotic	0.80	0.99	1.07	1.04	0.00103	0.0026
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 0.10	*SEM = 0.11*	*SEM = 0.12*	*SEM = 0.11*	*SEM = 0.0002*	*SEM = 0.0006*

[1]Treatments: CTRL = basal diet and gel application only; HS = hatchery synbiotic; DS = dietary synbiotic; HSDS = hatchery synbiotic and dietary synbiotic. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. SEM: standard error means.

Table 3.10. Effect of synbiotic application method on fold change of D7 and D21 ileum gene expression of nutrient transporters (PepT1, SGLT1). Includes interaction between diet*hatchery application, main effect of diet, and main effect of hatchery application method.

Interaction between Diet * Hatchery Application (*n* = 8 birds/trt)				
Trt[1]	D7 PepT1	**D21 PepT1**	D7 SGLT1	D21 SGLT1
1. CTRL	1.09	**0.84**[b]	0.43	0.61
2. HS	0.85	**1.11**[b]	1.99	1.06
3. DS	0.64	**1.04**[b]	3.20	0.97
4. HSDS	0.87	**6.36**[a]	0.23	1.28
	P > 0.10	***P ≤ 0.10***	*P > 0.10*	*P > 0.10*
	SEM = 0.16	***SEM = 1.37***	*SEM = 1.35*	*SEM = 0.26*
Main Effect of Diet (*n* = 16 birds/trt)				
Diet	D7 PepT1	D21 PepT1	D7 SGLT1	D21 SGLT1
1. Basal	0.97	0.97[b]	1.33	0.83
2. Synbiotic	0.75	3.67[a]	1.92	1.10
	P > 0.10	*P ≤ 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 0.11	*SEM = 0.97*	*SEM = 0.96*	*SEM = 0.18*
Main Effect of Hatchery Application (*n* = 16 birds/trt)				
Application	D7 PepT1	D21 PepT1	D7 SGLT1	D21 SGLT1
1. Gel Only	0.87	0.93[b]	2.01	0.79
2. Synbiotic	0.86	3.74[a]	1.24	1.15
	P > 0.10	*P ≤ 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 0.11	*SEM = 0.97*	*SEM = 0.96*	*SEM = 0.18*

[1]Treatments: CTRL = basal diet and gel application only; HS = hatchery synbiotic; DS = dietary synbiotic; HSDS = hatchery synbiotic and dietary synbiotic. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. SEM: standard error means.

Table 3.11. Effect of synbiotic application method on fold change of D7 and D21 ileum gene expression of immune markers (IL-10, TLR2, TLR4). Includes interaction between diet*hatchery application, main effect of diet, and main effect of hatchery application method.

Interaction between Diet * Hatchery Application (*n* = 8 birds/trt)						
Trt[1]	D7 IL-10	**D21 IL-10**	D7 TLR2	D21 TLR2	D7 TLR4	D21 TLR4
1. CTRL	1.35	**3.08**^{ab}	1.09	0.32	0.70	0.89
2. HS	0.88	**1.70**^{b}	1.38	0.29	0.84	0.98
3. DS	0.54	**1.52**^{b}	0.57	0.26	0.47	0.83
4. HSDS	0.42	**5.84**^{a}	0.59	0.41	0.44	1.17
	P > 0.10	***P ≤ 0.05***	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 0.26	***SEM = 1.35***	*SEM = 0.42*	*SEM = 0.07*	*SEM = 0.12*	*SEM = 0.24*
Main Effect of Diet (*n* = 16 birds/trt)						
Diet	**D7 IL-10**	D21 IL-10	D7 TLR2	D21 TLR2	D7 TLR4	D21 TLR4
1. Basal	**1.12**^{a}	2.39	1.25	0.31	**0.78**^{a}	0.94
2. Synbiotic	**0.49**^{b}	3.54	0.58	0.32	**0.46**^{b}	0.98
	P ≤ 0.05	*P > 0.10*	*P > 0.10*	*P > 0.10*	***P ≤ 0.05***	*P > 0.10*
	SEM = 0.18	*SEM = 0.95*	*SEM = 0.30*	*SEM = 0.01*	***SEM = 0.08***	*SEM = 0.17*
Main Effect of Hatchery Application (*n* = 16 birds/trt)						
Application	D7 IL-10	D21 IL-10	D7 TLR2	D21 TLR2	D7 TLR4	D21 TLR4
1. Gel Only	0.94	2.30	0.82	0.29	0.57	0.86
2. Synbiotic	0.68	3.63	1.01	0.35	0.67	1.07
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 0.18	*SEM = 0.95*	*SEM = 0.30*	*SEM = 0.05*	*SEM =0.08*	*SEM = 0.17*

[1]Treatments: CTRL = basal diet and gel application only; HS = hatchery synbiotic; DS = dietary synbiotic; HSDS = hatchery synbiotic and dietary synbiotic. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. SEM: standard error means.

CHAPTER IV

Determining optimum dose of *in ovo* probiotics or synbiotics for broiler hatchability and performance during necrotic enteritis

Abstract

Necrotic enteritis (NE) is a major enteric disease in poultry caused by opportunistic bacteria *Clostridium perfringens*. As subtherapeutic antibiotic use has declined, probiotics application to establish a balanced and beneficial microbiota may help reduce the harmful effects of NE, a method that may be more effective with earlier delivery during embryonic development. This 21-day study was conducted to determine optimum *in ovo* dosage level of a probiotic or synbiotic (PROB or SYNB) during a NE challenge for use in subsequent studies. In total, 392 embryonic day 18 (E18) Cobb 500 broiler eggs were randomly divided into 7 treatments: *in ovo* application of sterile water (**CTRL**), low (**PROB-L** or **SYNB-L**: $1x10^5$ CFU per bird), medium (**PROB-M** or **SYNB-M**: $1x10^6$ CFU per bird), or high (**PROB-H** or **SYNB-H**: $1x10^7$ CFU per bird) probiotic or synbiotic doses dissolved in sterile water. One day post-placement, a commercial coccidiosis vaccine was sprayed on feed and litter to induce a naturally-occurring NE challenge. Performance and feed intake measurements were taken at DOH, D4, D8, D14, and D21. On D8, ileal lesion scores and mucosal scrapings were sampled for lesion scoring and mRNA abundance of immune genes: interleukin (IL)-1β, Toll-like receptor (TLR)4, IL-10, avian β-defensin (AvBD)10, and AvBD13. Statistical analysis was performed in JMP with LS Means to separate means with significance assigned at $P \leq 0.05$ and trends set at $0.10 \leq P \leq 0.05$. *In ovo* application of PROB had no significant influence on hatchability, bird performance, or mRNA abundance of target genes ($n = 4$). In contrast, there was greater DOH-21 mortality and higher

FCR from D14-21 in SYNB-L compared to CTRL. The D21 BW, D14-21 FI, and D14-21 BWG were lower in SYNB-L and SYNB-M compared to CTRL and SYNB-H. Cumulatively, SYNB-H had a lower FCR compared to SYNB-M. A greater D8 IL-1β mRNA abundance was measured in SYNB-M and SYNB-H compared to SYNB-L. The D8 mRNA abundance for AvBD10 was greater in SYNB-H compared to the CTRL and SYNB-M. Overall, SYNB-H performed most similar to CTRL, and better than SYNB-L or SYNB-M. For subsequent experiments, the high doses of PROB and SYNB were selected for investigating *in ovo* application of probiotics during extended trials involving NE and additional parameters.

Keywords: probiotics, synbiotics, *in ovo*, performance, broiler, necrotic enteritis

Introduction

Two commercially important diseases in broilers are coccidiosis and necrotic enteritis (NE). Coccidiosis is a disease caused by various species of *Eimeria* protozoa that infect enterocytes, rupture host cells, and cause extensive damage and inflammation in young birds (Dalloul and Lillehoj, 2006). The gut damage inflicted by coccidiosis can lead to NE, caused by the opportunistic bacteria *Clostridium perfringens* (CP) that proliferates in the presence of compromised intestinal tissues and increased gut mucus production. Further damage to the small intestine occurs when CP produces an enterotoxin that induces hemorrhagic necrosis of intestinal tissues (Hargis, 2014). Historically, NE could be controlled by reducing coccidiosis via feeding anticoccidials and treating NE outbreaks by administering antibiotics in drinking water (Noack et al., 2009; Hargis, 2014). However, increasing bans on antibiotics and anticoccidial drugs has prompted the use of prebiotics and probiotics as a viable alternative in reducing these enteric diseases. Feeding beneficial probiotics to chicks can help establish a healthy gut microbiota, exclude pathogenic bacteria, and produce antimicrobials (Fioramonti et al., 2003; Yang et al.,

2009). Supplementing prebiotics can selectively encourage growth of beneficial gut bacteria, including bifidobacteria and lactobacteria (Baffoni et al., 2012).

Injecting prebiotics and probiotics *in ovo* allows for more precise delivery, reduces labor costs and post-hatch handling, and delivers the product to the chick a few days earlier than a post-hatch application (Ricks et al., 1999). Numerous studies have confirmed *in ovo* application of probiotics and prebiotics to be safe, have no impact on hatchability, stimulate the immune system, and provide protection against intestinal diseases (de Oliveira et al., 2014; Pruszynska-Oszmalek et al., 2015; Pender et al., 2016; Pender et al., 2017; Triplett et al., 2018). To measure potential immune modulation by probiotics, five genes were chosen to measure mRNA abundance of Toll-like receptor 4 (TLR4), interleukin (IL)-10, IL-1β, avian β-defensin (AvBD)10, and AvBD13. TLR4 senses lipopolysaccharide (LPS) components of the outer membrane in Gram-negative bacteria and activates innate immune responses (Akira and Takeda, 2004). In chickens, TLR4 forms a complex with myeloid differentiation protein-2 (MD-2) and activates a MyD88-dependent signaling pathway, ultimately activating NF-κB, a vital transcription factor for activating inflammatory genes in cells, including IL-1β (Keestra and van Putten, 2008; Liu et al., 2017; Qu et al., 2019). In response to bacterial challenges, pro-inflammatory IL-1β is upregulated, increasing cytokine and chemokine production (Gibson et al., 2014; Chen et al., 2016). Avian beta-defensins are antimicrobial peptides that antagonize pathogenic bacteria and parasites by disrupting membrane integrity (Casterlow et al., 2011). Here, AvBD10 and AvBD13 were selected because of their activities in the distal intestinal tract (Lynn et al., 2004; Cuperus et al., 2013). The host immune system maintains intestinal homeostasis by secreting anti-inflammatory mediators such as IL-10 that is produced by multiple

cell types. It contributes to negative feedback regulation to control inflammatory response (Manzanillo et al., 2015; Wu et al., 2016).

In this study, three doses of two different soluble probiotic products were injected *in ovo* into the amnion of broiler eggs at E18. After hatch, a commercial coccidiosis vaccine was applied to feed and litter to induce a naturally occurring NE challenge. The objective was to evaluate performance and immune response of broilers to the NE challenge after various levels of probiotics, and select the best performing probiotic dose for subsequent *in ovo* and disease challenge studies.

Materials and Methods

Birds and Experimental Treatments

This 21-day study was conducted under the approval of IACUC # 18-136-APSC at the Virginia Tech turkey farm research facilities. A total of 392 embryonic day 18 (E18) Cobb 500 eggs were obtained from a nearby commercial hatchery and transported to Virginia Tech under a closely monitored temperature similar to hatchery conditions (35.5°C). On the same day, embryos were randomly divided into seven treatments (56 eggs/treatment) and received an *in ovo* injection of either sterile water (**CTRL**) or water-soluble probiotics (Table 4.1). The remaining six treatments consisted of a probiotic or synbiotic product evaluated at low (**L**), medium (**M**), or high (**H**) doses. The first probiotic (**PROB**) contained a guaranteed analysis of $1x10^8$ CFU/gram containing *L. acidophilus*, *L. casei, Enterococcus faecium*, and *Bifidobacterium thermophilum* dissolved in water. The second product was a synbiotic (**SYNB**) containing guaranteed analysis of 5×10^9 CFU/gram of *Enterococcus faecium*, *Pediococcus acidilactici*, *Bidifobacterium animalis*, *Lactobacillus reuteri* as the dried products of *E. faecium*, *P. acidilactici*, *B. animalis*, *L. reuteri*, and the prebiotic inulin (a fructooligosaccharide). Both treatments were adjusted to

consist of low ($1x10^5$ CFU), medium ($1x10^6$ CFU), or high ($1x10^7$ CFU) doses dissolved in sterile water as a delivery medium.

In Ovo Injection and Incubation Conditions

To give the injection, each egg was candled, the air cell outlined, and a small hole was made using a sterile 18-gauge needle attached to a rubber stopper. A sterile 1½ inch long, 20-gauge needle and syringe were then used to inject 100 µL of designated probiotic or sterile water into the amnion. Immediately after injection, the small hole was sealed with clean tape, and eggs were returned to the incubator. Incubation temperature was maintained at 37.5°C with humidity between 60-70% until hatch. After hatch, 48 of the 56 birds/treatment were chosen based on average weight and outward appearance of health for transfer to an on-site growout facility.

Bird Housing and Management

Each of the seven treatments was further divided into 4 replicates/treatment (48 birds/treatment; 12 birds/replicate). Each replicate was raised in a 1.2 x 1.2 meter floor pen using clean pine shavings on top of concrete flooring. Water was supplied by adjustable nipple drinkers, and feed was provided by a height-adjustable metal feeder. Birds received ad libitum feed and water throughout the experimental period. A corn/soybean starter mash diet was fed from DOH-D14, and a corn/soy grower mash diet was fed from D14-D21 (Table 4.2). Temperature began at 36°C at DOH and was gradually lowered following industry standards until reaching 24°C by D21. Light was provided for 24 hours the first week, and gradually adjusted down to 20 hours of light and 4 hours of dark between D7-D14.

Performance Measurements

Observations and mortality were recorded twice daily. Feeder weight and individual bird weights were taken on DOH, D8, D14, and D21; group pen weights were taken on D4. Performance measurements were adjusted for mortality and reported on an average bird per pen basis for average body weight (BW), BW gain (BWG), total feed intake (FI), and feed conversion ratio (FCR).

Experimental NE Challenge Model

One day post-placement, the feed and litter of each pen was sprayed with 11X the recommended dosage of commercially available Coccivac-B52 vaccine containing live oocysts of *Eimeria acervulina*, *E. maxima*, *E. maxima MFP*, *E. mivati*, and *E. tenella*. The 11X dosage was chosen based on successful dosage levels used in various previous studies by the same lab in the same facility. The non-challenged treatment pens (NC) were placed at the front of the house, as separated as possible from challenged pens and upwind of the tunnel ventilation. Challenged treatment pens were placed in a randomized design throughout the house to reduce variability from location in the house. This coccidiosis challenge causes damage to the gut and increases mucus production, thus, promoting a NE outbreak in birds by the opportunistic bacteria *C. perfringens* ubiquitous to that poultry facility. Birds were closely monitored for peak mortality, and on D8, one bird per pen was euthanized by cervical dislocation and sampled. Lesions were scored for NE on a 0-4 scale as previously described (Prescott et al., 1978) with 0 = no lesions, 1 = thin-walled and friable intestines, 2 = focal necrosis or ulceration, 3 = large patches of necrosis 2-3 cm long, and 4 = severe necrosis.

mRNA Abundance in Ileal Tissues

On D8, an ileal mucosal scraping was taken from the same bird that was lesion scored. The ileum was immediately removed, rinsed with 10% ice-cold PBS, scrapings were immediately frozen on dry ice, and stored at −80°C until analysis. Tissue samples were homogenized using a TissueLyser (Qiagen) and RNA was extracted following standard manufacturer's protocol (Qiagen RNeasy Mini Kit). Optical density (OD) was used to assess RNA quality (OD 260/280) and concentration (OD 260) as measured by a NanoDrop-1000. The high-capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to make cDNA for qRT-PCR. Reactions (10 uL/well) were run in duplicate using Fast SYBR™ Green Master Mix on a 7500 FAST Real-Time PCR instrument (Applied Biosystems) with melt curves to determine product specificity. Target genes included immune response markers IL-1β, IL-10, TLR4, AvBD10, and AvBD13. Primer details are provided in Table 4.3. Changes in mRNA abundance of each target gene were calculated using glyceraldhyde-3-phosphate (GAPDH) as the endogenous control and the CTRL treatment as the calibrator. Results for each treatment are reported as average mRNA abundance relative to GAPDH using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Data for each probiotic were analyzed separate from one another to determine optimum dosage level of each in comparison to the control. Analysis of data was performed using JMP Pro 15 using one-way ANOVA using Fisher's least significant difference (LSD) method. Significance ($P \leq 0.05$) or a trend ($0.05 \leq P \leq 0.10$) between treatments was determined using a student's *t*-test. Results are reported as least square means (LS Means) with standard error means (SEM).

Results

Performance Measurements

Hatchability and Mortality. Hatchability was not affected by *in ovo* treatment type (Tables 4.4a and 4.4b). There was no post-hatch mortality observed in birds given sterile water or PROB *in ovo* injections throughout the experiment (Table 4.5a). Within the SYNB treatments, there were non-significant levels of mortality each week (Table 4.5b). However, cumulatively, SYNB-L had a significantly higher mortality ($P \leq 0.05$) compared to the other treatments.

Body Weight. Table 4.6a shows no significant difference in BW per bird between any of the PROB treatments and CTRL. Table 4.6b gives results for SYNB, where a significant difference in BW at DOH ($P \leq 0.01$) and trend on D21 ($P \leq 0.1$) were recorded. On DOH, SYNB-M and SYNB-L had higher BW than CTRL and SYNB-H. The birds in SYNB-H had lower BW when compared to all other treatments. No significant changes were observed at D4, D8, or D14. By D21, there was a trend for SYNB-L and SYNB-M to have lower BW compared to CTRL, but SYNB-H had a similar BW to the CTRL birds.

Body Weight Gain. There were no significant differences in BWG per bird for PROB doses (Table 4.7a); however, there was a trend ($P \leq 0.1$) for lower BWG in SYNB-L compared to CTRL (Table 4.7b). From D14-21, SYNB-L and SYNB-M had significantly lower BWG compared to CTRL and SYNB-H ($P \leq 0.05$). Cumulatively from DOH-D21, SYNB-L and SYNB-M exhibited less BWG compared to CTRL, and SYNB-H had similar BWG compared to CTRL ($P \leq 0.05$).

Feed Intake. Table 4.8a shows FI of PROB treatments. Although no significant differences were recorded, a trend ($P \leq 0.1$) was observed from DOH-D21 where PROB-M had lower FI

compared to other treatments. Within SYNB treatments, both SYNB-L and SYNB-M tended ($P \leq 0.1$) to have lower FI compared to the CTRL from D14-21 (Table 4.8b).

Feed Conversion Ratio. There were no significant differences within PROB treatments (Table 4.9a). A trend ($P \leq 0.1$) was recorded in SYNB treatments (Table 4.9b) from D8-14 where SYNB-L and SYNB-H had lower FCR compared to SYNB-M. From D14-21, there was a significant reduction ($P \leq 0.05$) in SYNB-H compared to SYNB-L and SYNB-M. Cumulatively, there was a trend ($P \leq 0.1$) from DOH-D21 in which SYNB-H had lower FCR compared to SYNB-M.

Lesion Scores

NE lesions were observed at D8, with higher lesions observed in the duodenum than the jejunum or ileum. No significant differences were observed in lesion scores among the treatment groups, but numerically, the higher doses of each product appeared to lower duodenum lesion scores compared to the CTRL and lower probiotic doses (Tables 4.10a and 4.10b).

mRNA Abundance

At the peak of infection, there were no differences observed in mRNA abundance of the target immune response genes among PROB treatments (Table 4.11a). Within SYNB treatments (Table 4.11b), a significant difference ($P \leq 0.05$) was observed in IL-1β where SYNB-M and SYNB-H had greater mRNA abundance compared to SYNB-L. AvBD10 mRNA levels were greater ($P \leq 0.1$) in SYNB-H compared to the CTRL and SYNB-M.

Discussion

Necrotic enteritis is a costly disease to the poultry industry, caused by *C. perfringens* bacteria ubiquitously found in soil, used poultry litter, dust, and even feed (Hargis, 2014). Annual global economic losses due to NE are estimated at $6 billion (Wade and Keyburn, 2015). Disease-causing strains often carry NetB genes, capable of producing a pore-forming toxin resulting in hemorrhagic necrosis in the intestines (Timbermont et al., 2009; Smyth and Martin, 2010). An outbreak of NE can be predisposed by several factors including slow feed passage in the GIT, feeding of certain grains high in non-starch polysaccharides (e.g. wheat, barley, rye), or an existing enteric disease that damages the intestinal epithelial layer (Williams, 2005; Hargis, 2014). Historically, this disease has been controlled by antimicrobials and anticoccidials to reduce damage to the gut that allows NE to occur (Tsiouris, 2016). As more restrictions are placed on antimicrobials and coccidiostats, alternative strategies will become increasingly important for maintaining bird health (Kim et al., 2011).

Rapid broiler intestinal development occurs just prior to hatch and for a few days post-hatch, as the birds transition to exogenous feed (Lji et al., 2001), and currently, chicks do not receive access to feed or water for up to 48 hours after hatch (Roto et al., 2016). The *in ovo* application of probiotics before hatch can contribute to the early establishment of a healthy gut. Probiotics can competitively exclude pathogens, produce bacteriostatic compounds, produce short-chain fatty acids as energy for the host, and modulate the host's mucosal immune system, while prebiotics support the growth of these beneficial probiotics (Dalloul et al., 2005; de Oliveira et al., 2014; Hegarty et al., 2016; Morrison and Preston, 2016; Teague et al., 2017; Triplett et al., 2018).

In this study, two different soluble products (probiotic or synbiotic) were injected into the amniotic fluid at E18 to determine the most effective dose. Hatchability was not impacted by *in ovo* application, in accordance with other studies (Pender et al., 2016; 2017; Teague et al., 2017). A coccidiosis application on DOH was used to promote an outbreak of naturally occurring NE. The impact by NE manifested in relatively low lesion scores, with an average lesion score of 2 in the duodenum, indicating a successful infection. Interestingly, there were no significant differences in performance within PROB treatments throughout the experiment. Overall, there was a trend for PROB-M to lower FI while maintaining similar BW and BWG suggesting possible improvement when given *in ovo* probiotics. Although lesion scores were not significantly different, the higher dose (PROB-H) did lower lesions in the duodenum. Other studies have demonstrated the ability of probiotics to reduce intestinal lesions following probiotic application in the feed, without affecting performance (Emami et al., 2019). Some studies recorded no change in performance past the first week of the experiment after *in ovo* probiotic application (Pender et al., 2017). Like this study, additional probiotic studies reported no change in BWG, FI, or FCR throughout the trial (Rahimi et al., 2011; Liu et al., 2016; Lin et al., 2017). Here, the PROB application did not adversely affect performance or modulate immune markers in this 21-day study. The mechanisms of action by probiotics can be complex, and a variety of factors can lead to discrepancies in function, including bacterial strain(s), dosage, animal and housing conditions, diet, and method of application, which may explain why limited changes were seen in this study compared to others (Mountzouris et al., 2007; Lin et al., 2017). Due to the nature of this pilot study, low replicates per treatment could also explain the lack of significant differences. Future larger scale studies should employ the high dose of PROB *in ovo*. A comparison with feed application could be beneficial in understanding long term

effects of these probiotics when carried out for a full growout period and including additional measurements and parameters.

Within SYNB, the experiment started with lower DOH BW in SYNB-H, but this difference was diffused by D4 and birds were statistically similar to the CTRL BW for the remainder of the experiment. Thus, this brief difference on DOH may be insignificant to the long-term goal of a uniform market weight at the end of the experiment. SYNB-L exhibited greater cumulative mortality, yet no difference in lesion scores compared to other treatments. This is likely due to the small scale of the study where few dead birds made a larger difference rather than disease causing the spike in mortality. Unlike SYNB-H, SYNB-L performance was often lower. Lower BW and BWG in the SYNB-L and SYNB-M treatments were not entirely unexpected based on results from a previous synbiotic study described in Chapter 3 of this dissertation. There, synbiotic use initially resulted in lower BW and BWG, but birds compensated by D21. Here, the SYNB-L and SYNB-M treatments had lower FI, cumulative BWG, and D21 BW along with higher FCR. Although this study did not demonstrate improvement by probiotics in certain performance parameters, it has been documented by others. Probiotics have been shown to decrease NE lesion scores and improve FCR compared to non-medicated controls (Knap et al., 2010; Eeckhaut et al., 2016). Wang et al. (2017) reported higher average daily gain and lower FCR in broilers fed probiotics during a NE challenge compared to the challenged controls. The improved D21 FCR in SYNB-H dose compared to the lower doses could indicate it was the most effective in improving nutrient digestibility (Zhou et al., 2009). Both prebiotics and probiotics have demonstrated the ability to increase SCFA production, which fuels enterocyte growth and proliferation, improving intestinal nutrient absorption (Pan and Yu, 2013). Improved nutrient digestibility was recently demonstrated by Emami et al. (2019) after measuring increased mRNA

abundance of an important amino acid transporter, PepT1, and a major glucose transporter, SGLT1, in the small intestine of NE-challenged broilers supplemented with probiotics in the feed (Miska et al., 2015; Shimizu et al., 2017). Other studies found improved FCR in broilers given probiotics *in ovo* (Khaksefidi and Ghoorchi, 2006). Such variations in findings emphasize the importance of continuing to study probiotic application.

NE elicits an immune reaction in response to the infection. The SYNB-H treatment elicited a greater immune response as indicated by greater levels of pro-inflammatory IL-1β and AvBD10 mRNA. Similarly, co-infection with *Eimeria* and *C. perfringens* resulted in greater IL-1β levels (Park et al., 2008). SYNB was able to induce a stronger inflammatory response against NE than the low and medium doses, potentially translating to better protection. Measuring mRNA abundance alone has certain limitations, as it does not always translate to similar protein levels or function in birds. Hong et al. (2012) demonstrated an upregulation in AvBD10 after NE challenge, while Su et al. (2017) observed less changes in AvBD expression after *Eimeria* infection. They attributed the impact on AvBD10 more to NE infection than infection with *Eimeria*. An upregulation of AvBD10 by SYNB-H could improve immune response. Although the results were not statistically significant, the higher probiotic dose had overall lower lesion scores compared to the other treatments. This could possibly be explained by increased innate immune activation to clear infection.

Probiotic application may be more successful when given as early as possible, since chicks are exposed to pathogens as soon as they hatch. Historically, hens confer protection to young chicks when they ingest parts of the healthy maternal microbiome from feces (Kabir, 2009). Applying probiotics *in ovo* while chicks are at the hatchery could allow for early establishment of a healthy microbiome and contribute to competitive exclusion of pathogens (Cox et al., 1992; McReynolds

et al., 2009). This study demonstrated no negative effects of PROB application. It also showed better performance with the larger doses of SYNB, and a greater AvBD10 response. In the future, large scale studies are needed to investigate the modes of action of these methods using the high doses of probiotics in conjunction with a NE challenge.

References

Akira, S. and K. Takeda. 2004. Toll-like receptor signaling. Nat. Rev. Immunol. 4:499-511.

Baffoni, L., F. Gaggìa, D. Di Gioia, C. Santini, L. Mogna, and B. Biavati. 2012. A Bifidobacterium-based synbiotic product to reduce the transmission of C. jejuni along the poultry food chain. Int. J. Food Microbiol. 157:156-161.

Casterlow, S., H. Li, E.R. Gilbert, R.A. Dalloul, A.P. McElroy, D.A. Emmerson, and E.A. Wong. An antimicrobial peptide is downregulated in the small intestine of *Eimeria maxima*-infected chickens. 2011. Poult. Sci. 90:1212-19.

Chen, W.T., W.Y. Huang, T. Chen, E.O. Salawu, D. Wang, Y.Z. Lee, Y.Y. Chang, L.W. Yang, S.C. Sue, X. Wang, and H.S. Yin. 2016. Structure and function of chicken interleukin-1 beta mutants: uncoupling of receptor binding and in vivo biological activity. Sci. Rep. 6:27729.

Cox, N.A., J.S. Bailey, L.C. Blankenship, and R.P. Gildersleeve. 1992. *In ovo* administration of a competitive exclusion culture treatment to broiler embryos. Poult. Sci. 71:1781-84.

Cuperus, T., M. Coorens, A. van Dijak, and H.P. Haagsman. 2013. Avian host defense peptides. Dev. Comp. Immun. 41:352-69.

Dalloul, R.A., H.S. Lillehoj, N.M. Tamim, T.A. Shellam, and J.A. Doerr. 2005. Induction of local protective immunity to *Eimeria acervulina* by a *Lactobacillus*-based probiotic. Comp. Immunol. Microbiol. Infect. Dis. 28:351-61.

Dalloul, R.A. and H.S. Lillehoj. 2006. Poultry coccidiosis: recent advancements in control measures and vaccine development. Expert Review of Vaccines. 5:143-163.

De Oliveira, J.E., E. van der Hoeven-Hangoor, I.B. van de Linde, R.C. Montijn, and J.M.B.M. van der Vossen. 2014. *In ovo* inoculation of chicken embryos with probiotic bacteria and its effect on posthatch Salmonella susceptibility. Poult. Sci. 93:818-829.

Eeckhaut, V., J. Wang, A.V. Parys, F. Haesebrouck, M. Joossens, G. Falony, J. Raes, R. Ducatelle, and F. Van Immerseel. 2016. The probiotic *Butyricicossus pullicaecorum* reduces feed conversion and protects from potentially harmful intestinal microorganisms and necrotic enteritis in broilers. Front. Microbiol. 7:1416.

Emami, N.K., A. Calik, M.B. White, M. Young, and R.A. Dalloul. 2019. Necrotic enteritis in broiler chickens: The role of tight junctions and mucosal immune responses in alleviating the effect of the disease. Microorganisms 7. doi 10.3390/microorganisms7080231

Fioramonti, J., V. Theodorou, and L. Bueno. 2003. Probiotics: what are they? What are their effects on gut physiology? Best Pract. Res. Clin. Gastroenterol. 17:711-24.

Gibson, M.S., P. Kaiser, and M. Fife. 2014. The chicken IL-1 family: evolution in the context of the studied vertebrate lineage. Immunogenetics 66:427-38.

Hargis, B.M. 2014. Overview of necrotic enteritis in poultry. Merck Veterinary Manual. https://www.merckvetmanual.com/poultry/necrotic-enteritis/overview-of-necrotic-enteritis-in-poultry.

Hegarty, J.W., C.M. Guinane, R.P. Ross, C. Hill, and P.D. Cotter. 2016. Bacteriocin production: a relatively unharnessed probiotic trait? F1000Res. 5:2587.

Hong, Y.H., W. Song, S.H. Lee, and H.S. Lillehoj. 2012. Differential gene expression profiles of beta-defensins in the crop, intestine, and spleen using a necrotic enteritis model in 2 commercial broiler chicken lines. Poult. Sci. 91:1081-88.

Keestra, A.M. and J.P.M. van Putten. 2008. Unique properties of the chicken TLR4/MD-2 complex: selective lipopolysaccharide activation of the MyD88-dependent pathway. J. Immunol. 181:4354-62.

Khaksefidi, A. and T. Ghoorchi. 2006. Effect of probiotic on performance and immunocompetence in broiler chicks. J. Poult. Sci. 43:296-300.

Kim, G.B., Y.M. Seo, C.H. Kim, and I.K. Paik. 2011. Effect of dietary prebiotic supplementation on the performance, intestinal microflora, and immune response of broilers. Poult. Sci. 90:75-82.

Knap, I., B. Lund, B. Kehler, C. Hofacre, and G. Mathis. 2009. *Bacillus licheniformis* prevents necrotic enteritis in broiler chickens. Avian Dis. 54:931-35.

Lin, Y., S. Xu, D. Zeng, X. Ni, M. Zhou, Y. Zeng, H. Wang, Y. Zhou, H. Zhu, K. Pan, and G. Li. 2017. Disruption in the cecal microbiota of chickens challenged with *Clostridium perfringens* and other factors was alleviated by *Bacillus licheniformis* supplementation. PLoS One. 12:e0182426.

Lji, P.A., Saki, A., and D.R. Tivey. 2001. Body and intestinal growth of broiler chicks on a commercial starter diet. 1. Intestinal weight and mucosal development. Brit. Poult. Sci. 42:505-13.

Liu, T., L. Zhang, D. Joo, and S.C. Sun. 2017. NF-κB signaling in inflammation. Signal Transduct. Target. Ther. 2:17023.

Lynn, D.J., R. Higgs, S. Gaines, J. Tierney, T. James, A.T. Lloyd, M.A. Fares, G. Mulcahy, and C. O'Farrelly. 2004. Bioinformatic discovery and initial characterization of nine novel antimicrobial peptide genes in the chicken. Immunogenetics 56:170-77.

Manzanillo, P., C. Eidenschenk, and W. Ouyang. 2015. Deciphering the crosstalk among IL-1 and IL-10 family cytokines in intestinal immunity. Trends Immunol. 36:471-78.

McReynolds, J., C. Waneck, J. Byrd, K. Genovese, S. Duke, and D. Nisbet. 2009. Efficacy of multistrain direct-fed microbial and phytogenic products in reducing necrotic enteritis in commercial broilers. Poult. Sci. 88:2075-80.

Miska, K.B., R.H. Fetterer, and E.A. Wong. 2015. mRNA expression of amino acid transporters, aminopeptidase, and the di- and tri-peptide transporter PepT1 in the intestine and liver of posthatch broiler chicks. Poult. Sci. 96:1323-32.

Mountzouris, K.C., P. Tsirtsikos, E. Kalamara, S. Nitsch, G. Schatzmayr, and K. Fegeros. 2007. Evaluation of the efficacy of a probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. Poult. Sci. 86:309-17.

Morrison, D.J., and T. Preson. 2016. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 7:189-200.

Noack, S., H.D. Chapman, and P.M. Selzer. 2009. Anticoccidial drugs of the livestock industry. Parasitol. Res. 8:2009-2026.

Pan, D. and Z. Yu. 2014. Intestinal microbiome of poultry and its interaction with host and diet. Gut Microbes 5:108-19.

Park, S.S., H.S. Lillehoj, P.C. Allen, D.W. Park, S. FitzCoy, D.A. Bautista, and E.P. Lillehoj. 2008. Immunopathology and cytokine responses in broiler chickens coinfected with *Eimeria maxima* and *Clostridium perfringens* with the use of an animal model of necrotic enteritis. Avian Dis. 52:14-22.

Pender, C.M., S. Kim, T.D. Potter, M.M. Ritzi, M. Young, and R.A. Dalloul. 2016. Effects of *in ovo* supplementation of probiotics on performance and immunocompetence of broiler chicks to an *Eimeria* challenge. Benef. Microbes 7:699-705.

Pender, C.M., S. Kim, T.D. Potter, M.M. Ritzi, M. Young, and R.A. Dalloul. 2017. *In ovo* supplementation of probiotics and its effects on performance and immune-related gene expression in broiler chicks. Poult. Sci. 96:1052-62.

Prescott, J.F., R. Sivendra, and D.A. Barnum. 1978. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. Can. Vet. J. 19:181-183.

Pruszynska-Oszmalek, E., P.A. Kolodziejski, K. Stadnicka, M. Sassek, D. Chalupka, B. Kuston, L. Nogowski, P. Mackowiak, G. Maiorano, J. Jankowski, and M. Bednarczyk. 2015. *In ovo* injection of prebiotics and synbiotics affects the digestive potency of the pancreas in growing chickens. Poult. Sci. 94:1909-16.

Qu, J., W. Wang, Q. Zhang, and L. Shu. 2019. Inhibition of lipopolysaccharide-induced inflammation of chicken liver tissue by selenomethionine via TLR4-NF-κB-NLRP3 signaling pathway. Biol. Trace Elem. Res. 195:205-14.

Ricks, C.A., A. Avakian, T. Bryan, R. Gildersleeve, E. Haddad, R. Ilich, S. King, L. Murray, P. Phelps, R. Poston, C. Whitfill, and C. Williams. 1999. *In ovo* vaccination technology. Adv. Vet. Med. 41:495-515.

Roto, S.M., Y.M. Kwon, and S.C. Ricke. 2016. Applications of *in ovo* technique for the optimal development of the gastrointestinal tract and the potential influence on the establishment of its microbiome in poultry. Front. Vet. Sci. 3:63.

Shimizu, K., Y. Komaki, N. Fukano, and T. Bungo. 2017. Transporter gene expression and transference of fructose in broiler chick intestine. J. Poult. Sci. 55:137-141.

Smyth, J.A. and T.G. Martin. 2010. Disease producing capability of *netB* positive isolates of *C. perfringens* recovered from normal chickens and a cow, and *netB* positive and negative isolates from chickens with necrotic enteritis. Vet. Microbiol. 146:76-84.

Su, S., D.M. Dwyer, K.B. Miska, R.H. Fetterer, M.C. Jenkins, and E.A. Wong. 2017. Expression of host defense peptides in the intestine of *Eimeria*-challenged chickens. Poult. Sci. 96:2421-27.

Teague, K.D., L.E. Graham, J.R. Dunn, H.H. Chang, N. Anthony, J.D. Latorre, A. Menconi, R.E. Woldenden, A.D. Wolfenden, B.D. Mahaffey, M. Baxter, X. Hernandex-Velasco, R. Merino-Guzman, L.R. Bielke, B.M. Hargis, and G. Tellez. 2017. *In ovo* evaluation of FloraMax-b11 on Marek's disease HVT vaccine protective efficacy, hatchability, microbiota composition, morphometric analysis, and *Salmonella enteritidis* infection in broiler chickens. Poult. Sci. 96:2074-2082.

Timbermont, L., A. Lanckriet, A.R. Gholamiandehkordi, F. Pasmans, A. Martel, F. Haesebrouck, R. Ducatelle, and F. Van Emmerseel. 2009. Origin of *Clostridium perfringens* isolates determines the ability to induce necrotic enteritis in broilers. Comp. Immunol. Microbiol. Inf. Dis. 32:503-512.

Triplett, M.D., W. Zhai, E.D. Peebles, C.D. McDaniel, and A.S. Kiess. 2018. Investigating commercial *in ovo* technology as a strategy for introducing probiotic bacteria to broiler embryos. Poult. Sci. 97:658-666.

Tsiouris, V. 2016. Poultry management: a useful tool for the control of necrotic enteritis in poultry. Avian Pathol. 45:323-325.

Villaluenga, C.M., M. Wardeska, R. Pilarski, M. Bednarczyk, and K. Gulewicz. 2004. Utilization of the chicken embryo model for assessment of biological activity of different oligosaccharides. Folia Biologica 52:135-142.

Wang, H., X. Ni, X. Qing, L. Liu, J. Lai, A. Khalique, G. Li, K. Pan, B. Jung, and D. Zeng. 2017. Probiotic enhanced intestinal immunity in broilers against subclinical necrotic enteritis. Front. Immunol. 8:1592.

Williams, R. 2005. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathol. 34:159-180.

Wu, Z., H. Tuanjun, L. Rothwell, L. Vervelde, P. Kaiser, K. Boulton, M.J. Nolan, F.M. Tomley, D.P. Blake, D.A. Hume. 2016. Analysis of the function of IL-10 in chickens using specific neutralizing antibodies and a sensitive capture ELISA. Dev. Comp. Immunol. 63:206-212.

Yang, Y., P.A. Iji, and M. Choct. 2009. Dietary modulation of gut microflora in broiler chickens: a review of the role of six kinds of alternatives to in-feed antibiotics. World's Poult. Sci. J. 65:97-114.

Zhou, T.X., Y.J. Chen, J.S. Yoo, Y. Huang, J.H. Lee, H.D. Jang, S.O. Shin, H.J. Kim, J.H. Cho, and I.H. Kim. 2009. Effects of chitooligosaccharide supplementation on performance, blood characteristics, relative organ weight, and meat quality in broiler chickens. Poult. Sci. 88:593-600.

Table 4.1. Treatment Groups.

Trt[1]	Description	Reps	Animals/ Rep	Animals/ Trt
1. CTRL	Control = basal (standard corn/soy) diets + 100µL sterile water	4	12	48
2. PROB-L	Basal + 100 µL 1 x 10^5 Probiotic 1	4	12	48
3. PROB-M	Basal + 100 µL 1 x 10^6 Probiotic 1	4	12	48
4. PROB-H	Basal + 100 µL 1 x 10^7 Probiotic 1	4	12	48
5. SYNB-L	Basal + 100 µL 1 x 10^5 Synbiotic 2	4	12	48
6. SYNB-M	Basal + 100 µL 1 x 10^6 Synbiotic 2	4	12	48
7. SYNB-H	Basal + 100 µL 1 x 10^7 Synbiotic 2	4	12	48

[1]Treatments: CTRL = basal diet and sterile water injection only; PROB-L = probiotic 1, low dose (1x10^5 CFU); PROB-M = probiotic 1, medium dose (1x10^6 CFU); PROB-H = probiotic 1, high dose (1x10^7 CFU). SYNB-L = probiotic 2, low dose (1x10^5 CFU); SYNB-M = probiotic 2, medium dose (1x10^6 CFU); SYNB-H = probiotic 2, high dose (1x10^7 CFU).

Table 4.2. Feed Composition of broiler mash starter diet (0-14 d) and grower mash diet (14-21 d).

Ingredient, g/kg	0-14 d	14-21 d
Corn	59.53	64.11
Soybean meal, CP 48%	33.50	28.81
Vegetable oil	2.18	2.59
Limestone	1.11	1.94
Dicalcium phosphate	2.05	1.01
DL-Methionine (98%)	0.38	0.37
L-Lysine HCl (78%)	0.37	0.34
L-Threonine	0.15	0.35
Salt	0.37	0.14
Vitamin-Mineral Premix (NB3000)*	0.36	0.36
Total	100.00	100.00
Chemical composition (Calculated)		
Dry Matter, %	87.75	87.61
Crude Protein, %	20.54	19.5
AME_n, kcal/kg	3000	3077
Lysine, %	1.33	1.20
Dig. Lysine, %	1.18	1.02
Methionine + cysteine, %	0.88	0.82
Dig. Methionine + cysteine, %	0.80	0.80
Threonine, %	0.90	1.02
Dig. Threonine, %	0.77	0.82
Calcium, %	0.98	0.84
Available phosphorus, %	0.52	0.42

*Vitamins per kg diet: retinol 3.33 mg, cholecalciferol 0.1 mg, α-tocopherol acetate 23.4 mg, vitamin K3 1.2 mg, vitamin B1 1.6 mg, vitamin b2 9.5 mg, niacin 40 mg, pantothenic acid 9.5 mg, vitamin B6 2 mg, folic acid 1 mg, vitamin B12 0.016 mg, biotin 0.05 mg, choline 556 mg. Minerals per kg diet: Mn 144 mg, Fe 72 mg, Zn 144 mg, Cu 16.2 mg, I 2.1 mg, Se 0.22 mg.

Table 4.3. Primers used for qRT-PCR mRNA abundance. Forward primer sequence is listed before reverse primer sequence.

Gene[a]	Primer Sequence (5' – 3')	NCBI Accession Number
GAPDH	CCT AGG ATA CAC AGA GGA CCA GGT T GGT GGA GGA ATG GCT GTC A	NM_204305
IL-1β	CCC GCC TTC CGC TAC A CAC GAA GCA CTT CTG GTT GAT C	XM_105297469.1
TLR4	CCACACACCTGCCTACATGAA GGATGGCAAGAGGACATATCAAA	NM_001030693
IL-10	CGC TGT CAC CGC TTC TTC A CGT CTC CTT GAT CTG CTT GAT G	NM_001004414
AvBD10	CAG ACC CAC TTT TCC CTG ACA CCC AGC ACG GCA GAA ATT	NM_001000609.2
AvBD13	CAG CTG TGC AGG AAC AAC CA CAG CTC TCC ATG TGG AAG CA	NM_001001780.1

[a]**GAPDH** = Glyceraldehyde 3-phosphate dehydrogenase. **IL** = Interleukin. **TLR** = Toll-like receptor. **AvBD** = Avian β-defensin.

Table 4.4a. Effect of probiotic *in ovo* dosage on hatchability.

Hatchability (Eggs Hatched/Eggs Placed)	
Trt[1]	DOH
1. CTRL	50/52
2. PROB-L	49/52
3. PROB-M	49/52
4. PROB-H	48/52

[1]Treatments: CTRL = basal diet and sterile water injection only; PROB-L = probiotic 1, low dose ($1x10^5$ CFU); PROB-M = probiotic 1, medium dose ($1x10^6$ CFU); PROB-H = probiotic 1, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.4b. Effect of synbiotic *in ovo* dosage on hatchability.

Hatchability (Eggs Hatched/Eggs Placed)	
Trt[1]	DOH
1. CTRL	50/52
2. SYNB-L	46/52
3. SYNB-M	48/52
4. SYNB-H	49/52

[1]Treatments: CTRL = basal diet and sterile water injection only; SYNB-L = probiotic 2, low dose ($1x10^5$ CFU); SYNB-M = probiotic 2, medium dose ($1x10^6$ CFU); SYNB-H = probiotic 2, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.5a. Effect of probiotic *in ovo* dosage on mortality (%).

Mortality (%) (*n* = 48 birds/trt)					
Trt[1]	DOH-4	D4-8	D8-14	D14-21	D0-21
1. CTRL	0	0	0	0	0
2. PROB-L	0	0	0	0	0
3. PROB-M	0	0	0	0	0
4. PROB-H	0	0	0	0	0
	N/A	*N/A*	*N/A*	*N/A*	*N/A*
	[2]SEM = 0	*SEM = 0*	*SEM = 0*	*SEM = 0*	*SEM = 0*

[1]Treatments: CTRL = basal diet and sterile water injection only; PROB-L = probiotic 1, low dose ($1x10^5$ CFU); PROB-M = probiotic 1, medium dose ($1x10^6$ CFU); PROB-H = probiotic 1, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.5b. Effect of synbiotic *in ovo* dosage on mortality (%).

Mortality (%) (*n* = 48 birds/trt)					
Trt[1]	DOH-4	D4-8	D8-14	D14-21	D0-21
1. CTRL	0.0	0.0	0.0	0.0	**0.0[b]**
5. SYNB-L	5.6	3.0	3.3	3.3	**13.9[a]**
6. SYNB-M	2.8	0.0	0.0	0.0	**2.8[b]**
7. SYNB-H	2.8	0.0	0.0	0.0	**2.8[b]**
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	***P ≤ 0.05***
	[2]SEM = 2.2	*SEM = 1.4*	*SEM = 1.5*	*SEM = 1.4*	***SEM = 2.2***

[1]Treatments: CTRL = basal diet and sterile water injection only; SYNB-L = probiotic 2, low dose ($1x10^5$ CFU); SYNB-M = probiotic 2, medium dose ($1x10^6$ CFU); SYNB-H = probiotic 2, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.6a. Effect of probiotic *in ovo* dosage on body weight (BW) per bird.

Body Weight (g/bird) (*n* = 48 birds/trt)					
Trt[1]	DOH	D4	D8	D14	D21
1. CTRL	41	85	146	360	785
2. PROB-L	41	82	144	345	748
3. PROB-M	41	76	136	337	725
4. PROB-H	41	86	146	357	772
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*
	[2]SEM = 0.5	*SEM = 4.9*	*SEM = 6.1*	*SEM = 11.0*	*SEM = 19.0*

[1]Treatments: CTRL = basal diet and sterile water injection only; PROB-L = probiotic 1, low dose ($1x10^5$ CFU); PROB-M = probiotic 1, medium dose ($1x10^6$ CFU); PROB-H = probiotic 1, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.6b. Effect of synbiotic *in ovo* dosage on body weight (BW) per bird.

Body Weight (g/bird) (*n* = 48 birds/trt)					
Trt[1]	DOH	D4	D8	D14	D21
1. CTRL	**41[b]**	85	146	360	**785[a]**
5. SYNB-L	**44[a]**	75	124	327	**700[b]**
6. SYNB-M	**44[a]**	85	142	340	**713[b]**
7. SYNB-H	**40[c]**	79	135	345	**755[ab]**
	P ≤ 0.01	*P > 0.10*	*P > 0.10*	*P > 0.10*	***P ≤ 0.10***
	[2]SEM = 0.5	*SEM = 6.5*	*SEM = 8.5*	*SEM = 13.0*	***SEM = 22.0***

[1]Treatments: CTRL = basal diet and sterile water injection only; SYNB-L = probiotic 2, low dose ($1x10^5$ CFU); SYNB-M = probiotic 2, medium dose ($1x10^6$ CFU); SYNB-H = probiotic 2, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.7a. Effect of probiotic *in ovo* dosage on body weight gain (BWG) per bird.

Body Weight Gain (g/bird) (*n* = 48 birds/trt)					
Trt[1]	DOH-4	D4-8	D8-14	D14-21	DOH-21
1. CTRL	44	61	201	425	744
2. PROB-L	42	61	192	403	707
3. PROB-M	35	60	188	388	684
4. PROB-H	46	60	198	414	731
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*
	[2]SEM = 4.8	*SEM = 2.9*	*SEM = 6.4*	*SEM = 11.0*	*SEM = 19.0*

[1]Treatments: CTRL = basal diet and sterile water injection only; PROB-L = probiotic 1, low dose ($1x10^5$ CFU); PROB-M = probiotic 1, medium dose ($1x10^6$ CFU); PROB-H = probiotic 1, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.7b. Effect of synbiotic *in ovo* dosage on body weight gain (BWG) per bird.

Body Weight Gain (g/bird) (*n* = 48 birds/trt)					
Trt[1]	DOH-4	D4-8	D8-14	D14-21	DOH-21
1. CTRL	44	**61**^{a}	201	**425**^{a}	**744**^{a}
5. SYNB-L	32	**45**^{b}	187	**362**^{b}	**645**^{b}
6. SYNB-M	41	**55**^{ab}	185	**373**^{b}	**669**^{b}
7. SYNB-H	39	**54**^{ab}	198	**410**^{a}	**714**^{ab}
	P > 0.10	***P ≤ 0.10***	*P > 0.10*	***P ≤ 0.05***	***P ≤ 0.05***
	[2]SEM = 6.3	***SEM = 3.7***	*SEM = 8.3*	***SEM = 1.0***	***SEM = 2.3***

[1]Treatments: CTRL = basal diet and sterile water injection only; SYNB-L = probiotic 2, low dose ($1x10^5$ CFU); SYNB-M = probiotic 2, medium dose ($1x10^6$ CFU); SYNB-H = probiotic 2, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.8a. Effect of probiotic *in ovo* dosage on feed intake (FI) per bird.

Feed Intake (g/bird) (*n* = 48 birds/trt)					
Trt[1]	DOH-4	D4-8	D8-14	D14-21	DOH-21
1. CTRL	68	87	260	590	**1005**[a]
2. PROB-L	61	84	247	550	**942**[ab]
3. PROB-M	57	78	246	546	**926**[b]
4. PROB-H	67	84	268	576	**995**[ab]
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	***P ≤ 0.10***
	[2]*SEM = 3.9*	*SEM = 4.1*	*SEM = 7.1*	*SEM = 1.3*	***SEM = 24.0***

[1]Treatments: CTRL = basal diet and sterile water injection only; PROB-L = probiotic 1, low dose ($1x10^5$ CFU); PROB-M = probiotic 1, medium dose ($1x10^6$ CFU); PROB-H = probiotic 1, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.8b. Effect of synbiotic *in ovo* dosage on feed intake (FI) per bird.

Feed Intake (g/bird) (*n* = 48 birds/trt)					
Trt[1]	DOH-4	D4-8	D8-14	D14-21	DOH-21
1. CTRL	68	87	260	**590**[a]	1,005
5. SYNB-L	55	69	236	**532**[b]	892
6. SYNB-M	65	87	261	**532**[b]	945
7. SYNB-H	61	76	243	**550**[ab]	930
	P > 0.10	*P > 0.10*	*P > 0.10*	***P ≤ 0.10***	*P > 0.10*
	[2]*SEM = 6.4*	*SEM = 6.1*	*SEM = 1.2*	***SEM = 1.6***	*SEM = 3.4*

[1]Treatments: CTRL = basal diet and sterile water injection only; SYNB-L = probiotic 2, low dose ($1x10^5$ CFU); SYNB-M = probiotic 2, medium dose ($1x10^6$ CFU); SYNB-H = probiotic 2, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.9a. Effect of probiotic *in ovo* dosage on feed conversion ratio (FCR) per bird.

Feed Conversion Ratio (per bird) (*n* = 48 birds/trt)					
Trt[1]	DOH-4	D4-8	D8-14	D14-21	DOH-21
1. CTRL	1.56	1.44	1.30	1.39	1.35
2. PROB-L	1.49	1.38	1.29	1.37	1.33
3. PROB-M	2.10	1.30	1.30	1.40	1.35
4. PROB-H	1.46	1.42	1.36	1.39	1.36
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*
	[2]SEM = 0.31	*SEM = 0.08*	*SEM = 0.04*	*SEM = 0.02*	*SEM = 0.02*

[1]Treatments: CTRL = basal diet and sterile water injection only; PROB-L = probiotic 1, low dose ($1x10^5$ CFU); PROB-M = probiotic 1, medium dose ($1x10^6$ CFU); PROB-H = probiotic 1, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.9b. Effect of synbiotic *in ovo* dosage on feed conversion ratio (FCR) per bird.

Feed Conversion Ratio (per bird) (*n* = 48 birds/trt)					
Trt[1]	DOH-4	D4-8	D8-14	D14-21	DOH-21
1. CTRL	1.56	1.44	**1.30^{ab}**	**1.39^{bc}**	**1.35^{ab}**
5. SYNB-L	2.79	1.54	**1.26^{b}**	**1.47^{a}**	**1.38^{ab}**
6. SYNB-M	1.60	1.60	**1.42^{a}**	**1.43^{ab}**	**1.41^{a}**
7. SYNB-H	1.60	1.42	**1.23^{b}**	**1.34^{c}**	**1.30^{b}**
	P > 0.10	*P > 0.10*	***P ≤ 0.10***	***P ≤ 0.05***	***P ≤ 0.10***
	[2]SEM = 0.60	*SEM = 0.13*	***SEM = 0.04***	***SEM = 0.02***	***SEM = 0.02***

[1]Treatments: CTRL = basal diet and sterile water injection only; SYNB-L = probiotic 2, low dose ($1x10^5$ CFU); SYNB-M = probiotic 2, medium dose ($1x10^6$ CFU); SYNB-H = probiotic 2, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.10a. Effect of probiotic *in ovo* dosage on D8 NE lesion scores.

	D8 NE Lesion Scores (*n* = 4 birds/trt)		
Trt[1]	Duodenum	Jejunum	Ileum
1. CTRL	1.63	0.88	0.25
2. PROB-L	2.13	1.13	0.13
3. PROB-M	1.83	1.33	0.83
4. PROB-H	1.38	1.25	0.25
	P > 0.10	*P > 0.10*	*P > 0.10*
	[2]SEM = 0.34	*SEM = 0.32*	*SEM = 0.18*

[1]Treatments: CTRL = basal diet and sterile water injection only; PROB-L = probiotic 1, low dose ($1x10^5$ CFU); PROB-M = probiotic 1, medium dose ($1x10^6$ CFU); PROB-H = probiotic 1, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.10b. Effect of synbiotic *in ovo* dosage on D8 NE lesion scores.

	D8 NE Lesion Scores (*n* = 4 birds/trt)		
Trt[1]	Duodenum	Jejunum	Ileum
1. CTRL	1.63	0.88	0.25
2. SYNB-L	1.67	0.67	0.17
3. SYNB-M	1.67	1.33	0.00
4. SYNB-H	1.00	0.67	0.00
	P > 0.10	*P > 0.10*	*P > 0.10*
	[2]SEM = 0.37	*SEM = 0.28*	*SEM = 0.09*

[1]Treatments: CTRL = basal diet and sterile water injection only; SYNB-L = probiotic 2, low dose ($1x10^5$ CFU); SYNB-M = probiotic 2, medium dose ($1x10^6$ CFU); SYNB-H = probiotic 2, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.11a. Effect of probiotic *in ovo* dosage on mRNA abundance of immune genes.

D8 Fold Change (*n* = 4 birds/trt)					
Trt[1]	IL-1β	TLR4	IL-10	AvBD10	AvBD13
1. CTRL	1.08	1.23	1.05	1.20	1.08
2. PROB-L	1.82	1.65	1.38	1.27	1.89
3. PROB-M	1.69	1.52	1.34	1.83	2.00
4. PROB-H	1.10	1.84	0.89	1.21	0.91
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*
	SEM = 0.32	*SEM = 0.41*	*SEM = 0.24*	*SEM = 0.69*	*SEM = 0.48*

[1]Treatments: CTRL = basal diet and sterile water injection only; PROB-L = probiotic 1, low dose ($1x10^5$ CFU); PROB-M = probiotic 1, medium dose ($1x10^6$ CFU); PROB-H = probiotic 1, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 4.11b. Effect of synbiotic *in ovo* dosage on mRNA abundance of immune genes.

D8 Fold Change (*n* = 4 birds/trt)					
Trt[1]	IL-1β	TLR4	IL-10	AvBD10	AvBD13
1. CTRL	**1.08**^{ab}	1.23	1.05	**1.20**^{b}	1.08
5. SYNB-L	**0.56**^{b}	0.80	0.76	**2.41**^{ab}	1.86
6. SYNB-M	**1.31**^{a}	2.06	1.07	**0.74**^{b}	0.96
7. SYNB-H	**1.32**^{a}	1.61	1.14	**6.45**^{a}	1.59
	P ≤ 0.05	*P > 0.10*	*P > 0.10*	***P ≤ 0.10***	*P > 0.10*
	[2]SEM = 0.18	*SEM = 0.44*	*SEM = 0.19*	***SEM = 1.41***	*SEM = 0.55*

[1]Treatments: CTRL = basal diet and sterile water injection only; SYNB-L = probiotic 2, low dose ($1x10^5$ CFU); SYNB-M = probiotic 2, medium dose ($1x10^6$ CFU); SYNB-H = probiotic 2, high dose ($1x10^7$ CFU). Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

CHAPTER V

In ovo application of synbiotics and post-hatch necrotic enteritis in broilers: performance and intestinal responses

Abstract

Reducing the negative effects of the bacterial disease necrotic enteritis (NE) without the use of antibiotics has prompted interest in natural alternatives, including the early *in ovo* application of synbiotics, a blend of probiotics and prebiotics to support growth of probiotics in the gut. The objective of this 42-day study was to apply a synbiotic *in ovo* and assess performance, day 7 (D7) intestinal lesion scores, mRNA abundance of various intestinal genes, short-chain fatty acid production in the ceca, and D42 carcass composition. A total of 1,630 fertilized Ross 708 eggs were injected with either water or synbiotic on embryonic day 18 (E18) and further divided after hatch into six treatments: negative control (**NC**): sterile water *in ovo* fed basal corn/soybean meal mash diet without NE challenge; antibiotic growth-promoter (**AGP+**): sterile water *in ovo* fed basal diet with virginiamycin (0.5 kg/MT) as an (AGP) with NE challenge; **NC+**: same as NC plus NE challenge; **SI+**: synbiotic *in ovo* fed the basal diet and NE challenged; **SD+**: sterile water *in ovo* fed basal diet supplemented with synbiotic (0.5 kg/MT feed) and NE challenged; and **SID+**: synbiotic *in ovo* fed basal diet with synbiotic (0.5 kg/MT feed) with NE challenge. On day-of-hatch, feed and litter were sprayed with a commercial coccidiosis vaccine as a predisposing factor to NE. Data were analyzed in JMP with LS Means to separate means and significance assigned at $P \leq 0.05$ and trends at $0.10 \leq P \leq 0.05$. There were no effects of *in ovo* application on hatchability or cumulative mortality. As expected, AGP+ often had higher BW, BWG, FI, and lower FCR. Cumulatively, SI+, SD+, and SID+ had similar BW and BWG to NC+. SID+ had lower cumulative FI compared to NC+, and a lower FCR. D42 lean:fat ratio was

improved in SD+ and SID+ birds compared to NC, NC+, and SI+. *In ovo* injections of synbiotics affected mRNA abundance at multiple points. It led to greater DOH CLD1 and less TLR4, TLR2, and GPR43 mRNA. The combination of synbiotics (SID+) often had greater impact on mRNA abundance than SD+ or SI+ alone. On D14, LEAP2 mRNA was greater in the CT of SID+ than SD+ or SI+. By D42, ileal TLR2, TLR4, AvBD8, AvBD10, and GPR43 mRNA were greater in SID+ compared to SD+ or SI+. Fatty acid analysis results of cecal contents indicated higher D7 propionate, isobutyrate, and valerate in SID+ compared to all other treatments, and higher isovalerate than other treatments except SI+. These results indicate the subclinical NE challenge was best controlled by AGP+, but the application of *in ovo*, in-feed, or combined *in ovo* and in-feed application of synbiotics was often able to improve some measurements compared to NC+, suggesting a potential benefit in broiler production from the use of synbiotics.

Keywords: synbiotics, *in ovo*, broiler, necrotic enteritis, mRNA, SCFA

Introduction

Clostridium perfringens (CP) bacteria are commonly found in the gut of broilers and do not always cause disease, but are considered opportunistic pathogens. Exposure to *Eimeria* parasites often predisposes a bird to necrotic enteritis (NE) (George et al., 1982; Cravens et al., 2013). *Eimeria* parasites invade and reproduce within enterocytes, cause intestinal damage, and increase mucus production, in an attempt to flush out the parasites. This damage and increased mucogenesis fuels CP growth and proliferation (Collier et al., 2008). Toxins from CP cause lesions, stunt microvilli, slow nutrient absorption, and stunt bird growth (McReynolds et al., 2004). Historically, antibiotics have been used to prevent or control outbreaks of NE caused by

toxin-producing strains of CP (Moore, 2016). Alternatives to antibiotics that may reduce disease and improve health have gained interest since the European Union's ban on antibiotic-growth promoters (AGPs), negative public perception of antibiotic use in animals, and the Veterinary Feed Directive in the US (Castanon, 2007; Veterinary Feed Directive, 2015). Synbiotics, a combination of probiotics and prebiotics, are considered a natural, viable alternative to antibiotics (Alloui et al., 2013). Probiotics are beneficial microbes capable of establishing themselves as part of the healthy microbiota in the gut, and commonly include strains from genera *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, and *Enterococcus* (Fuller, 1989; Kabir, 2009). Prebiotics are typically substances that promote growth of probiotic bacteria in the gut. Common products include non-starch polysaccharides and various oligosaccharides, such as inulin (Gibson and Roberfroid, 1995). A synbiotic may protect from disease by secreting antimicrobial substances, modulating immune response, competitive exclusion of pathogens, producing bacteriostatic short-chain fatty acids (SCFA) as a byproduct of fermentation in the ceca, and improving gut integrity (Cherrington et al., 1990; Mountzouris et al., 2007, 2010; Gadde et al., 2017; Sobolewska et al., 2017).

Common lactic acid-producing probiotics lower gut pH, inhibiting growth of other bacteria. In a recent study, probiotics *Enterococcus faecium*, *Pediococcus acidilactici*, and *Lactobacillus reuteri* were capable of adhering to chicken ileum epithelial cells and competitively excluding bacterial pathogens such as *Escherichia coli* and *Salmonella* (Reuben et al., 2019). These strains are also capable of producing bacteriocins (Hegarty et al., 2016; Tarradas et al., 2020). Along with *Bifidobacterium*, these probiotics make SCFA as a byproduct of fermentation from carbohydrates (Pessione, 2012). Studies have demonstrated that host colonocytes uptake the SCFA butyrate and use it as a main energy source, resulting in improved gut epithelia and

increased cell turnover, while acetate and propionate production enters portal circulation, passing through the liver before distribution to the body (Corrier et al., 1990; Blottiere et al., 2003). Higher levels of SCFA induce expression of G-protein coupled receptor 43 (GPR43), a primary receptor for SCFA absorption (Macia et al., 2015).

The constant contact of the gastrointestinal tract (GIT) with the external environment naturally increases its encounters with pathogens. The GIT is considered to be the largest lymphoid organ in the body, and maintaining intestinal integrity is important to overall animal health (Kogut and Arsenault, 2016). Optimal GIT function is effective in digestion, absorption, housing normal microbiota, and guarding against pathogens by maintaining a strong barrier (Ritzi et al., 2014; Celi et al., 2017). One method of assessing the gut's ability to defend against pathogens and absorb nutrients is to monitor mRNA abundance of various genes. The gut maintains a tight barrier between epithelial cells due to tight junction proteins. These proteins, such as claudin-1 (CLD1) interact with other tight junction proteins to regulate permeability between cells and aid in ion passage (Mitchell and Koval, 2010). Avian Toll-like receptor (TLR) proteins are transmembrane receptors that monitor the luminal environment, sampling for both normal microflora and pathogenic bacteria (Tarradas et al., 2020). TLR2 recognizes lipopeptides and TLR4 recognizes bacterial lipopolysaccharides (LPS) from Gram-positive bacteria (Keestra et al., 2007, 2013). Bacterial recognition triggers activation of the MyD88 signaling pathway within a cell, activating NF-κB, which promotes synthesis of inflammatory cytokines (Keestra et al., 2013). Probiotics and their production of SCFA can increase protective mucin production by goblet cells in the intestine, which acts as a protective layer between microbes in the gut and the epithelium (Seifi et al., 2017). This effect can be measured by monitoring levels of MUC2 expression. SCFA may also increase expression of host defense peptides to protect the host from

pathogenic bacteria (Xiong et al., 2016). In avian species, host defense peptides are part of an innate immune response, and include avian β-defensins (AvBD) and liver expressed antimicrobial peptide-2 (LEAP2). They are able to induce leukocyte differentiation and recruitment, inhibit LPS-induced effects, and show varying patterns of upregulation and downregulation in response to pathogens (Semple and Dorin, 2012; Cuperus et al., 2013; Su et al., 2017; Tarradas et al., 2020).

The *in ovo* application of synbiotics could potentially lead to earlier colonization of the gut with beneficial microbes. *In ovo* injection has been successfully and safely used against Marek's disease since the 1980s (Sharma and Burmester, 1982; Peebles, 2018). Previously, *in ovo* injection of probiotics has demonstrated efficient protection against post-hatch infection with *Salmonella* (Cox et al., 1992; de Oliveira et al, 2014; Teague et al., 2017). While numerous studies showed improvements as a result of synbiotic application, others reported no change (Kabir et al., 2004; Nayebpor et al., 2007, Rahimi et al., 2011; Sen et al., 2012; Getachew, 2016). The objective of this study was to evaluate the effects of *in ovo* application of synbiotics during a post-hatch NE challenge by analyzing performance, lesion scores, mRNA abundance in the ileum, fatty acid concentrations in the ceca, and overall body composition of market age broilers.

Materials and Methods

Incubation Management and In ovo Treatments

A 42-day study was conducted under the approval of IACUC # 18-136-APSC at the Virginia Tech turkey farm research facilities. A total of 1,630 fertilized Ross 708 broiler eggs were obtained from a commercial hatchery and were approximately 3 days old from a laying flock of 39 weeks in age. Embryonic day 0 (E0) egg weights were recorded, the egg flats were placed in

incubators, and eggs were pre-warmed at 26.6℃ and 55% humidity for 10 hours. Next, incubators were set to industry standard temperature of 37.6℃ and 53% humidity until E18. Eggs were automatically turned at a 45 degree-angle every 2 hours between E0-E18. On E17, egg flats were briefly removed from the incubator and candled to remove early dead and infertile ones. Prior to hatch, eggs were divided into two *in ovo* injection treatments with either sterile water or soluble synbiotic. At E18, eggs were individually *in ovo*-injected, transitioned to hatch trays, and returned to the incubator. Temperature was reduced to 35.6℃ and relative humidity increased to 75% until hatch.

Synbiotic Preparation and In ovo Injections

Sterile deionized water or a synbiotic mixture were prepared just before injection. For the synbiotic, 3 g water-soluble commercial synbiotic was dissolved in 150 mL sterile water per manufacturer's recommendations to reach a dilution of 1 x 10^7 CFU/mL. This dose was selected based on results of previous testing in Chapter 4. The synbiotic contained the dried products of *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, *Lactobacillus reuteri*, and the prebiotic inulin.

For *in ovo* injection, one flat at a time (30 eggs/flat) was removed to reduce time out of the incubator. Each egg was candled, and the air cell outlined. The injection site was cleaned with 70% ethanol and 10% sodium hypochlorite. A small pilot hole was made in the center of the air cell with a short 18G needle. A smaller 22G needle 3.81 cm (1.5 inch) long attached to a 1 cc syringe was used to inject 100 µL sterile water or synbiotic into the amniotic fluid of each egg. Each egg was individually injected perpendicular to the amniotic fluid. The sterile water treatment (1,084 eggs) was performed prior to the synbiotic injections (542 eggs). The pilot hole was sealed with clean tape, and eggs were returned to the incubator. After all eggs were injected,

they were transitioned to hatch trays at the same time. Treatments were kept separated from each other to reduce any cross-contamination of synbiotic to the control group.

Post-Hatch Treatments and Bird Management

Treatments were further divided after hatch based on *in ovo* injection type, diet application, and presence or absence of NE challenge (Table 5.1). The six treatments were as follows: **NC**: sterile water *in ovo* fed basal corn/soybean meal mash diet without NE challenge; **AGP+**: sterile water *in ovo* fed basal diet with virginiamycin as an AGP (0.5 kg/MT) with NE challenge; **NC+**: same as NC plus NE challenge; **SI+**: synbiotic *in ovo* fed the basal diet and NE challenged; **SD+**: sterile water *in ovo* fed basal diet supplemented with synbiotic (0.5 kg/MT feed) and NE challenged; and **SID+**: synbiotic *in ovo* fed basal diet with synbiotic (0.5 kg/MT feed) with NE challenge. Diets were formulated to Ross nutritional recommendations. A mash starter diet was fed from DOH – D14, grower diet from D14 – D28, and a finisher diet from D28 – D42 (Table 5.2).

On DOH, birds were removed from hatch trays, inspected for health, feather-sexed, and placed in the appropriate treatment with a proportional number of males and females in each pen based on the sex ratio at hatch (Table 5.1). Each treatment contained 8 replicate pens (232 birds/treatment; 29 birds/replicate). Birds were housed in 2.4 x 1.2-meter concrete floor pens with fresh pine shavings, adjustable nipple drinkers and metal feeders. Water and feed were available ad libitum throughout the experiment. Temperature began at 36℃ and gradually adjusted down per industry standards until reaching 24℃ at D21 where it was maintained for the remainder of the experiment. Light was provided for 24 hours during the first week, and gradually adjusted down from D7 through D14 to 4 hours of dark and 20 hours of light where it remained until the end of the experiment.

Experimental NE Challenge Model

On DOH, shortly after bird placement, the Coccivac-B52 commercial coccidiosis vaccine containing live oocysts of *Eimeria acervulina*, *E. maxima*, *E. maxima MFP*, *E. mivati*, and *E. tenella* was sprayed on feed and litter of all treatments except NC at 11X the recommended dose to induce a naturally-occurring NE outbreak endemic to the poultry facility. The 11X dosage was chosen based on successful dosage levels used in various previous studies by the same lab in the same facility. The non-challenged treatment pens (NC) were placed at the front of the house, as separated as possible from challenged pens and upwind of the tunnel ventilation. Challenged treatment pens were placed in a randomized design throughout the house to reduce variability from location in the house. Lesion scores were recorded from 3 birds/pen during the peak of the outbreak (D7) using a 0-4 scale previously described (Prescott et al., 1978) with 0 = no lesions, 1 = thin-walled and friable intestines, 2 = focal necrosis or ulceration, 3 = large patches of necrosis 2-3 cm long, and 4 = severe necrosis. Each bird was euthanized by cervical dislocation and lesions were recorded from the duodenum, jejunum, and ileum.

Performance Measurements

Before incubation, eggs were arranged in flats to assure flat weights were similar to one another during incubation. Hatchability was recorded on DOH. Pen weights and feed weights were recorded on DOH, D7, D14, D28, and D42 to calculate average body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR). Observations and mortality were recorded twice daily to adjust performance measurements.

Body Composition

At the end of the experiment, D42 body composition was measured using Dual Energy X-Ray Absorptiometry (DXA). Lean and fat percentages were measured on individual birds (24

birds/treatment). Birds were first euthanized by cervical dislocation, their feathers plucked, and carcasses chilled overnight until analysis of individual bird body mass, lean tissue mass, and fat tissue mass (GE Healthcare, Lunar Prodigy Advance program). Results are presented as average carcass weight, percent fat, and percent lean of birds in each treatment.

mRNA Abundance in Ileal and Cecal Tonsil Tissues

Ileal and cecal tonsil samples were collected at different time points during the experiment for mRNA abundance of Toll-like receptor (TLR) 2, TLR4, claudin-1 (CLD1), mucin 2 (MUC2), tumor necrosis factor-alpha (TNF-α), liver-expressed antimicrobial peptide 2 (LEAP2), avian β-defensin (AvBD) 8, AvBD10, AvBD13, and G-protein coupled receptor 43 (GPR43). On DOH, before bird placement, 8 birds of average weight were selected from the group that received sterile water *in ovo*, and 8 were selected from the synbiotic-injected group. On D7, D14, and D42, ileal and cecal tonsil samples were taken from 1 bird/pen. Since probiotics colonize the distal areas of the gut, sections of the ileum and cecal tonsils were chosen to monitor potential effects in these areas. Birds were euthanized by cervical dislocation, a section of ileum or cecal tonsils was excised and gently rinsed out with cold PBS, immediately frozen on dry ice, and stored at −80°C until analysis. Tissue samples were homogenized using a TissueLyser (Qiagen) and RNA was extracted following standard manufacturer's protocol (Qiagen RNeasy Mini Kit). Optical density (OD) was used to assess RNA quality (OD 260/280) and concentration (OD 260) as measured by a NanoDrop-1000. The high-capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to make cDNA for qRT-PCR. Reactions (10 µL/well) were run in duplicate using Fast SYBR™ Green Master Mix on a 7500 FAST Real-Time PCR instrument (Applied Biosystems) with melt curves to determine product specificity. Primer details are provided in Table 5.3. Changes in mRNA abundance of each target gene were calculated using

glyceraldehye-3-phosphate (GAPDH) as the endogenous control and the NC treatment as the calibrator. Results for each treatment are reported as average mRNA abundance relative to GAPDH using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Quantification of Volatile Fatty Acids from Cecal Contents

To evaluate fatty acid composition in cecal contents and potential production of SCFA by the *in ovo*-injected or in-feed synbiotics, cecal content was collected from 1 bird/pen on D7, D14, and D42. After collection in sterile microcentrifuge tubes, samples were immediately frozen on dry ice before storage at −80°C until analysis. A gas chromatography-mass spectrophotometry (GC-MS) method was used based on procedures described by Richardson et al. (1989) and modifications from Morrison et al. (2004). In brief, samples were homogenized in a mixture of water and hydrochloric acid before centrifugation. The supernatant was placed in a new tube and vortexed with diethyl ether before centrifugation again. The resulting supernatant was placed in a new vial with acetonitrile and MTBSTFA (*N*-methyl-*N*-(*t*-butyldimethylsilyl)-trifluoroacetamide), and incubated for 1 hour at 80°C. Samples were cooled briefly and placed into gas chromatography vials for analysis. Results are presented as mmol/kg of sample.

Statistical Analysis

SAS JMP Pro 15 was used to perform one-way ANOVA using Fisher's least significant difference (LSD) method on all results except hatchability. Results are presented as least square means (LS Means) with standard error means (SEM). Significance was set at $P \leq 0.05$ as being significant and trends set at $0.05 \leq P \leq 0.10$.

Results

Performance Measurements

Hatchability and Mortality. There was no impact on hatchability after *in ovo* application (Table 5.4). As shown in Table 5.5, mortality was only significant ($P \leq 0.05$) from D7-14 where SID+ had higher mortality compared to no mortality in any other treatments. Cumulatively, no differences in mortality were observed between treatments.

Body Weight. There were significant differences in BW per bird at each time point (Table 5.6). On DOH, NC, AGP+, NC+, and SD+ had significantly lower starting BW ($P \leq 0.05$) compared to SI+. On D7, NC birds were significantly heavier ($P \leq 0.01$) compared to the challenged treatments, and both SI+ and AGP+ weighed less than NC+ and SID+. By D14, SD+ was similar to both NC and AGP+ ($P \leq 0.05$). There was a significant difference ($P \leq 0.01$) on D28 where AGP+ birds had greater BW than all other treatments, but SD+ had greater BW compared to SI+ birds. Although not significantly different, both SD+ and SID+ had higher BW compared to NC+. At the end of the experiment, AGP+ had the highest D42 BW, followed by the non-challenged NC birds, and the challenged NC+, SI+, SD+, and SID+ birds had lower BW compared to AGP+ and NC ($P \leq 0.01$).

Body Weight Gain. Similar to BW, each time period measured had significantly different BWG between the treatments (Table 5.7). During the first week, both NC and NC+ had higher growth rates compared to AGP+, SI+, and SID+ ($P \leq 0.01$). From D7-14, NC+ had the lowest BWG and AGP+ had the highest ($P \leq 0.05$), while SD+ was higher than NC+ and statistically similar to NC and AGP+. On D14-28, AGP+ had the highest BWG, and SI+ had the lowest ($P \leq 0.01$). Both SD+ and SID+ were similar to NC and NC+. Lastly, NC and AGP+ were similar from D28-42,

and SI+ was similar to NC and NC+ ($P \leq 0.01$). There was less BWG observed in SD+ and SID+ compared to NC, AGP+, and NC+. Cumulatively from DOH-42, AGP+ exhibited the greatest BWG, followed by NC, then NC+, SI+, SD+, and SID+ all had similar BWG to one another, but were less than NC and AGP+ ($P \leq 0.01$).

Feed Intake. Each week reported a significant difference in FI per bird based on treatment (Table 5.8). In week 1, NC birds had higher feed intake compared to the challenged treatments except NC+, in which it was equal ($P \leq 0.01$). Similarly, from D7-14 NC had higher FI compared to all other treatments ($P \leq 0.05$). During D14-28, FI was highest in NC and AGP+ and lowest in SI+ and SID+ ($P \leq 0.05$). From D28-42, FI was higher ($P \leq 0.01$) in NC, AGP+, and NC+ than SD+ and SID+, while SI+ was statistically similar to NC+. Overall, FI from DOH-42 was lower in SI+, SD+, and SID+ compared to NC and AGP+, though both SI+ and SD+ were also similar to NC+ ($P \leq 0.01$).

Feed Conversion Ratio. Table 5.9 reports FCR per bird. From D7-14, AGP+ and SD+ had lower FCR than NC, or NC+ ($P \leq 0.05$). On D14-28, AGP+ had lower FCR than all other treatments, while SD+ and SID+ were both lower than NC, NC+, and SI+ ($P \leq 0.01$). Overall, NC and NC+ had the highest FCR from DOH-42, SD+ and SID+ had lower FCR than NC and NC+, while AGP+ had the lowest ($P \leq 0.01$).

Lesion Scores

On D7, NE lesions were observed from the duodenum, jejunum, and ileum (Table 5.10). In the duodenum and jejunum, NC had significantly lower lesion scores compared to challenged treatments ($P \leq 0.05$; $P \leq 0.01$). Among the challenged treatments, there were no differences observed in the duodenum, and AGP+ had lower lesions compared to SI+, SD+ and SID+. No significant differences were observed in the ileum.

Body Composition

D42 bird weight, percent fat per bird, and percent lean per bird are presented in Table 5.11. Carcass weights of sampled birds were significantly different between treatments ($P \leq 0.05$). However, this difference was comparable to the difference of D42 BW results of live birds from performance results. AGP+ had a similar BW to NC, and was higher compared to other challenged treatments. Both SD+ and SID+ birds had higher lean ($P \leq 0.05$) and lower fat ($P \leq 0.05$) than NC, NC+, and SI+.

mRNA Abundance

Ileum and Cecal Tonsils on DOH. Differences were observed in the ileum of birds that received water *in ovo* on E18 compared to those that received *in ovo* synbiotics (Table 5.12a). The targets included TLR2, TLR4, CLD1, MUC2, TNF-α, LEAP2, AvBD8, AvBD10, AvBD13, and GPR43. Birds that received *in ovo* synbiotics had less mRNA abundance of TLR4, TLR2, and GPR43 on DOH ($P \leq 0.10$; $P \leq 0.05$; $P \leq 0.05$) and greater mRNA abundance of CLD1 ($P \leq 0.10$). There were no significant differences in mRNA abundance levels of various targets measured in cecal tonsils on DOH (Table 5.12b).

TLR2. Table 5.13a gives results for mRNA abundance in both the ileum and cecal tonsils. No significant differences were seen in the ileum until D42, where SID+ had significantly greater levels than NC, SD+, or SI+ ($P \leq 0.05$). In the cecal tonsils, SID+ had greater mRNA abundance compared to NC, NC+, AGP+, and SI+ on D42 ($P \leq 0.05$).

TLR4. In Table 5.13b, there was a significant difference in the ileum at D14 ($P \leq 0.05$) where SI+ and SID+ had lower mRNA abundance than NC+. By D42, SID+ had greater abundance than NC, AGP+, SD+, SI+, and SID+ ($P \leq 0.05$). In the cecal tonsils, NC+ had greater abundance on D14 compared to all other treatments ($P \leq 0.05$).

CLD1. No significant differences were observed in the ileum (Table 5.13c). There was a trend ($P \leq 0.10$) on D14 in the cecal tonsils, where SID+ had similar mRNA abundance to NC+ and AGP+. SD+ and SI+ were both lower than AGP+.

MUC2. In Table 5.13d, no significant differences were seen in the ileum; however, on D14 in the cecal tonsils, both NC and NC+ had greater mRNA abundance of MUC2 compared to other treatments ($P \leq 0.05$). On D42, there was a trend ($P \leq 0.10$) for NC to have the lowest MUC2 level while SID+ had the greatest.

TNF-α. There was a trend ($P \leq 0.10$) in the ileum on D7, in which mRNA abundance was greater in SD+ and SI+ compared to AGP+ (Table 5.13e). On D14, NC had significantly greater abundance than other treatments ($P \leq 0.01$). By D42, NC had the lowest abundance, and SID+ had greater abundance relative to SD+, AGP+, NC+, and NC ($P \leq 0.05$). No significant differences were observed in the cecal tonsils.

LEAP2. No significant effects were observed in the ileum (Table 5.13f). In the cecal tonsils on D14, SD+ and SI+ had lower abundance compared to NC+ and SID+ ($P \leq 0.05$).

AvBD8. Table 5.13g reports mRNA abundance in the ileum and cecal tonsils. Although there were no effects in the cecal tonsils, there was an effect in the ileum on D42 where SID+ had greater abundance than all other treatments ($P \leq 0.05$).

AvBD10. There were significant differences in both the ileum and cecal tonsils (Table 5.13h). On D14 in the ileum, there was a trend ($P \leq 0.10$) for SI+ and SID+ to have lower abundance than NC and NC+. By D42, NC, AGP+, SD+, and SI+ had lower mRNA abundance than the greatest level in treatment SID+ ($P \leq 0.05$). In the cecal tonsils on D14, both NC+ and SID+ tended to have greater abundance than SD+ or SI+ ($P \leq 0.10$).

AvBD13. In the ileum on D7, SD+ had significantly greater ($P \leq 0.05$) mRNA abundance compared to other treatments (Table 5.13i). On D14, AGP+, SI+, and SID+ were greater in the ileum compared to NC ($P \leq 0.05$). There were no differences observed by D42 in the ileum, or at any time point in the cecal tonsils.

GPR43. Significant differences were observed between treatments in both the ileum and cecal tonsils (Table 5.13j). In ileal D7 samples, SD+ had greater abundance compared to other treatments ($P \leq 0.05$). On D14, ileal abundance was lower in SI+ and SID+ compared to NC and NC+ ($P \leq 0.05$). By D42, ileal abundance was greater in NC+ compared to all treatments except SID+, and lower in NC, AGP+, and SI+ compared to NC+ and SID+ ($P \leq 0.05$) . In the cecal tonsils, no differences were observed on D7. By D14, there was a trend ($P \leq 0.10$) for SD+ and SI+ to have lower fold change compared to NC+ and AGP+.

Fatty Acids from Cecal Contents

D7 Fatty Acids. Propionate, isobutyrate, and valerate concentrations were significantly higher in SID+ ($P \leq 0.05$) compared to all other treatments (Table 5.14a). There was a trend ($P \leq 0.10$) in higher isovalerate from SID+ birds compared to NC, NC+, AGP+, and SD+.

D14 Fatty Acids. Table 5.14b shows D14 results. AGP+ had significantly higher ($P \leq 0.05$) isobutyrate and isovalerate than other treatments. Valerate was higher in AGP+ and SID+ compared to NC ($P \leq 0.05$).

D42 Fatty Acids. As shown in Table 5.14c, AGP+ and SD+ tended to have higher isovalerate compared to NC+ ($P \leq 0.10$). There were no significant differences in other fatty acids.

Discussion

This study applied a synbiotic *in ovo*, in post-hatch feed, or in combination in order to measure effects compared to control treatments during a NE challenge. The *in ovo* injection of probiotics may raise additional concerns about hatchability, which can already be reduced by various factors: nutritional imbalances in the flock, lower- or higher-than-average egg weight, unusual shell shape, breeder flock age, preincubation storage time longer than 5 days, and incorrect incubation environment (Narushin and Romanov, 2002; Reijrink et al., 2009; Grochowska et al., 2019). This study used eggs that had been stored for no more than 3 days from a 39-week old flock, and hatchability was not reduced by *in ovo* injection. Other studies have reported similar hatch rates from flocks of this approximate age, including 86% hatchability from flocks aged 31-36 weeks (Yassin et al., 2008), 89% hatchability from flocks aged 31-50 weeks (Grochowska et al., 2019), and 85.9% hatchability from a 36-week-old flock (Abudabos, 2010). Additionally, other *in ovo* studies confirm that hatchability was not affected by injection with similar strains of probiotic bacteria used in this study. A mix of *Lactobacillus acidophilus* did not affect hatchability during *in ovo* injection (Edens et al., 1997; Triplett et al., 2018). A commercial blend containing *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* also had no negative impacts on hatchability (Pender et al., 2016, 2017). Although these studies have demonstrated the safety of *in ovo* application, it is important to note that a common in-feed probiotic, *Bacillus subtilis*, increased late embryo mortality and pipped eggs, reduced hatchability, and resulted in lower BW of chicks (Triplett et al., 2018), emphasizing the importance of continued research into *in ovo* application.

In terms of performance, there were no effects on mortality, BW, or BWG from any method of synbiotic application in this experiment. Starting BW on DOH was higher in birds that received

in ovo injections of synbiotics. This was in contrast to Triplett et al. (2018) who found slightly decreased DOH BW in chicks injected with 50 µL either *Lactobacillus animalis* or *Bifidobacterium animalis*, but reported that chicks quickly compensated at the grower facility. Perhaps the synbiotic-application chicks in this study had higher BW at hatch than the water-application chicks due to the addition of synbiotic powder dissolved in water before *in ovo* injection. This may have added more weight than water injection alone. However, it is more likely this is a minor finding, resulting from data within a tight range of numbers and low standard error means. The average weight between the water-injected (39.56 g) and synbiotic-inject chicks (40.46 g) was only 0.9 grams.

As expected, AGP+ often had the highest BW and BWG, and lowest FCR. It is well documented that in-feed antibiotics increase growth rate and decrease FCR in poultry and other livestock (Rosen, 1995; Cravens et al., 2013). In this study, synbiotic application was unable to outperform AGP+, but still improved some aspects of performance in comparison to the challenged control. Specifically, synbiotics decreased cumulative FI, particularly in SID+, lowered FCR in SD+ and SID+, yet ended with similar BW and BWG as NC+. Other studies have found improved FCR in probiotic-fed broilers during NE challenge compared to controls, without improvements in average daily gain (Midilli et al., 2008; Emami et al., 2019). Awad et al. (2009) reported higher BW, average daily gain, FCR, and carcass yield percentage in broilers fed synbiotics compared to probiotics or controls. Mookiah et al. (2013) found that synbiotic supplementation improved BWG and FCR of broilers from DOH-42. Previous studies have documented the ability of *Lactobacillus* to produce beneficial digestive enzymes, such as amylase, protease, and lipase, which could explain improved FCR (Jin et al., 1996). In contrast, others found no significant effects on performance of broilers consuming in-feed synbiotics (Biggs et al., 2007; Jung et al.

2008). In this study, lean:fat ratio was increased with in-feed application of synbiotics. Another probiotic study reported decreased abdominal fat with increasing levels of in-feed *Lactobacillus johnsonnii* BS15 in male broilers (Liu et al., 2016). This variation in performance between studies could be due to the type of synbiotic used, the dosage, the overall health of the birds, and their environment.

The probiotic strains in this study, *Enterococcus*, *Pediococcus*, *Lactobacillus*, and *Bifidobacterium*, make fatty acids as byproducts of fermentation from carbohydrates (Pessione, 2012). In addition to being an energy source for enterocytes, butyrate has also been implicated as an important promoter of mucin-encoding genes, including MUC2 (Finnie et al., 1995; Gaudier et al., 2004). Herein, the application of synbiotics *in ovo* or in-feed was not enough to alter volatile fatty acids (VFA) between treatments, yet the combination of in-feed and *in ovo* synbiotics was more beneficial. It was enough to increase D7 levels of propionate, isobutyrate, isovalerate, and valerate. This suggests an added benefit from the combination of synbiotics *in ovo* and continuing it in the feed after hatch. A similar study by Mookiah et al. (2013) applied either prebiotics, probiotics, or synbiotics to broilers and evaluated VFA content at D21 and D42. In contrast to this study, they reported higher total VFA levels on D21 and D42 in birds that received high levels of synbiotic compared to the control. This current study failed to see an increase in VFA content past D7 with use of synbiotics. In this study, AGP+ had the highest levels of isobutyrate, isovalerate, and valerate on D14. Rehman et al. (2008a,b) reported higher butyrate in cecal digesta after using inulin, the same prebiotic used in this study. By D42, little difference was observed between treatments in this study. Broilers hatch with minimum microbiota in the small intestine, but colonize rapidly after hatch. There is a small presence of microbiota in the ceca at hatch, and it is said to be well established between 20 and 30 days of

age (Amit-Romach et al., 2004; Liao et al., 2020). Perhaps in young birds, addition of synbiotics *in ovo* and in the feed boosted D7 VFA production due to the probiotic bacteria and inulin. This difference may have decreased with time, as birds in other treatments developed a more mature microbiome over the growing period.

On DOH, CLD1 was increased in birds that received synbiotic injections, suggesting a tighter potential barrier against pathogens with the increase in mRNA abundance of this tight junction protein (Morrison and Preston, 2016). Although increased levels of four VFAs were recorded on D7 in SID+, there was no effect on MUC2 or GPR43 in the intestine that corresponded with SID+ treatment. GPR43, a receptor for SCFA, was lower in the ileum of broilers that received *in ovo* probiotics on DOH, but higher than all other treatments in SI+ on D7. A possible explanation could be that probiotics are not producing SCFA on DOH prior to an exogenous carbohydrate feed source, but the presence of the synbiotic temporarily altered normal expression of the receptor. By D7, *in ovo* injection may have increased SCFA-producing bacteria, thus, leading to greater expression of the GPR43 receptor compared to other treatments. Some SCFA, particularly butyrate, have shown the ability to increase MUC2 abundance (Gaudier et al., 2004). Though some VFAs were increased in SID+ on D7, there was no change in MUC2 abundance. It has been reported that co-infection with NE and *Eimeria* decreases MUC2 expression in broilers, but in contrast to this study, antibiotic treatments were able to prevent the MUC2 decrease in that study (Forder et al., 2012). Only measuring mRNA abundance has limitations, since presence of mRNA alone does not always indicate presence of protein or function.

TLR2 and TLR4 are important innate transmembrane receptors for LPS on Gram-negative bacteria (Kannaki et al., 2015). TLRs activate the transcription factor NF-κB and induce

inflammatory cytokines including TNF-α (Akira and Takeda, 2004; Wu et al., 2018). Another valuable piece of innate immunity in avian species includes host defense peptides, such as AvBD and LEAP2. At the peak of challenge in this study (D7), ileal TNF-α was high in SI+ and SD+ had greater AvBD13 in the ileum and greater TLR2 in the cecal tonsils compared to controls, suggesting that synbiotics were promoting an inflammatory immune response. There was no difference in TNF-α between the NC, NC+, and AGP+, which is in agreement with a previous study evaluating effects of an *Eimeria*/CP co-infection (Forder et al., 2012). In SID+ birds, there was greater abundance of ileal and cecal tonsil TLR2, ileal TLR4, TNF-α, AvBD8, and AvBD10 on D42, suggesting enhanced innate immune activation as a result of combined *in ovo* and in-feed synbiotic application. Su et al. (2017) reports in detail the upregulation and downregulation of multiple AvBDs in the three segments of the small intestine of D28 broilers after infection with various strains of *Eimeria*. There, none of the *Eimeria* strains tested (*E. acervulina*, *E. tenella*, and *E. maxima*) altered AvBD expression in the ileum on D28 of age. After challenge with *E. maxima*, LEAP2 was downregulated in the ileum (Su et al., 2017). There was little difference in LEAP2 in this study, although Sumners et al. (2011) also reported decreased levels of LEAP2 and TLR4 in the intestine of *Eimeria*-infected birds during the first week after infection. In this study, the additional NE challenge could have altered expression of host defense peptides, or the probiotics may have increased their expression in some cases.

In summary, a synbiotic is a combination of a non-viable prebiotic intended to support viable, beneficial probiotic bacteria. The prebiotic in this study included the oligosaccharide inulin, and the probiotics derived from *E. faecium*, *P. acidilactici*, *B. animalis*, and *L. reuteri*. *In ovo* application on E18 did not affect hatchability or mortality. The combined application of *in ovo* and in-feed synbiotics was able to reduce FI and FCR compared to the challenged control, and

improve lean:fat ratios in the D42 carcass. Prior to hatch, synbiotic *in ovo* application resulted in greater CLD1 and lower TLR2, TLR4, and GPR43 mRNA abundance in the ileum. Measuring mRNA abundance does not always indicate function or equal protein levels, which may give better insight into whether the mRNA abundance translated to actual function in the bird. The combination of *in ovo* and in-feed synbiotics resulted in increased volatile fatty acids on D7. On D42, various immune markers were increased in the ileum of SID+ birds. There was increased ileal TNF-α in SI+ and AvBD13 in SD+ on D7 during peak infection, possibly increasing the inflammatory response to disease. Overall, the different synbiotic application methods led to increased immune response to NE challenge on D7 and D42, improved carcass lean:fat ratio, and reduced FCR.

References

Abudabos, A. 2010. The effect of broiler breeder strain and parent flock age on hatchability and fertile hatchability. Int. J. Poult. Sci. 9:231-35.

Akira, S. and K. Takeda. 2004. Toll-like receptor signaling. Nat. Rev. Immunol. 4:499-511.

Alloui, M.N., W. Szczurek, and S. Swiatkiewicz. 2013. The usefulness of prebiotics and probiotics in modern poultry nutrition: a review. Annals of An. Sci. 13:17-32.

Amit-Romach, E., D. Sklan, and Z. Uni. 2004. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. Poult. Sci. 83:1093-98.

Awad, W.A., K. Ghareeb, S. Abdel-Raheem, and J. Bohm. 2009. Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. Poult. Sci. 88:49-56.

Biggs, P., C. Parsons, and G.C. Fahey. 2007. The effects of several oligosaccharides on growth performance, nutrient digestibilities, and cecal microbial populations in young chicks. Poult. Sci. 86:2327-36.

Blottiere, H.M., B. Buecher, J.P. Galmiche, and C. Cherbut. 2003. Molecular analysis of the effect of short-chain fatty acids on intestinal cell proliferation. Proc. Nutr. Soc. 62:101-106.

Castanon, J.I. 2007. Hisstory of the use of antibiotic as growth promoters in European poultry feeds. Poult. Sci. 86:2466-71.

Celi, P., A.J. Cowieson, F. Fru-Nji, R.E. Steinert, A.M. Kluenter, and V. Verlhac.2017. Gastrointestinal functionality in animal nutrition and health: new opportunities for sustainable animal production. Anim. Feed Sci. Technol. 234:88-100.

Cherrington, C.A., M. Hinton, and I. Chopra. 1990. Effects of short-chain organic acids on macromolecular synthesis in *Escherichia coli*. J. Bacteriol. 68:69-74.

Collier, C.T., C.L. Hofacre, A.M. Payne, D.B. Anderson, P. Kaiser, R.I. Mackie, and H.R. Gaskins. 2008. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. Vet. Immunol. Immunopathol. 122:104-115.

Corrier, D.E., A. Hinton, Jr, R.L. Ziprin, and J.R. DeLoach. 1990. Effect of dietary lactose on *Salmonella* colonization of market-age broiler chickens. Avian Dis. 34:668-76.

Cox, N.A., J.S. Bailey, L.C. Blankenship, and R.P. Gildersleeve. 1992. *In ovo* administration of a competitive exclusion culture treatment to broiler embryos. Poult. Sci. 71:1781-84.

Cravens, R.L., G.R. Goss, F. Chi, E.D. De Boer, S.W. Davis, S.M. Hendrix, J.A. Richardson, and S.L. Johnston. 2013. The effects of necrotic enteritis, aflatoxin B_1, and virginiamycin on growth performance, necrotic enteritis lesion scores, and mortality in young broilers. Poult. Sci. 92:1997-2004.

Cuperus, T. M. Coorens, A. van Dijk, and H.P. Haagsman. 2013. Avian host defense peptides. Dev. Comp. Immunol. 41:352-69.

de Oliveira, J.E., E. van der Hoeven-Hangoor, I.B. van de Linde, R.C. Montijn, and J.M.B.M. van der Vossen. 2014. *In ovo* inoculation of chicken embryos with probiotic bacteria and its effects on posthatch Salmonella susceptibility. Poult. Sci. 93:818-29.

Edens, F.W., C.R. Parkhurst, I.A. Casas, and W.J. Dobrogosz. 1997. Principles of ex ovo competitive exclusion and *in ovo* administration of *Lactobacillus reuteri*. Poult. Sci. 76:179-196.

Emami, N.K., A. Calik, M.B. White, M. Young, and R.A. Dalloul. 2019. Necrotic enteritis in broiler chickens: the role of tight junctions and mucosal immune responses in alleviating the effect of the disease. Microorganisms 7:231.

Finnie, I.A., A.D. Dwarakanath, B.A. Taylor, and J.M. Rhodes. 1995. Colonic mucin synthesis is increased by sodium butyrate. Gut 36:93-99.

Forder, R.E.A., G.S. Nattrass, M.S. Geier, R.J. Hughes, and P.I. Hynd. 2012. Quantitative analyses of genes associated with mucin synthesis of broiler chickens with induced necrotic enteritis. Poult. Sci. 91:1335-41.

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66:365-78.

Gaudier, E., A. Jarry, H.M. Blottiere, P. de Coppet, M.P. Buisine, J.P. Aubert, C. Laboisse, C. Cherbut, and C. Hoebler. 2004. Butyrate specifically modulates *MUC* gene expression in intestinal epithelial goblet cells deprived of glucose. Muc. Biol. 287:G1168-74.

George, B.A., C.L. Quarles, and D.J. Fagerberg. 1982. Virginiamycin effects on controlling necrotic enteritis infection in chickens. Poult. Sci. 61:447-450.

Getachew, T. 2016. A review on the effects of probiotic supplementation in poultry performance and cholesterol levels of egg and meat. J. World Poult. Res. 6:31-36.

Gibson, G.R. and M.B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125:1401-12.

Grochowska, E., A. Kinal, Z. Sobek, I. Siatkowski, and M. Bednarczyk. 2019. Field study of the factors affecting egg weight loss, early embryonic mortality, hatchability, and chick mortality with the use of classification tree technique. Poult. Sci. 98:3626-36.

Hegarty, J.W., C.M. Guinane, R.P. Ross, C. Hill, and P.D. Cotter. 2016. Bacteriocin production: a relatively unharnessed probiotic trait? F1000Res. 5:2587.

Jin, L.Z., Y.W. Ho, N. Abdullah, and S. Jalaludin. 1996. Effects of *Lactobacillus* culture on the digestive enzymes in chicken intestine. *Proceedings of the 8th Animal Science Congress, Tokyo,* Japanese Society of Zootechnical Science, Tokyo, pp. 224-225.

Jung, S.J. R. Houde, B. Baurhoo, X. Zhao, and B.H. Lee. 2008. Effects of galacto-oligosaccharides and a *Bifidobacteria lactis*-based probiotic strain on the growth performance and fecal microflora of broiler chickens. Poult. Sci. 87:1694-99.

Kabir, S.M.L. 2009. The role of probiotics in the poultry industry. Int. J. Mol. Sci. 10:3531-46.

Kannaki, T.R., M.R. Reddy, P.C. Verma, and M. Shanmugam. 2015. Differential Toll-like receptor (TLR) mRNA expression patterns during chicken embryological development. Anim. Biotechnol. 26:130-35.

Keestra, A.M., M.R. de Zoete, L.I. Bouwman, M.M. Vaezirad, and J.P.M. van Putten. 2013. Unique features of the chicken Toll-like receptors. Dev. Comp. Immunol. 413:316-323.

Keestra, A.M., M.R. de Aoete, R.A.M.H. van Aubel, and J.P.M. van Putten. 2007. The central Leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2. J. Immunol. 178:7110-7119.

Kogut, M.H. and R.J. Arsenault. 2016. Editorial: gut health: the new paradigm in food animal production. Front. Vet. Sci. 3:71.

Liao, X., Y. Shao, G. Sun, Y. Yang, L. Zhang, Y. Guo, X. Luo, and L.Lu. 2020. The relationship among gut microbiota, short-chain fatty acids, and intestinal morphology of growing and healthy broilers. Poult. Sci. 99:5883-95.

Liu, L., X. Ni, D. Zeng, H. Wang, B. Jing, Z. Yin, and K. Pan. 2016. Effect of a dietary probiotic, *Lactobacillus johnsonii* BS15, on growth performance, quality traits, antioxidant ability, and nutritional and flavor substances of chicken meat. An. Prod. Sci. 57:920-26.

Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C(T)) Method. Methods 25:402-08.

Macia, L., J. Tan, A.T. Vieira, K. Leach, D. Stanley, S. Luong, M. Maruya, C.I. McKenzie, A. Hijikata, C. Wong, L. Binge, A.N. Thorburn, N. Chevalier, C. Ang, E. Marino, R. Robert, S. Offermanns, M.M. Teixeira, R.J. Moore, R.A. Flavell, S. Fagarasan, and C.R. Mackay. 2015. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. Nat. Comm. 6:6734.

Meslin, C., C. Desert, I. Callebaut, A. Djari, C. Klopp, F. Pitel, S. Leroux, P. Martin, P. Froment, and E. Guilbert. 2015. Expanding duplication of free fatty acid receptor-2 (GPR43) genes in the chicken genome. Genome Biol. Evol. 7:1332-48.

Midilli, M., M. Alp, N. Kocabach, O.H. Muglah, N. Turan, H. Yilmaz, and S. Cakir. 2008. Effects of dietary probiotic and prebiotic supplementation on growth performance and serum IgG concentration of broilers. S. Afr. J. Anim. Sci. 38:21-27.

Mitchell, L.A. and M. Koval. 2010. Specificity of interaction between *Clostridium perfringens* enterotoxin and claudin-family tight junction proteins. Toxins 2:1595-1611.

Morrison, D.J. and T. Preston. 2016. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 7:189-200.

Mountzouris, K.C., P. Tsirtsikos, E. Kalamara, E. Nitsch, G. Schatzmayr, and K. Fegeros. 2007. Evaluation of the efficacy of a probiotic containing *Lactobacillus, Bifidobacterium, Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. Poult. Sci. 86:309-317.

Mountzouris, K.C., P. Tsirtsikos, I. Palamidi, A. Arvaniti, M. Mohnl, G. Schatzmayr, and K. Fegeros. 2010. Effects of probiotic inclusion levels in broiler nutrition on growth performance, nutrient digestibility, plasma immunoglobulins, and cecal microflora composition. Poult. Sci. 89:58-67.

Morrison, D.J., K. Cooper, S. Waldron, C. Slater, L.T. Weaver, and T. Preston. 2004. A streamlined approach to the analysis of volatile fatty acids and its application to the measurement of whole-body flux. Rapid Commun. Mass Spectrom. 18:2593-2600.

Narushin, V.G. and M.N. Romanov. 2002. Egg physical characteristics and hatchability. Worlds Poult. Sci. J. 58:297-303.

Nayebpor, M., P. Farhoman, and A. Hashemi. 2007. Effects of different levels of direct fed microbials (PrimaLac) on growth performance and humoral response in broiler chickens. J. Anim. Vet. Adv. 6:1308-1313.

Peebles, E.D. 2018. *In ovo* applications in poultry: a review. Poult. Sci. 97:2311-38.

Pender, C.M., S. Kim, T.D. Potter, M.M. Ritzi, M. Young, and R.A. Dalloul. Effects of *in ovo* supplementation of probiotics on performance and immunocompetence of broiler chicks to an *Eimeria* challenge. 2016. Ben. Microbes 7:699-705.

Pender, C.M., S. Kim, T.D. Potter, M.M. Ritzi, M. Young, and R.A. Dalloul. 2017. *In ovo* supplementation of probiotics and its effects on performance and immune-related gene expression in broiler chicks. Poult. Sci. 96:1052-62.

Pessione, E. 2012. Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. Front. Cell Infect. Microbiol. 2:86.

Prescott, J.F., R. Sivendra, and D.A. Barnum. 1978. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. Can. Vet. J. 19:181-183.

Ramini, S., S. Kathariou, J.L Grimes, and R.M. Siletzky. 2011. Effect of direct-fed microbials on performance and *Clostridium perfringens* colonization of turkey poults. Poult. Sci. 90:2656-2562.

Rehman, H., J. Bohm, and J. Zentek. 2008a. Effects of differentially fermentable carbohydrates on the microbial fermentation profile of the gastrointestinal tract of broilers. J. An. Physiol. An. Nutr. 92:471-80.

Rehman, H., P. Hellweg, D. Taras, and J. Zentek. 2008b. Effects of dietary inulin on the intestinal short chain fatty acids and microbial ecology in broiler chickens as revealed by denaturing gradient gel electrophoresis. Poult. Sci. 87:783-89.

Reijrink, I.A.M., R. Meijerhof, B. Kemp, E.A.M. Graat, and H. van den Brand. 2009. Influence of prestorage incubation on embryonic development, hatchability, and chick quality. Poult. Sci. 88:2649-60.

Reuben, R.C., P.C. Roy, S.L. Sarkar, R. Alam, and I.K. Jahid. 2019. Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics. BMC Microbiol. 19:253.

Richardson, A.J., A.G. Calder, C.S. Stewart., and A. Smith. 1989. Simultaneous determination of volatile and non-volatile acidic fermentation products of anaerobes by capillary gas chromatography. Letters in Appl. Microbiol. 9:5-8.

Ritzi, M.M., W. Abdelrahman, M. Mohnl, and R.A. Dalloul. 2014. Effects of probiotics and application methods on performance and response of broiler chickens to an *Eimeria* challenge. Poult. Sci. 93:2772-78.

Rosen, G.D. 1995. Antibacterials in poultry and pig nutrition. Pages 132-172 in Biotechnology in Animal Feeds and Animal Feeding. R.J. Wallace and A. Chesson, eds. Weinheim, Germany.

Seifi, K., M.A. Karimi-Torshizi, and H. Deldar. 2017. Probiotics intake from proximal or distal gastrointestinal tract: The investigation on intestinal morphology and performance of Japanese quail. J. Anim. Physiol. Anim. Nutr. 102:e509-512.

Semple, F. and J.R. Dorin. 2012. Beta-defensins: multifunctional modulators of infection, inflammation, and more? J. Innate Immun. 4:337-48.

Sen, S., S.L. Ingale, Y.W. Kim, J.S. Kim, K.H. Kim, J.D. Lohakare, E.K. Kim, H.S. Kim, M.H. Ryu, I.K. Kwon, and B.J. Chae. 2012. Effect of supplementation of *Bacillus subtilis* LS 1-2 to

broiler diets on growth performance, nutrient retention, caecal microbiology and small intestinal morphology. Res. Vet. Sci. 93:264-68.

Sobolewska, A., G. Elminowska-Wenda, J. Bogucka, A. Dankowiakowska, A. Kulakowska, A. Szczerba, K. Stadnicka, M. Szpinda, and M. Bednarczyk. 2017. The influence of *in ovo* injection with the prebiotic DiNovo® on the development of histomorphological parameters of the duodenum, body mass and productivity in large-scale poultry production conditions. J. An. Sci. Biotech. 8:45.

Sharma, J.M. and B.R. Burmester. 1982. Resistance to Marek's Disease at hatching in chickens vaccinated as embryos with the turkey herpesvirus. Avian Dis. 26:134-49.

Su, S., D.M. Dwyer, K.B. Miska, R.H. Fetterer, M.C. Jenkins, and E.A. Wong. 2017. Expression of host defense peptides in the intestine of *Eimeria*-challenged chickens. Poult. Sci. 96:2421-27.

Sumners, L.H., K.B. Miska, M.C. Jenkins, R.H. Fetterer, C.M. Cox, S. Kim, and R.A. Dalloul. 2011. Expression of Toll-like receptors and antimicrobial peptides during *Eimeria praecox* infection in chickens. Exp. Parasitol, 127:714-18.

Tarradas, J., N. Tous, E. Esteve-Garcia, and J. Brufau. 2020. The control of intestinal inflammation: a major objective in the research of probiotic strains as alternatives to antibiotic growth promoters in poultry. Microorganisms 8:148.

Teague, K.D., L.E. Graham, J.R. Dunn, H.H. Chang, N. Anthony, J.D. Latorre, A. Menconi, R.E. Wolfenden, A.D. Wolfenden, B.D. Mahaffey, M. Baxter, X. Hernandez-Velasco, R. Merino-Guzman, L.R. Bielke, B.M. Hargis, and G. Tellez. 2017. *In ovo* evaluation of FloraMax®-B11 on Marek's disease HVT vaccine protective efficacy, hatchability, microbiota composition, morphometric analysis, and *Salmonella* Enteritidis infection in broiler chickens. Poult. Sci. 96:2074-82.

Triplett, M.D., W. Zhai, E.D. Peebles, C.D. McDaniel, and A.S. Kiess. 2018. Investigating commercial *in ovo* technology as a strategy for introducing probiotic bacteria to broiler embryos. Poult. Sci. 97:658-66.

Veterinary Feed Directive: A Rule by the Food and Drug Administration on 06/03/2015. 2015. Pages 31707-31735. Published by the Food and Drug Administration, HHS. https://www.federalregister.gov/documents/2015/06/03/2015-13393/veterinary-feed-directive.

Wu, Y., Y. Shao, B. Song, W. Zhen, Z. Wang, Y. Guo, M.S. Shahid, and W. Nie. 2018. Effects of *Bacillus coagulans* supplementation on the growth performance and gut health of broiler chickens with *Clostridium perfringens*-induced necrotic enteritis. J. Anim. Sci. Biotech. 9:9.

Yamawaki, R.A., E.L. Milbradt, M.P. Coppola, J.C.Z. Rodrigues, R.L. Andreatti Filho, C.R. Padovani, and A.S. Okamoto. 2013. Effect of immersion and inoculation *in ovo* of *Lactobacillus* spp. in embryonated chicken eggs in the prevention of *Salmonella* Enteritidis after hatch. Poult. Sci. 92:1560-63.

Yassin, H., A.G.J. Velthuis, M. Boerjan, J. van Riel, and R.B.M. Huirne. 2008. Field study on broiler eggs hatchability. Poult. Sci. 87:2408-17.

Xiong, H., B. Guo, Z. Gan, D. Song, Z. Lu, H. Yi, Y. Wu, Y. Wang, and H. Du. 2016. Butyrate upregulates endogenous host defense peptides to enhance disease resistance in piglets via histone deacetylase inhibition. Sci. Rep. 6:27070.

Table 5.1. Treatment Groups.

Trt[1]	Diet	*In ovo* Application	NE Challenge	Replicate pens	Birds/ pen	Birds/ Trt
1. NC	Control = basal (standard corn/soy) diets	100µL water	-	8	29	232
2. AGP+	Control w/ virginiamycin	100µL water	+	8	29	232
3. NC+	Control	100µL water	+	8	29	232
4. SI+	Control	100µL synbiotic	+	8	29	232
5. SD+	Control w/ synbiotic	100µL water	+	8	29	232
6. SID+	Control w/ synbiotic	100µL synbiotic	+	8	29	232

[1]Treatments: NC = basal diet, sterile water injection, no NE challenge; AGP+ = basal diet with virginiamycin (0.5 kg/MT), sterile water injection, NE challenge; NC+ = basal diet, sterile water injection, NE challenge; SI+ = basal diet, synbiotic *in ovo* injection, NE challenge; SD+ = basal diet with synbiotic (0.5 kg/MT), sterile water injection, NE challenge; SID+ = basal diet with synbiotic (0.5 kg/MT), synbiotic *in ovo* injection, NE challenge.

Table 5.2. Feed Composition of starter (0-14 d), grower (14-28 d), and finisher (28-42 d) mash diets.

Ingredient, g/kg	0-14 d	14-28 d	28-42 d
Corn	56.86	60.75	65.19
Soybean meal, CP 48%	35.63	32.62	27.55
Vegetable oil	2.18	2.40	3.60
Limestone	1.15	0.80	0.60
Dicalcium phosphate	2.25	1.90	1.80
DL-Methionine (98%)	0.45	0.25	0.20
L-Lysine HCl (78%)	0.40	0.34	0.12
L-Threonine	0.22	0.05	0.04
Salt	0.15	0.25	0.25
Sodium Bicarbonate	0.35	0.35	0.35
Vitamin-Mineral Premix (NB3000)*	0.36	0.30	0.30
Total	100.00	100.00	100.00
Chemical composition (Calculated)			
Dry Matter, %	87.11	87.14	87.16
Crude Protein, %	21.36	20.23	18.08
AME_n, kcal/kg	2962	3050	3190
Lysine, %	1.41	1.29	1.15
Dig. Lysine, %	1.25	1.15	0.99
Methionine + cysteine, %	1.09	0.97	0.91
Dig. Methionine + cysteine, %	0.96	0.86	0.80
Threonine, %	0.98	0.88	0.82
Dig. Threonine, %	0.87	0.78	0.72
Calcium, %	1.05	0.82	0.81
Available phosphorus, %	0.56	0.50	0.47

*Vitamins per kg diet: retinol 3.33 mg, cholecalciferol 0.1 mg, α-tocopherol acetate 23.4 mg, vitamin K3 1.2 mg, vitamin B1 1.6 mg, vitamin b2 9.5 mg, niacin 40 mg, pantothenic acid 9.5 mg, vitamin B6 2 mg, folic acid 1 mg, vitamin B12 0.016 mg, biotin 0.05 mg, choline 556 mg. Minerals per kg diet: Mn 144 mg, Fe 72 mg, Zn 144 mg, Cu 16.2 mg, I 2.1 mg, Se 0.22 mg.

Table 5.3. Primers used for qRT-PCR mRNA abundance. Forward primer sequence is listed before reverse primer sequence.

Gene[a]	Primer Sequence (5' – 3')	Accession Number
GAPDH	CCT AGG ATA CAC AGA GGA CCA GGT T GGT GGA GGA ATG GCT GTC A	NM_204305
TLR2	GCG AGC CCC CAC GAA GGA GTC GTT CTC ACT GTA GGA GAC A	NM_204278
TLR4	CCA CAC ACC TGC CTA CAT GAA GGA TGG CAA GAG GAC ATA TCA AA	NM_001030693
CLD1	GTG TTC AGA GGC ATC AGG TAT C CTC AGG TCA AAC AGA GGT ACA A	NM_001013611.2
MUC2	TTC ATG ATG CCT GCT CTT GTG CCT GAG CCT TGG TAC ATT CTT GT	XM_421035
TNF-α	CCC ATC CCT GGT CCG TAA C ATA CGA AGT AAA GGC CGT CCC	MF000729.1
LEAP2	TGT GCT TCC CTG CAC CAA GGC GTC ATC CGC TTC AGT	AY534899
AvBD8	ATG CGC GTA CCT AAC AAC GA TGC CCA AAG GCT CTG GTA TG	NM_001001781.1
AvBD10	CAG ACC CAC TTT TCC CTG ACA CCC AGC ACG GCA GAA ATT	NM_00100609.2
AvBD13	CAG CTG TGC AGG AAC AAC CA CAG CTC TCC ATG TGG AAG CA	NM_001001780.1
GPR43	GCC CCA TAG CAA ACT TCT GGG CAG CCA TAA AGA GAG	Meslin et al., 2015

[a] **GAPDH** = *Glyceraldehyde 3-phosphate dehydrogenase.* ***TLR*** *= Toll-like receptor.* ***CLD1*** *= Claudin-1.* ***MUC2*** *= Mucin 2.* ***TNF-α*** *= Tumor necrosis factor alpha.* ***LEAP2*** *= Liver-expressed antimicrobial peptide 2.* ***AvBD*** *= Avian β-defensin.* ***GPR43*** *= G-protein coupled receptor 43.*

Table 5.4. Effect of treatment on hatchability.

	No. Eggs Hatched/Eggs Placed	% Hatchability
Trt[1]	DOH	DOH
Water	1114/1289	86.42
Synbiotic	489/515	95.00

[1]Treatments: Water = Sterile water *in ovo* treatment. Synbiotic = Synbiotic *in ovo* treatment. Data presented as number of eggs that hatched per number of eggs placed at start.

Table 5.5. Effect of treatment on mortality (%).

Mortality (%) (*n* = 232 birds/trt)					
Trt[1]	DOH-7	D7-14	D14-28	D28-42	DOH-42
1. NC	0.4	**0.0**[b]	0.5	0.0	0.9
2. AGP+	1.3	**0.0**[b]	0.0	0.5	1.7
3. NC+	0.4	**0.0**[b]	0.0	0.0	0.4
4. SI+	0.9	**0.0**[b]	0.5	0.0	1.3
5. SD+	0.0	**0.0**[b]	0.5	0.0	0.4
6. SID+	1.7	**0.4**[a]	1.1	0.5	3.5
	P > 0.10	***P ≤ 0.05***	*P > 0.10*	*P > 0.10*	*P > 0.10*
	[2]*SEM = 0.6*	***SEM = 0.2***	*SEM = 0.5*	*SEM = 0.3*	*SEM = 0.8*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.6. Effect of treatment on body weight (BW) per bird.

Body Weight (g/bird) (*n* = 232 birds/trt)					
Trt[1]	DOH	D7	D14	D28	D42
1. NC	39.7^{bc}	134^{a}	356^{a}	1216^{b}	2681^{b}
2. AGP+	39.6^{c}	113^{c}	345^{ab}	1322^{a}	2805^{a}
3. NC+	39.5^{c}	126^{b}	325^{bc}	1151^{cd}	2574^{c}
4. SI+	40.7^{a}	118^{c}	324^{bc}	1135^{d}	2555^{c}
5. SD+	39.6^{c}	119^{bc}	342^{abc}	1196^{bc}	2559^{c}
6. SID+	40.3^{ab}	114^{c}	318^{c}	1176^{bcd}	2499^{c}
	P ≤ 0.05	*P ≤ 0.01*	*P ≤ 0.05*	*P ≤ 0.01*	*P ≤ 0.01*
	[2]SEM = 0.2	*SEM = 2.7*	*SEM = 8.5*	*SEM = 19.3*	*SEM = 31.9*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.7. Effect of treatment on body weight gain (BWG) per bird.

Body Weight Gain (g/bird) (*n* = 232 birds/trt)					
Trt[1]	DOH-7	D7-14	D14-28	D28-42	DOH-42
1. NC	94[a]	206[abc]	830[b]	1458[ab]	2635[b]
2. AGP+	73[c]	215[a]	948[a]	1483[a]	2765[a]
3. NC+	86[ab]	187[c]	798[bc]	1423[b]	2534[c]
4. SI+	77[c]	192[bc]	784[c]	1407[bc]	2502[c]
5. SD+	80[bc]	209[ab]	826[b]	1356[cd]	2513[c]
6. SID+	74[c]	189[bc]	829[b]	1317[d]	2452[c]
	P ≤ 0.01	*P ≤ 0.05*	*P ≤ 0.01*	*P ≤ 0.01*	*P ≤ 0.01*
	[2]SEM = 2.7	*SEM = 7.1*	*SEM = 12.5*	*SEM = 20.7*	*SEM = 32.8*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.8. Effect of treatment on feed intake (FI) per bird.

Feed Intake (g/bird) (*n* = 232 birds/trt)					
Trt[1]	DOH-7	D7-14	D14-28	D28-42	DOH-42
1. NC	130[a]	299[a]	1312[ab]	2397[a]	4137[a]
2. AGP+	110[c]	275[b]	1359[a]	2381[a]	4126[a]
3. NC+	124[ab]	260[b]	1277[bc]	2328[ab]	3988[ab]
4. SI+	116[bc]	259[b]	1239[c]	2282[bc]	3896[bc]
5. SD+	111[c]	263[b]	1257[bc]	2238[cd]	3869[bc]
6. SID+	111[c]	254[b]	1232[c]	2185[d]	3782[c]
	P ≤ 0.01	*P ≤ 0.01*	*P ≤ 0.01*	*P ≤ 0.01*	*P ≤ 0.01*
	[2]SEM =3.0	*SEM = 8.3*	*SEM = 20.6*	*SEM = 30.2*	*SEM = 53.9*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.9. Effect of treatment on feed conversion ratio (FCR) per bird.

Feed Conversion Ratio (per bird) (*n* = 232 birds/trt)					
Trt[1]	DOH-7	D7-14	D14-28	D28-42	DOH-42
1. NC	1.39	**1.46**[a]	**1.58**[a]	1.65	**1.57**[a]
2. AGP+	1.52	**1.28**[b]	**1.44**[c]	1.61	**1.49**[c]
3. NC+	1.44	**1.41**[a]	**1.60**[a]	1.64	**1.57**[a]
4. SI+	1.51	**1.35**[ab]	**1.58**[a]	1.62	**1.56**[ab]
5. SD+	1.41	**1.27**[b]	**1.52**[b]	1.65	**1.54**[b]
6. SID+	1.52	**1.35**[ab]	**1.49**[b]	1.66	**1.54**[b]
	P > 0.10	***P ≤ 0.01***	***P ≤ 0.01***	*P > 0.10*	***P ≤ 0.01***
	[2]SEM = 0.05	***SEM = 0.04***	***SEM = 0.01***	*SEM = 0.02*	***SEM = 0.01***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.10. Effect of treatment on D7 NE lesion scores.

	D7 NE Lesion Scores (*n* = 24 birds/trt)		
Trt[1]	Duodenum	Jejunum	Ileum
1. NC	**0.50**[b]	**0.08**[c]	0.00
2. AGP+	**1.00**[a]	**0.40**[b]	0.10
3. NC+	**1.00**[a]	**0.58**[ab]	0.10
4. SI+	**1.08**[a]	**0.63**[a]	0.13
5. SD+	**0.98**[a]	**0.65**[a]	0.17
6. SID+	**1.02**[a]	**0.65**[a]	0.15
	P ≤ 0.01	***P ≤ 0.01***	*P > 0.10*
	[2]SEM = 0.12	***SEM = 0.08***	*SEM = 0.04*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.11. Effect of treatment on D42 body composition using DXA.

D42 DXA Analysis (*n* = 24 birds/trt)			
Trt[1]	Bird Wt (g)	% Fat per Bird	% Lean per Bird
1. NC	**2658**[ab]	**16.8**[a]	**82.0**[b]
2. AGP+	**2799**[a]	**16.5**[ab]	**82.3**[ab]
3. NC+	**2599**[bc]	**16.8**[a]	**82.0**[b]
4. SI+	**2459**[c]	**16.7**[a]	**82.1**[b]
5. SD+	**2461**[bc]	**15.5**[b]	**83.3**[a]
6. SID+	**2475**[bc]	**15.5**[b]	**83.2**[a]
	P ≤ 0.01	***P ≤ 0.05***	***P ≤ 0.05***
	[2]SEM = 70.8	***SEM = 0.4***	***SEM = 0.4***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.12a. Effect of synbiotic application method on DOH mRNA levels in the ileum.

DOH Ileum (*n* = 8 birds/trt)										
[1]Trt	TLR2	TLR4	CLD1	MUC2	TNFa	LEAP2	AvBD8	AvBD10	AvBD13	GPR43
Water	**1.04[a]**	**1.80[a]**	**1.45[b]**	1.24	1.88	1.84	0.43	3.31	1.08	**1.11[a]**
Synb.	**0.30[b]**	**0.54[b]**	**1.88[a]**	1.70	1.22	3.91	0.15	1.78	1.59	**0.40[b]**
P-value	***≤0.01***	***≤0.10***	***≤0.10***	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	***≤0.05***
[2]*SEM*	***0.12***	***0.18***	***0.57***	*0.44*	*0.91*	*0.97*	*0.091*	*1.43*	*0.43*	***0.20***

[1]Treatments: Water = *In ovo* injection with sterile water on E18; Synb. = *In ovo* injection with synbiotic on E18. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 5.12b. Effect of synbiotic application method on DOH mRNA levels in the cecal tonsils.

DOH Cecal Tonsils (*n* = 8 birds/trt)										
[1]Trt	TLR2	TLR4	CLD1	MUC2	TNFa	LEAP2	AvBD8	AvBD10	AvBD13	GPR43
Water	1.10	1.14	1.19	1.06	1.18	1.60	0.43	2.95	1.48	1.18
Synb.	0.73	0.64	2.14	2.22	1.02	1.03	0.27	6.35	0.32	0.32
P-value	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*
[2]*SEM*	*0.25*	*0.29*	*0.84*	*1.05*	*0.45*	*0.58*	*0.05*	*2.87*	*0.58*	*0.58*

[1]Treatments: Water = *In ovo* injection with sterile water on E18; Synb. = *In ovo* injection with synbiotic on E18. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 5.13a. Effect of synbiotic application method on TLR2 mRNA levels in the ileum and cecal tonsils at three time periods.

TLR2 Fold Change (n = 8 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	2.68	1.54	**0.71^{b}**	**1.27^{b}**	1.11	**0.71^{b}**
NC+	6.57	2.00	**1.67^{ab}**	**2.21^{b}**	0.73	**0.82^{b}**
AGP+	22.68	0.99	**1.69^{ab}**	**4.80^{b}**	0.77	**0.63^{b}**
SD+	1.52	0.89	**1.16^{b}**	**337.04^{a}**	0.44	**1.18^{ab}**
SI+	0.85	0.92	**0.80^{b}**	**32.95^{b}**	0.52	**0.56^{b}**
SID+	1.61	0.62	**2.81^{a}**	**5.76^{b}**	0.77	**1.97^{a}**
	P > 0.10	*P > 0.10*	***P ≤ 0.05***	***P ≤ 0.10***	*P > 0.10*	***P ≤ 0.05***
	[2]*SEM = 8.45*	*SEM = 0.37*	***SEM = 0.41***	***SEM = 99.63***	*SEM = 0.19*	***SEM = 0.28***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.13b. Effect of synbiotic application method on TLR4 mRNA levels in the ileum and cecal tonsils at three time periods.

TLR4 Fold Change (n = 8 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.286	**1.487^{ab}**	**0.431^{b}**	1.412	**1.072^{b}**	0.703
NC+	4.056	**1.631^{a}**	**0.974^{ab}**	16.955	**2.040^{a}**	0.823
AGP+	5.584	**0.784^{abc}**	**0.780^{b}**	4.715	**0.820^{b}**	0.694
SD+	2.671	**0.922^{abc}**	**0.542^{b}**	13.959	**0.646^{b}**	0.760
SI+	2.732	**0.353^{c}**	**0.604^{b}**	23.514	**0.674^{b}**	0.573
SID+	2.817	**0.659^{bc}**	**1.604^{a}**	4.000	**0.792^{b}**	1.179
	P > 0.10	***P ≤ 0.05***	***P ≤ 0.05***	*P > 0.10*	***P ≤ 0.05***	*P > 0.10*
	[2]*SEM = 1.23*	***SEM = 0.30***	***SEM = 0.25***	*SEM = 9.36*	***SEM = 0.31***	*SEM = 0.14*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.13c. Effect of synbiotic application method on CLD1 mRNA levels in the ileum and cecal tonsils at three time periods.

CLD1 Fold Change (*n* = 8 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.098	1.359	0.714	1.759	**1.151[b]**	0.743
NC+	1.461	1.380	1.614	8.577	**2.539[ab]**	0.849
AGP+	1.933	0.683	1.217	24.129	**3.268[a]**	0.993
SD+	2.322	0.808	0.958	171.577	**0.743[b]**	0.664
SI+	1.265	1.103	1.182	57.667	**0.645[b]**	0.720
SID+	2.169	0.843	1.996	3.714	**1.338[ab]**	1.221
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	***P ≤ 0.10***	*P > 0.10*
	[2]*SEM = 0.57*	*SEM = 0.35*	*SEM = 0.34*	*SEM = 48.84*	***SEM = 0.75***	*SEM = 0.19*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.13d. Effect of synbiotic application method on MUC2 mRNA levels in the ileum and cecal tonsils at three time periods.

MUC2 Fold Change (*n* = 8 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.154	1.017	0.750	1.618	**1.386[a]**	**0.565[c]**
NC+	1.139	1.066	0.463	2.960	**1.411[a]**	**1.519[ab]**
AGP+	1.064	0.886	0.337	4.657	**0.569[b]**	**0.699[bc]**
SD+	1.392	1.171	0.345	3.777	**0.441[b]**	**0.964[abc]**
SI+	1.917	1.004	0.804	7.638	**0.325[b]**	**1.083[abc]**
SID+	1.308	1.037	0.599	0.918	**0.576[b]**	**1.833[a]**
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	***P ≤ 0.05***	***P ≤ 0.10***
	[2]*SEM = 0.30*	*SEM = 0.15*	*SEM = 0.17*	*SEM = 2.56*	***SEM = 0.27***	***SEM = 0.31***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.13e. Effect of synbiotic application method on TNF-α mRNA levels in the ileum and cecal tonsils at three time periods.

TNF-α Fold Change (*n* = 8 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	**1.33^{bc}**	**1.48^{a}**	**0.72^{c}**	1.61	1.29	0.77
NC+	**4.80^{abc}**	**0.36^{b}**	**1.00^{bc}**	7.78	1.07	0.88
AGP+	**1.06^{c}**	**0.20^{b}**	**0.94^{bc}**	9.13	0.82	0.79
SD+	**5.63^{ab}**	**0.20^{b}**	**1.03^{bc}**	57.23	1.19	1.33
SI+	**6.50^{a}**	**0.11^{b}**	**1.19^{ab}**	19.72	0.56	0.62
SID+	**2.13^{bc}**	**0.19^{b}**	**1.60^{a}**	75.45	1.67	1.70
	P ≤ 0.10	***P ≤ 0.01***	***P ≤ 0.05***	*P > 0.10*	*P > 0.10*	*P > 0.10*
	[2]SEM = 1.54	***SEM = 0.17***	***SEM = 0.16***	*SEM = 35.20*	*SEM = 0.45*	*SEM = 0.29*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.13f. Effect of synbiotic application method on LEAP2 mRNA levels in the ileum and cecal tonsils at three time periods.

LEAP2 Fold Change (*n* = 8 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.49	1.18	0.72	1.36	**1.20^{ab}**	0.57
NC+	1.44	1.12	0.71	5.45	**1.56^{a}**	0.57
AGP+	1.73	0.62	0.75	16.12	**0.63^{ab}**	0.50
SD+	4.38	0.88	0.67	229.55	**0.49^{b}**	0.61
SI+	2.84	0.93	0.56	41.89	**0.51^{b}**	0.60
SID+	3.02	0.63	0.60	2.77	**1.58^{a}**	0.97
	P > 0.10	*P > 0.10*	*P > 0.10*	*P > 0.10*	***P ≤ 0.05***	*P > 0.10*
	[2]SEM = 0.89	*SEM = 0.17*	*SEM = 0.12*	*SEM = 65.64*	***SEM = 0.33***	*SEM = 0.14*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.13g. Effect of synbiotic application method on AvBD8 mRNA levels in the ileum and cecal tonsils at three time periods.

AvBD8 Fold Change (n = 8 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.38	1.63	**0.76^{b}**	3.76	1.04	1.02
NC+	1.55	2.49	**1.50^{b}**	3647.56	6.50	1.36
AGP+	1.36	1.01	**1.46^{b}**	4360.88	3.19	1.93
SD+	1.80	1.49	**1.17^{b}**	22153.09	1.69	5.19
SI+	1.67	0.57	**0.85^{b}**	13125.64	3.62	1.30
SID+	2.34	1.22	**3.06^{a}**	315.05	126.63	4.40
	P > 0.10	*P > 0.10*	***P ≤ 0.01***	*P > 0.10*	*P > 0.10*	*P > 0.10*
	[2]*SEM = 0.49*	*SEM = 0.48*	***SEM = 0.40***	*SEM = 6600.01*	*SEM = 54.01*	*SEM = 1.90*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.13h. Effect of synbiotic application method on AvBD10 mRNA levels in the ileum and cecal tonsils at three time periods.

AvBD10 Fold Change (n = 8 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.61	**1.92^{a}**	**0.92^{bc}**	1.07	**1.40^{ab}**	1.32
NC+	3.14	**2.02^{a}**	**2.49^{ab}**	24.04	**5.49^{a}**	0.67
AGP+	1.74	**1.00^{ab}**	**1.42^{bc}**	5.0	**3.37^{ab}**	0.27
SD+	3.57	**1.37^{ab}**	**1.29^{bc}**	3.56	**0.77^{b}**	0.37
SI+	1.74	**0.43^{b}**	**0.75^{c}**	29.71	**1.17^{b}**	1.20
SID+	2.71	**0.22^{b}**	**3.94^{a}**	6.85	**5.36^{a}**	2.09
	P > 0.10	***P ≤ 0.10***	***P ≤ 0.01***	*P > 0.10*	***P ≤ 0.10***	*P > 0.10*
	[2]*SEM = 0.77*	***SEM = 0.50***	***SEM = 0.61***	*SEM = 11.79*	***SEM = 1.45***	*SEM = 0.57*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.13i. Effect of synbiotic application method on AvBD13 mRNA levels in the ileum and cecal tonsils at three time periods.

AvBD13 Fold Change (*n* = 8 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	**1.43^{b}**	**2.31^{a}**	16.16	1.40	1.14	1.47
NC+	**1.83^{b}**	**1.79^{ab}**	2.34	280.91	5.33	1.54
AGP+	**1.01^{b}**	**0.35^{bc}**	1.84	658.50	5.61	1.92
SD+	**6.32^{a}**	**1.33^{abc}**	0.89	127.78	5.50	1.56
SI+	**2.45^{b}**	**0.23^{c}**	0.84	228.87	6.27	0.94
SID+	**1.68^{b}**	**0.42^{bc}**	4.05	88.79	4.25	1.43
	P ≤ 0.05	***P ≤ 0.05***	*P > 0.10*	*P > 0.10*	*P > 0.10*	*P > 0.10*
	[2]***SEM = 1.12***	***SEM = 0.53***	*SEM = 6.45*	*SEM = 197.74*	*SEM = 1.53*	*SEM = 0.46*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.13j. Effect of synbiotic application method on GPR43 mRNA levels in the ileum and cecal tonsils at three time periods.

GPR43 Fold Change (*n* = 8 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	**1.25^{b}**	**1.62^{ab}**	**0.71^{c}**	1.67	**1.19^{ab}**	**0.67^{b}**
NC+	**1.73^{b}**	**1.93^{a}**	**3.62^{a}**	8.46	**2.57^{a}**	**1.03^{a}**
AGP+	**2.28^{b}**	**0.44^{bc}**	**1.10^{c}**	12.53	**3.14^{a}**	**0.71^{b}**
SD+	**8.22^{a}**	**1.32^{abc}**	**1.30^{bc}**	41.26	**0.55^{b}**	**0.60^{b}**
SI+	**3.02^{b}**	**0.24^{c}**	**0.92^{c}**	88.35	**0.43^{b}**	**0.57^{b}**
SID+	**1.31^{b}**	**0.40^{c}**	**3.38^{ab}**	39.76	**1.76^{ab}**	**0.83^{ab}**
	P ≤ 0.05	***P ≤ 0.05***	***P ≤ 0.05***	*P > 0.10*	***P ≤ 0.10***	***P ≤ 0.10***
	[2]***SEM = 1.49***	***SEM = 0.42***	***SEM = 0.72***	*SEM = 26.59*	***SEM = 0.70***	***SEM = 0.11***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.14a. Effect of synbiotic application method on cecal fatty acid measurements on D7.

D7 Cecal Content: Fatty Acids (mmol/kg sample) (*n* = 8 birds/trt)						
[1]Trt	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate
NC	82	**2^{b}**	**6^{b}**	16	**5^{b}**	**2^{b}**
NC+	92	**2^{b}**	**4^{b}**	15	**3^{b}**	**2^{b}**
AGP+	91	**1^{b}**	**4^{b}**	14	**4^{b}**	**2^{b}**
SD+	76	**2^{b}**	**6^{b}**	17	**5^{b}**	**3^{b}**
SI+	127	**2^{b}**	**5^{b}**	13	**6^{ab}**	**4^{b}**
SID+	125	**3^{a}**	**10^{a}**	20	**13^{a}**	**9^{a}**
	P > 0.10 [2] *SEM = 26.7*	***P ≤ 0.01*** ***SEM = 0.3***	***P ≤ 0.05*** ***SEM = 1.5***	*P > 0.10* *SEM = 3.4*	***P ≤ 0.10*** ***SEM = 2.5***	***P ≤ 0.05*** ***SEM = 1.5***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.14b. Effect of synbiotic application method on cecal fatty acid measurements on D14.

D14 Cecal Content: Fatty Acids (mmol/kg sample) (*n* = 8 birds/trt)						
[1]Trt	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate
NC	68	3	**6^{b}**	32	**4^{b}**	**9^{c}**
NC+	73	3	**5^{b}**	43	**5^{b}**	**11^{bc}**
AGP+	81	4	**16^{a}**	48	**17^{a}**	**18^{a}**
SD+	72	3	**5^{b}**	41	**4^{b}**	**13^{bc}**
SI+	74	3	**6^{b}**	40	**6^{b}**	**12^{bc}**
SID+	77	2	**6^{b}**	60	**5^{b}**	**14^{ab}**
	P > 0.10 [2] *SEM = 8.1*	*P > 0.10* *SEM = 0.4*	***P ≤ 0.01*** ***SEM = 1.9***	*P > 0.10* *SEM = 7.1*	***P ≤ 0.01*** ***SEM = 2.4***	***P ≤ 0.01*** ***SEM = 1.6***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

Table 5.14c. Effect of synbiotic application method on cecal fatty acid measurements on D42.

D42 Cecal Content: Fatty Acids (mmol/kg sample) (*n* = 8 birds/trt)						
[1]Trt	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate
NC	236	14	26	197	**15**^{ab}	45
NC+	247	9	17	172	**11**^{b}	32
AGP+	210	13	31	179	**26**^{a}	45
SD+	175	12	323	112	**27**^{a}	34
SI+	196	10	21	122	**15**^{ab}	32
SID+	173	10	25	125	**20**^{ab}	35
	P > 0.10 [2] *SEM = 28.9*	*P > 0.10* *SEM = 1.9*	*P > 0.10* *SEM = 4.8*	*P > 0.10* *SEM = 26.9*	***P ≤ 0.10*** ***SEM = 4.3***	*P > 0.10* *SEM = 6.7*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; SI+ = basal diet, synbiotic injection, and NE challenge; SD+ = basal diet with synbiotic, sterile water injection, and NE challenge. SID+ = basal diet with synbiotic, synbiotic injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another.
[2]SEM: standard error means.

CHAPTER VI

Comparison between *in ovo* and feed application of probiotics during post-hatch necrotic enteritis: performance and intestinal responses.

Abstract

Antibiotic alternatives to address major poultry diseases like necrotic enteritis (NE) continue to draw interest based on their specific application method, mechanisms of action, and ability to improve bird health. In this 42-day study, Ross 708 broiler eggs were injected with either water or water-soluble probiotic on embryonic day 18 (E18). After hatch, birds were further divided into one of five treatments: negative control (**NC**): sterile water *in ovo*, fed basal corn/soybean meal mash diet without NE challenge; antibiotic growth-promoter (**AGP+**): sterile water *in ovo*, fed basal diet with virginiamycin (0.5 kg/MT of feed) as AGP with NE challenge; **NC+**: NC treatment, with NE challenge; **PI+**: probiotic *in ovo*, fed basal diet, with NE challenge; **PD+**: sterile water *in ovo*, fed basal diet supplemented with probiotic (1.3 kg/MT of feed), with NE challenge. On day-of-hatch, a commercial coccidiosis vaccine was used to induce a naturally occurring outbreak of NE. Data were collected on performance measurements, NE lesion scores, cecal volatile fatty acid (VFA), D42 body composition, and mRNA abundance of TLR2, TLR4, CLD1, MUC2, TNF-α, LEAP2, AvBD8, AvBD10, AvBD13, and GPR43 in the ileum (ILE) and cecal tonsils (CT). Data were analyzed in JMP with LS Means to separate means with significance assigned at $P \leq 0.05$. There were no effects of *in ovo* application on hatchability; however, PI+ experienced heat stress in the incubator on E19, greatly reducing hatchability. Cumulatively, PI+ had lower overall BW, BWG, FI, and higher FCR. Performance of PD+ was similar to NC+, and AGP+ had higher overall performance. *In ovo* probiotic application did not reduce lesion scores measured at the peak outbreak. PD+ had higher mRNA abundance of D14

TLR2 and TNF-α, and D42 LEAP2 in the CT compared to controls. PI+ had higher mRNA abundance of D7 and D42 MUC2, D7 LEAP2 and D42 TNF-α in the ILE, and D7 TLR2 and D42 TNF-α in the CT. On D14, higher VFA concentrations of isobutyrate, isovalerate, and valerate were found in AGP+ compared to other treatments. On D42, lower levels of acetate, propionate, and butyrate were found in PI+ and PD+ compared to AGP+ and NC. Overall, the use of probiotics in this study had little effect on performance outcome, body composition, and ceca VFA levels, but it increased mRNA abundance of various immune markers in the gut throughout the experiment.

Keywords: probiotics, *in ovo*, broiler, necrotic enteritis, mRNA abundance, heat stress, VFA

Introduction

Antibiotic growth promoters (AGP) have been used to reduce disease and improve performance in livestock for decades (Dibner and Richards, 2005). Antibiotics are often broad spectrum, and do not target a single individual organism, but work to reduce numerous potentially harmful bacteria. By decreasing overall levels of bacteria in the gut, there may be increased nutrient availability to the host, since maintaining a large microbiome uses more dietary nutrients (Butaye et al., 2003). AGP improve body weight gains (BWG), reduce feed conversion ratios (FCR), and keep disease incidence lower, making livestock production more efficient and affordable (Izat et al., 1990; Belay and Teeter, 1996; Van Immerseel et al., 2009). However, concerns over antimicrobial resistance in human medicine have prompted both voluntary reduction and government regulation on the use of medically important antibiotics and AGP in animals (Castanon, 2007). In 2006, the European Union banned the use of AGP, and the Veterinary Feed Directive in the US has greatly raised restrictions on antibiotic use. As AGP in feed decreased, a rise in diseases soon followed (Van Immerseel et al., 2009; Veterinary Feed Directive, 2015).

One of the costliest diseases to the poultry industry is necrotic enteritis (NE), averaging $6 billion worldwide each year (Wade and Keyburn, 2015). It is caused by Gram-positive, spore-forming, *Clostridium perfringens* (CP) bacteria ubiquitous in the environment (Clavijo and Florez, 2018). This normal part of the intestinal microbiota can become an opportunistic pathogen, proliferating rapidly when pre-existing damage to the gut occurs, often caused by toxins in the feed, increased mucogenesis, increased digesta viscosity, intestinal dysbiosis from feed changes, lower pH, or parasites (Williams, 2005). One of the most common predisposing factors to NE in broilers is coccidiosis, the active disease caused by one or more *Eimeria* species (Cravens et al., 2013). These parasites invade enterocytes, reproduce within, and rupture the host cell upon exit, causing lesions and increasing mucus production in an attempt to flush out parasites. CP proliferates in the presence of increased mucus, anaerobic conditions, and nutrients leaking out of the host's ruptured cells (Collier et al., 2008). Toxin-producing strains cause intestinal lesions and damage the microvilli, inhibiting nutrient absorption, stunting growth, causing watery diarrhea, and death in severe cases (To et al., 2017).

Disease impairs the gastrointestinal tract's capacity to maintain homeostasis, which includes the ability to optimally digest and absorb nutrients, act as an effective barrier to the external environment, and balance potentially damaging inflammation resulting from normal reactions to disease (Broom and Kogut, 2018). Tight junction proteins, including claudin-1 (CLD1) are important to maintaining a tight barrier. These inter-epithelial connections function to seal the paracellular space between enterocytes and prevent pathogens from penetrating; however, CP toxins are capable of binding to these tight junction proteins and forming pores, breaking down the important barrier between internal and external environment (Mitchell and Koval, 2010; Robinson et al., 2015; Emami et al., 2019). In addition to the epithelial cells themselves, a

barrier of protective mucus produced by goblet cells lines the gastrointestinal tract (Turner, 2009; Robinson et al., 2015). When responding to bacterial diseases like NE, the intestine senses evolutionarily conserved patterns on the outer wall of bacteria, called microbe-associated molecular patterns (MAMPs) which can include lipopolysaccharides (LPS) and lipoproteins (Janeway and Medzhitov, 2002; Keestra et al., 2007, 2013). Transmembrane receptors on cells that monitor for MAMPs include Toll-like receptor 2 (TLR2) and TLR4. Activation of TLRs triggers innate immune reactions and an inflammatory response through an internal MyD88 signaling pathway that ultimately leads to production of pro-inflammatory cytokines including tumor necrosis factor (TNF)-α.

A large antimicrobial aspect of the innate immune system involves host defense peptides (HDPs), specifically avian β-defensins (AvBDs) and liver-expressed antimicrobial peptide-2 (LEAP2), cationic proteins capable of killing bacteria, fungi, parasites, and viruses (Xiao et al., 2004; Robinson et al., 2015; Su et al., 2017). They kill by disrupting the cell membrane, forming pores, and inhibiting pathogen protein, DNA, and RNA synthesis (Brogden, 2005). These AvBDs are expressed in epithelial cells of the skin, gastrointestinal, and respiratory tract of many animals and humans (Selsted and Ouellette, 2005). Activation of pathogen recognition receptors increases secretion of AvBDs and mucin, increasing the protective mucous layer and lining it with antimicrobial host defense peptides (Kabat et al., 2014; McDermott and Huffnagle, 2014).

Probiotic bacteria are considered one possible natural alternative to antibiotics in poultry that may modulate gastrointestinal microbial populations and immune responses (Cox and Dalloul, 2015; Calik et al., 2019). A probiotic is a live microorganism capable of surviving passage through the upper gastrointestinal tract, establishing itself in the lower gut, and providing benefits to the host (Fuller, 1989). Common species of probiotics known to provide a benefit

include those from *Lactobacillus*, *Bacteroides*, *Enterococcus*, and *Bifidobacteria* (Parvez et al., 2006). Commensal bacteria in the gut can competitively exclude pathogens by occupying attachments sites, producing bacteriocins, modulating the host immune system, producing pH-lowering substances (i.e. lactic acid), and making beneficial byproducts from fermentation (i.e. short-chain fatty acids) that the host can metabolize (Cherrington et al., 1990; Mountzouris et al., 2007, 2010; Gadde et al., 2017; Sobolewska et al., 2017). This study applied probiotics *in ovo* at E18 and evaluated its effectiveness against a NE challenge by measuring performance, lesion scores, body composition, mRNA abundance of various genes in the ileum and cecal tonsils, and concentrations of volatile fatty acids in the ceca.

Materials and Methods

Incubation Management and In Ovo Treatments

A 42-day study was conducted under the approval of IACUC # 18-136-APSC at the Virginia Tech turkey farm research facilities. A total of 1,630 fertilized Ross 708 broiler eggs were obtained from a commercial hatchery and were approximately 3 days old from a laying flock of 39 weeks in age. Embryonic day 0 (E0) egg weights were recorded, the egg flats were placed in incubators, and eggs were pre-warmed at 26.6℃ and 55% humidity for 10 hours. Next, incubators were set to industry standard temperature of 37.6℃ and 53% humidity until E18. Eggs were automatically turned at a 45˚ angle every 2 hours between E0-E18. On E17, egg flats were briefly removed from the incubator and candled to remove early dead and infertile ones. Prior to hatch, eggs were divided into two *in ovo* injection treatments with either sterile water or soluble synbiotic. At E18, eggs were individually *in ovo*-injected, transitioned to hatch trays, and returned to the incubator. Temperature was reduced to 35.6℃ and relative humidity increased to 75% until hatch.

Probiotic Preparation and In Ovo Injections

Sterile deionized water or a probiotic mixture were prepared just before injection. For the probiotic, 3 g water-soluble commercial probiotic was dissolved in 150 mL sterile water per manufacturer's recommendations to reach a dilution of 1 x 10^7 CFU. This dose was selected based on results from a previous experiment in Chapter 4. The probiotic contained a mixture of dried *Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus faecium*, and *Bifidobacterium thermophilum*.

For *in ovo* injection, one flat at a time (30 eggs/flat) was removed to reduce time out of the incubator. Each egg was candled, and the air cell outlined. The injection site was cleaned with 70% ethanol and 10% sodium hypochlorite. A small pilot hole was made in the center of the air cell with a short 18G needle. A smaller 22G needle 3.81 cm (1.5 inch) long attached to a 1 cc syringe was used to inject 100 µL sterile water or probiotic into the amniotic fluid of each egg. Each egg was individually injected perpendicular to the amniotic fluid. The sterile water treatment (1,084 eggs) was performed prior to the probiotic injections (542 eggs). The pilot hole was sealed with clean tape, and eggs were returned to the incubator. After all eggs were injected, they were transitioned to hatch trays at the same time. Treatments were kept separated from each other to reduce any cross-contamination of probiotic to the control group.

Post-Hatch Treatments and Bird Management

Treatments were further divided after hatch based on *in ovo* injection type, diet application, and presence or absence of NE challenge (Table 6.1). The five treatments were as follows; **NC:** sterile water *in ovo*, basal corn and soybean meal mash diet, no NE challenge; **AGP+**: sterile water *in ovo*, basal diet with virginiamycin as an antibiotic growth promoter (0.5 kg/MT), with NE challenge; **NC+**: same as NC plus NE challenge; **PI+**: probiotic *in ovo*, basal diet, with NE

challenge; **PD+**: sterile water *in ovo*, basal diet with probiotic in feed (1.3 kg/MT), with NE challenge. Originally, a 6th treatment was intended to include dietary and in ovo application of synbiotics with NE challenge (**PID+**), however, low hatchability of birds injected with probiotics eliminated this treatment in favor for more replicates of **PI+**. Diets were formulated to Ross nutritional recommendations. A mash starter diet was fed from DOH – D14, grower diet from D14 – D28, and a finisher diet from D28 – D42 (Table 6.2).

On DOH, birds were removed from hatch trays, inspected for health, feather-sexed, and placed in the appropriate treatment with a proportional number of males and females in each pen based off the sex ratio at hatch (Table 6.1). Each treatment contained 6 replicate pens (102 birds/treatment; 17 birds/replicate). Birds were housed in 2.4 x 1.2-meter concrete floor pens with fresh pine shavings, adjustable nipple drinkers and metal feeders. Water and feed were available ad libitum throughout the experiment. Temperature began at 36℃ and gradually adjusted down per industry standards until reaching 24℃ at D21 where it was maintained for the remainder of the experiment. Light was provided for 24 hours during the first week, and gradually adjusted down from D7 – D14 to 4 hours of dark and 20 hours of light where it remained for the remainder of the experiment.

Experimental NE Challenge Model

On DOH, shortly after bird placement, the Coccivac-B52 commercial coccidiosis vaccine containing live oocysts of *Eimeria acervulina*, *E. maxima*, *E. maxima MFP*, *E. mivati*, and *E. tenella* was sprayed on feed and litter of all treatments except NC at 11X the recommended dose to induce a naturally-occurring NE outbreak endemic to the poultry facility. The 11X dosage was chosen based on successful dosage levels used in various previous studies by the same lab in the same facility that yielded desired levels of a NE challenge. The unchallenged treatment pens

(NC) were placed at the front of the house, as separated as possible from challenged pens and upwind of the tunnel ventilation. Challenged treatment pens were placed in a randomized design throughout the house to reduce variability from location in the house. Lesion scores were recorded from 3 birds/pen during the peak of the outbreak (D7) using a 0-4 scale previously described (Prescott et al., 1978) with 0 = no lesions, 1 = thin-walled and friable intestines, 2 = focal necrosis or ulceration, 3 = large patches of necrosis 2-3 cm long, and 4 = severe necrosis. Each bird was euthanized by cervical dislocation and lesions were recorded from the duodenum, jejunum, and ileum.

Performance Measurements

Before incubation, eggs were arranged in flats to assure flat weights were similar to one another during incubation. Hatchability was recorded on DOH. Pen weights and feed weights were recorded on DOH, D7, D14, D28, and D42 to calculate average body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR). Observations and mortality were recorded twice daily to adjust performance measurements.

After *in ovo* injection on E18, incubator temperature became uncalibrated during the night. Actual temperature was higher than the display and set point, resulting in heat stress and a reduction in hatch of chicks that received probiotics. Treatments were equally divided to reflect the reduction in available birds for the experiment.

Body Composition

At the end of the experiment, D42 body composition was measured using Dual Energy X-Ray Absorptiometry (DXA). Lean and fat percentages were measured on individual birds (24 birds/treatment). Birds were first euthanized by cervical dislocation, their feathers plucked, and

carcasses chilled overnight until analysis of individual bird body mass, lean tissue mass, and fat tissue mass (GE Healthcare, Lunar Prodigy Advance program). Results are presented as average body weight, percent fat, and percent lean of birds in each treatment.

mRNA Abundance in Ileal and Cecal Tonsil Tissues

Ileal and cecal tonsil samples were taken at different time points during the experiment for mRNA abundance of Toll-like receptor (TLR) 2, TLR4, claudin-1 (CLD1), mucin 2 (MUC2), tumor necrosis factor-alpha (TNF-α), liver-expressed antimicrobial peptide 2 (LEAP2), avian β-defensin (AvBD) 8, AvBD10, AvBD13, and G-protein coupled receptor 43 (GPR43). On DOH, before bird placement, 8 birds of average weight were selected from the group that received sterile water *in ovo*, and 8 were selected from the synbiotic-injected group. On D7, D14, and D42, ileal and cecal tonsil samples were taken from 1 bird/pen. Since probiotics colonize the distal areas of the gut, sections of ileum and cecal tonsils were chosen to monitor potential effects in these areas. Birds were euthanized by cervical dislocation, a section of ileum or cecal tonsils was excised and gently rinsed out with cold PBS, immediately frozen on dry ice, and stored at −80°C until analysis. Tissue samples were homogenized using a TissueLyser (Qiagen) and RNA was extracted following standard manufacturer's protocol (Qiagen RNeasy Mini Kit). Optical density (OD) was used to assess RNA quality (OD 260/280) and concentration (OD 260) as measured by a NanoDrop-1000. The high-capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to make cDNA for qRT-PCR. Reactions (10 µL/well) were run in duplicate using Fast SYBR™ Green Master Mix on a 7500 FAST Real-Time PCR instrument (Applied Biosystems) with melt curves to determine product specificity. Primer details are provided in Table 6.3. Changes in mRNA abundance of each target gene were calculated using glyceraldehye-3-phosphate (GAPDH) as the endogenous control and the NC treatment as the

calibrator. Results for each treatment are reported as average mRNA abundance relative to GAPDH using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Quantification of Volatile Fatty Acids from Cecal Contents

To evaluate fatty acid composition in cecal contents and potential production of short-chain fatty acids (SCFA) by the injected or in-feed synbiotics, cecal content was collected from 1 bird/pen on D7, D14, and D42. After collection in sterile microcentrifuge tubes, samples were immediately frozen on dry ice before storage at −80°C until analysis. A gas chromatography-mass spectrophotometry (GC-MS) method was used based on procedures described by Richardson et al. (1989) and modifications from Morrison et al. (2004). In brief, samples were homogenized in a mixture of water and hydrochloric acid before centrifugation. The supernatant was placed in a new tube and vortexed with diethyl ether before centrifugation again. The resulting supernatant was placed in a new vial with acetonitrile and MTBSTFA (*N*-methyl-*N*-(*t*-butyldimethylsilyl)-trifluoroacetamide), and incubated for 1 hour at 80°C. Samples were cooled briefly and placed into gas chromatography vials for analysis. Results are presented as mmol/kg of sample.

Statistical Analysis

SAS JMP Pro 15 was used to perform one-way ANOVA using Fisher's least significant difference (LSD) method on all results except hatchability. Results are presented as least square means (LS Means) with standard error means (SEM). Significance was determined using student's *t*-test with $P \leq 0.05$ being significant and trends set at $0.05 \leq P \leq 0.10$.

Results

Performance Measurements

Hatchability and Mortality. Hatchability was not affected by *in ovo* application of water (86.42%) but those embryos receiving *in ovo* probiotics had 22% hatchability (Table 6.4). A temperature calibration error was detected and corrected, but the impact by heat stress in the incubators was enough to severely reduce hatchability. Mortality (Table 6.5) was significantly higher in PI+ birds from DOH-D7 ($P \leq 0.01$). No differences were observed after the first week, but from DOH-42, PI+ had overall higher mortality compared to other treatments ($P \leq 0.01$).

Body Weight. PI+ had higher BW ($P \leq 0.01$) on DOH compared to other treatments, but lower BW compared to all other treatments at D7, D14, and D28 (Table 6.6). On D7, NC had the highest BW, and NC+ was higher than both AGP+ and PD+ ($P \leq 0.01$). By D14, D28, and D42, BW of AGP+ birds was higher than all other treatments ($P \leq 0.01$).

Body Weight Gain. In Table 6.7, BWG per bird was significantly different between treatments at each time point measured ($P \leq 0.01$). PI+ had lower BWG compared to all other treatments from DOH-7, D7-14, and cumulatively, from DOH-42. From D14-28, PI+ was statistically similar to NC+, but all were lower than AGP+. During the last week, PI+ and PD+ gains were lower compared to NC, NC+, and AGP+. Overall from DOH-D42, AGP+ had the highest BWG, PI+ had the lowest, and PD+ was similar to NC+.

Feed Intake. There were significant differences in FI per bird between treatments at each time point measured ($P \leq 0.01$) yet there was no overall difference between treatments from DOH-42 due to the variation each week (Table 6.8). During the first week, FI was highest in non-challenged birds (NC). NC+ had higher FI than PI+ and AGP+. From D7-14, PI+ had

significantly higher FI than other treatments, and PD+ was higher than NC, NC+, and AGP+. In contrast, PI+ had lower FI compared to NC and AGP+ from D14-28 and D28-42.

Feed Conversion Ratio. Table 6.9 shows FCR throughout the study. From DOH-7, D7-14, and overall from DOH-42, PI+ and PD+ had higher FCR compared to NC and NC+ ($P \leq 0.01$). This differed from D14-28, where both PI+ and PD+ had lower FCR compared to NC+ ($P \leq 0.01$) and by D28-42, no differences were observed.

Lesion Scores

Table 6.10 reports D7 NE lesions from the duodenum, jejunum, and ileum. Lesions were most frequent in the duodenum, and least common in the ileum. There was no significant difference between treatments in the duodenum. In the jejunum, PI+ and PD+ had higher lesion scores compared to other treatments, and AGP+ and NC+ had higher scores than NC ($P \leq 0.01$). In the ileum, PI+ had higher lesions compared to NC, while PD+ was statistically similar to AGP+ and NC+ ($P \leq 0.05$).

Body Composition

The birds sampled for body composition showed no difference in % fat or % lean between treatments, but the weights were significantly different ($P \leq 0.01$) (Table 6.11). AGP+ had higher weight than PI+ and PD+. Both PI+ and PD+ were statistically similar to NC+.

mRNA Abundance

Ileum and Cecal Tonsils on DOH. The targets included TLR2, TLR4, CLD1, MUC2, TNF-α, LEAP2, AvBD8, AvBD10, AvBD13, and GPR43. In the ileum, only mRNA abundance of TLR2 was significantly different (Table 6.12a). Birds that received *in ovo* injections of water on E18

had higher TLR2 abundance than birds that received probiotic ($P \leq 0.05$). No differences were found in CT (Table 6.12b).

TLR2. Table 6.13a shows results for both CT and ileal mRNA abundance on D7, D14, and D42. No significant differences were found on D7 or D14. On D42, TLR2 abundance was lower in NC and PI+ compared to AGP+ ($P \leq 0.10$); however, PI+ had the greatest abundance in CT ($P \leq 0.05$).

TLR4. No differences were found in the ileum (Table 6.13b). On D14, PD+ had higher levels of TLR4 in CT compared to NC, NC+, and AGP+ ($P \leq 0.05$).

CLD1. As reported in Table 6.13c, there were no significant differences among treatment in CLD1 mRNA abundance.

MUC2. On D7, ileal mRNA abundance of MUC2 was higher ($P \leq 0.01$) in PI+ compared to other treatments (Table 6.13d). On D42, abundance was still greater ($P \leq 0.01$) in PI+ compared to other challenged treatments, but it was similar in NC. AGP+ had lower expression of MUC2 compared to NC and PI+. In the CT on D42, NC+ and PI+ had higher ($P \leq 0.05$) abundance compared to NC and AGP+.

TNF-α. Table 6.13e reports ileal and CT mRNA abundance on D7, D14, and D42. In the ileum, D14 abundance of TNF-α was decreased in challenged treatments compared to the non-challenged control ($P \leq 0.01$). By D42, ileal abundance was greatest in PD+ and PI+ compared to other treatments ($P \leq 0.01$). In the CT, D14 abundance was greater in PD+ compared to NC, NC+, and AGP+ ($P \leq 0.10$). On D42, CT abundance was greater in PI+ compared to other treatments ($P \leq 0.05$).

LEAP2. As shown in Table 6.13f, D7 ileal LEAP2 abundance was high in PI+ compared to NC, NC+, and AGP+ ($P \leq 0.05$), while NC and NC+ had lower abundance compared to both PD+ and PI+ on D7. On D42, ileal abundance was lower in PD+ and PI+ compared to other treatments ($P \leq 0.05$). On D14, there was a trend ($P \leq 0.10$) in the CT, in which AGP+ and PI+ had higher abundance relative to NC+. By D42, CT abundance was higher in both PD+ and PI+ compared to NC+ and AGP+ ($P \leq 0.05$).

AvBD. No significant differences were observed in mRNA abundance of AvBD8 (Table 6.13g), AvBD10 (Table 6.13h) or AvBD13 (Table 6.13i).

GPR43. In Table 6.13j, ileal GPR43 mRNA abundance was higher in NC+ than other treatments on D42 ($P \leq 0.05$). On D14, a trend was observed ($P \leq 0.10$) in the CT in which PD+ and PI+ had lower abundance compared to AGP+. Both PD+ and PI+ were similar to NC.

Volatile Fatty Acids in Cecal Contents

No differences were observed in VFA levels on D7 (Table 6.14a). On D14, AGP+ had significantly higher levels of isobutyrate ($P \leq 0.01$), isovalerate ($P \leq 0.01$), and valerate ($P \leq 0.01$) compared to all other treatments (Table 6.14b). Results for D42 are shown in Table 6.14c. Both NC and NC+ had higher levels of acetate compared to PI+ and PD+ ($P \leq 0.05$). Propionate and butyrate were higher in NC and AGP+ compared to PI+ and PD+ ($P \leq 0.05$; $P \leq 0.05$). Valerate tended to be lower in PI+ compared to NC and AGP+ ($P \leq 0.10$).

Discussion

In this study involving Ross broilers, probiotics were applied *in ovo* at E18 or supplemented in the feed after hatch to monitor their effects during a NE challenge induced by infection with *Eimeria*. Bird performance, lesion scores, mRNA abundance of various genes, and cecal VFAs were measured at multiple timepoints throughout the experiment. At the end, D42 body composition was measured by DXA for amount of lean and fat in carcasses.

Hatchability was not affected by *in ovo* injection with water, as supported by previous work in Chapter 4 and Chapter 5 of this dissertation, and by others (Li, 2018; Pender et al., 2016, 2017). Previous studies have documented normal hatchability using *Lactobacillus* strains, but decreased hatchability with increasing levels of *in ovo Bacillus subtilis* in the same experiment, leading to more late-embryo mortality and lower BW at hatch (Triplett et al., 2018). In this experiment, reduced hatchability in the probiotic group can be attributed to a period of heat stress on E19, rather than the probiotic injection itself. Incubator temperature was calibrated daily throughout the experiment; however, at some point during the transition on E18 after *in ovo* injections, the incubator malfunctioned resulting in chicks incubated at approximately 39.4°C, in contrast to the recommended 35.5°C temperature from E18-DOH. Hatchability from that incubator was around 22% and affected only the PI+ treatment. Eggs from other treatments were not affected by heat stress, since they hatched in a separate incubator, in an effort to reduce potential cross-contamination of probiotic bacteria into the treatments receiving water on E18.

Elevated incubator temperature from E14 to E17 decreased the size of the gizzard, proventriculus, small intestine, and heart (Leksrisompong et al., 2007). In agreement with this study, others reported high incubator temperature resulted in chicks that often have a whiter color due to poor absorption of yolk sacs, red hocks, low intestinal weights, unhealed navels,

poorly internalized yolk sacs, increased lethargy, and decreased BW at hatch (Gladys et al., 2000; Lourens et al., 2005; Barri, 2008). Yalcin and Siegel (2003) reported an average hatchability of 81.5% in an incubation temperature study that involved exposing incubating eggs to cooler (36.9°C) or warmer (39.6°C) temperatures for short periods (6 hours/day) prior to E18. Since the increase in temperature in our study occurred after E18, the effects were more severe. Usually during hatch, incubator temperatures are typically lowered.

Mortality was increased in PI+ (6%) compared to other treatments (<1.3%) during DOH-D7, and although subsequent weeks had similar mortalities between treatments, this difference was enough to result in significantly higher cumulative (DOH-42) mortality in PI+. The increased mortality is likely a residual effect of the heat stress from the incubator. As reported in Chapter 4, there were no mortalities recorded after *in ovo* injection of the same probiotic.

Spraying a high dose of live coccidiosis vaccine was able to induce NE in broilers, as indicated by moderate NE lesions in the duodenum and jejunum, but application of probiotic did not reduce lesion scores or lower mortality, although other studies have shown decreased lesions and mortality due to probiotic application (Pender et al., 2016; Emami et al., 2019). It is proposed that probiotics in the gut can reduce levels of pathogenic bacteria by lowering gut pH through production of lactic acid and SCFA, producing bacteriocins, and using up nutrients making them unavailable to pathogenic bacteria (Gadde et al., 2017).

DOH BW was higher in PI+, but this could be explained by the heat stress. In that treatment, it is likely that only the most fit chicks survived. Joseph et al. (2006) found that high incubation temperature during E18-DOH decreased chick yield and BWG during the first week after hatch. In Chapter 3, the same probiotic injection did not alter BW of chicks. Other studies found no change in DOH BW after *in ovo* injection of probiotics (Pender et al., 2016; Li, 2018). The NE

challenge lowered BW and BWG in all challenged treatments during the first week, but only the AGP+ was able to improve BW and BWG after the second week. Probiotic supplementation did not change cumulative FI or improve cumulative FCR. Interestingly, PI+ birds were able to catch up and had similar BWG as those in NC+ and PD+ groups during the 3rd and 4th weeks of the trial. It is likely that the early heat stress stunted growth in PI+, not the dietary probiotic, since PD+ was similar to the NC+ in most performance measurements. Other studies have found no change in FI after dietary probiotic application (Mookiah et al., 2013; Gohain and Sapcota, 1998; Willis et al., 2007; Yalcinkaya et al., 2008), no increase in BWG by dietary probiotic supplementation (Jung et al., 2008; Midilli et al., 2008), and Li (2018) found no significant effect on BW, BWG, or FI in broilers after *in ovo* injection with probiotics. In this study, probiotic supplementation did not change carcass composition, despite the high incubation temperature in PI+. Joseph et al. (2006) also reported no changes in carcass or processing yields compared to controls despite high incubation temperature prior to hatch.

Inflammation is an important and necessary response to disease, with the goal of identifying and clearing infection, while minimizing damage to the host (Broom and Kogut, 2018). In this study, mRNA abundance of certain immune markers was greater in PI+ and PD+ compared to controls. This increase could mean the birds had a more active immune response, though there was no decrease in lesion scores. However, this increase in immune activity could explain reduced performance in the PI+ and PD+ treatments. A robust immune reaction uses up nutritional resources and energy that would have otherwise gone towards growth (Gross and Siegel, 1997; Broom and Kogut, 2018).

In response to enteric disease, increased mucous production may help flush out parasites, damaged cells, and bacteria. Upregulation of a mucin-related gene, MUC2, in PI+ in the ILE on

D7 and D42 could provide protection to the bird by increased mucus production. Collier et al. (2008) showed that NE increased MUC2 gene expression, in an effort to increase the protective mucus layer, although mucus is also a nutritional substrate for CP. In this study, MUC2 abundance was higher in PI+, and so were NE lesions in the jejunum and ileum, suggesting increased mucus production may have increased the ability of CP to proliferate and cause damage.

In this study, TLR2 was lower on DOH by *in ovo* injection with probiotics, similar to the lower DOH TLR4 after synbiotic application in Chapter 4 of this dissertation. By D42, PI+ had greater TLR2 and PD+ had greater TLR4 in CT. Other studies have reported greater mRNA levels of TLR2 and TLR4 in broilers after probiotic supplementation (Bai et al., 2013). Increased TLR abundance triggers activation of the transcription factor NF-κB via MyD88-dependent pathways, promoting production of inflammatory cytokines, which may explain greater D42 TNF-α mRNA in the ileum of PI+ and PD+ birds, and in the CT of PI+ birds (Akira and Takeda, 2004; Wu et al., 2018). Another significant part of innate immunity are host defense peptides, highly conserved proteins secreted by numerous epithelial cells, including enterocytes, to protect against microbial infection (Brogden et al., 2003). They can distinguish between host cells and microbial cells based on differing cell membrane characteristics and kill microbial pathogens. Bacteria are killed in various ways, including pore formation in the membrane, blocking intracellular functions, and scrambling lipid distribution on the cell wall to interrupt normal membrane function (Zasloff, 2002). This study did not see a change in AvBD8, 10, or 13. During peak infection, both PI+ and PD+ led to greater LEAP2 mRNA compared to NC+, suggesting increased innate immune activation to NE; however, lesion scores were not reduced. LEAP2 mRNA was lower in PI+ and PD+ by D42, which contrasted D42 CT, where LEAP2 abundance

was higher than controls. Others have found consistent downregulation of LEAP2 in the small intestine after *Eimeria* infection (Casterlow et al., 2011; Sumners et al., 2011; Su et al., 2015, 2017). Tian et al. (2016) reported an upregulation of LEAP2 early during NE infection and a downregulation at later stages of infection as the infection began to resolve.

The probiotic in this study contained a mix of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus faecium*, and *Bifidobacterium thermophilum*. These bacteria produce SCFA, including butyrate, propionate, and acetate, as a byproduct of fermentation of non-starch polysaccharides, including sources such as inulin, oat bran, wheat bran, and cellulose (Sarra et al., 1992; Champ, 2004; Mishra et al., 2019). GPR43 is a SCFA receptor found concentrated in the ileum, close to the site of SCFA production in the ceca (Ang et al., 2015). The expression of this receptor may be directly regulated by levels of SCFA present, thus, if low levels of SCFA are being produced, GPR43 expression should be low (Venegas et al., 2019). This finding is supported by this study, in which low D14 (isobutyrate, isovalerate, and valerate) and D42 (acetate, propionate, butyrate, and valerate) levels of fatty acids coincided with significantly lower levels of GPR43 in D14 CT and D42 ileum. An unexpected finding in this study was the low levels of volatile fatty acids in PI+ and PD+ compared to AGP+ on D14 and D42. Probiotics are known to produce SCFA (Pessione, 2012; al-Khalaifah, 2018). Calik et al. (2017) recorded increasing concentrations of cecal butyrate with increasing levels of *Lactobacillus* measured in the ceca after probiotic supplementation. Another study found that feeding *Lactobacillus* in feed increased D42 levels of SCFA (Peng et al., 2016).

Overall, the *in ovo* or in-feed application of a probiotic during a NE challenge did not improve BW, BWG, FI, FCR, or carcass composition in comparison to controls. A moderate NE challenge was achieved after application of a live coccidiosis vaccine, as indicated by moderate

intestinal lesions. Intestinal mRNA abundance of TLR2, TLR4, MUC2, TNF-α, and LEAP2 were occasionally increased in probiotic-supplemented treatments. Measuring mRNA abundance does not always indicate protein levels, which would give better insight into whether the mRNA translated to actual function in the bird. Unexpectedly, volatile fatty acids were lower in probiotic-supplemented groups, which corresponded to low expression of SCFA receptor GPR43 in the ileum and CT. This general increase in immune activity in the intestine could explain the reduced performance in probiotic-supplemented birds, since mounting an immune response is costly and diverts resources away from growth. Future research should involve microbial quantification in the gut, to better understand the levels of different bacteria that contribute to the results of this study.

References

Al-Khalaifah, H.S. 2018. Benefits of probiotics and/or prebiotics for antibiotic-reduced poultry. Poult. Sci. 97:3807-3815.

Ang, Z. J.Z. Er, and J.L. Ding. 2015. The short-chain fatty acid receptor *GPR43* is transcriptionally regulated by XBP1 in human monocytes. Sci. Rep. 5:8134.

Bai, S.P., A.M. Wu, X.M. Ding, Y. Lei, J. Bai, Y.K. Zhang, and J.S. Chio. 2013. Effects of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens. Poult. Sci. 92:663-670.

Barri, A. 2008. Effects of incubation temperature and transportation stress on yolk utilization, small intestine development, and post-hatch performance of high-yield broiler chicks. Ph.D. Dissertation. Virginia Tech. Blacksburg, Virginia.

Beelay, T. and R.G. Teeter. 1996. Virginiamycin and caloric density effects on live performance, blood serum metabolite concentration, and carcass composition of broilers reared in thermoneutral and cycling ambient temperatures. Poult. Sci. 75:1383-1392.

Butaye, P., L.A. Devriese, and F. Haesebrouck. 2003. Antimicrobial growth promoters used in animal feed: effects of less well-known antibiotics on Gram-positive bacteria. Clin. Microbiol. Rev. 16:175-188.

Brogden, K.A., M. Ackermann, P.B. McCray, Jr., and B.F. Tack. 2003. Antimicrobial peptides in animals and their role in host defenses. Int. J. Antimicrob. Agents. 22:465-478.

Brogden, K.A. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3:238-250.

Broom, L.J. and M.H. Kogut. 2018. Inflammation: friend or foe for animal production? Poult. Sci. 97:510-514.

Calik, A., A. Ceylan, B. Ekim, S.G. Adabi, F. Dilber, A.G. Bayraktaroglu, T. Tekinay, D. Ozen, and P. Sacakli. 2017. The effect of intra-amniotic and posthatch dietary synbiotic administration on the performance, intestinal histomorphology, cecal microbial population, and short-chain fatty acid composition of broiler chickens. Poult. Sci. 96:169-183.

Calik, A., I.I. Omara, M.B. White, N.P. Evans, T.P. Karnezos, and R.A. Dalloul. 2019. Dietary non-drug feed additive as an alternative for antibiotic growth promoters for broilers during a necrotic enteritis challenge. Microorganisms 7:257.

Castanon, J.I. 2007. History of the use of antibiotic as growth promoters in European poultry feeds. Poult. Sci. 86:2466-2471.

Casterlow, S., H. Li, E.R. Gilber, R.A. Dalloul, A.P. McElroy, D.A. Emmerson, and E.A. Wong. 2011. An antimicrobial peptide is downregulated in the small intestine of *Eimeria maxima*-infected chickens. Poult. Sci. 90:1212-1219.

Champ, M.M.J. 2004. Physiological aspects of resistant starch and *in vivo* measurements. J. AOAC Int. 87:49-55.

Cherrington, C.A., M. Hinton, and I. Chopra. 1990. Effects of short-chain organic acids on macromolecular synthesis in *Escherichia coli*. J. Bacteriol. 68:69-74.

Clavijo, V. and M.J.V. Florez. 2018. The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. Poult. Sci. 97:1006-1021.

Collier, C.T., C.L. Hofacre, A.M. Payne, D.B. Anderson, P. Kaiser, R.I. Mackie, and H.R. Gaskins. 2008. Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting *Clostridium perfringens* growth. Vet. Immunol. Immunopathol. 122:104-115.

Cox, C.M. and R.A. Dalloul. 2015. Immunomodulatory role of probiotics in poultry and potential *in ovo* application. Benef. Microbes 6:45-52.

Cravens, R.L., G.R. Goss, F. Chi, E.D. De Boer, S.W. Davis, S.M. Hendrix, J.A. Richardson, and S.L. Johnston. 2013. The effects of necrotic enteritis, aflatoxin B_1, and virginiamycin on growth performance, necrotic enteritis lesion scores, and mortality in young broilers. Poult. Sci. 92:1997-2004.

Dibner, J.J. and J.D. Richards. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poult. Sci. 84:634-643.

Emami, N.K., A. Calik, M.B. White, M. Young, and R.A. Dalloul. 2019. Necrotic enteritis in broiler chickens: the role of tight junctions and mucosal immune responses in alleviating the effect of the disease. Microorganisms 7:231.

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. 66:365-78.

Gadde, U., W. Kim, S. Oh, and H. Lillehoj. 2017. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. Anim. Health Res. Rev. 18:1-20.

Gladys, G.E., D. Hill, R. Meijerhof, T.M. Saleh, and R.M. Hulet. 2000. Effect of embryo temperature and age of breeder flock on broiler post hatch performance. Poult. Sci. 79:123.

Gohain, A. and D. Sapcota. 1998. Effect of probiotic feeding on the performance of broilers. Indian J. Poult. Sci. 33:101-105.

Gross, W.B. and P.B. Siegel. 1997. Why some get sick. J. Appl. Poult. Res. 6:453-460.

Izat, A.L., M. Colberg, M.A. Reiber, M.H. Adams, J.T. Skinner, M.C. Cabel, H.L. Stilborn, and P.W. Waldroup. 1990. Effects of different antibiotics on performance, processing characteristics, and parts yield of broiler chickens. Poult. Sci. 69:1787-1791.

Janeway, C.A. and R. Medzhitov. 2002. Innate immune recognition. Annu. Rev. Immunol. 20:197-216.

Joseph, N.S., A. Lourens, and E.T. Moran, Jr. 2006. The effects of suboptimal eggshell temperature during incubation on broiler chick quality, live performance, and further processing yield. Poult. Sci. 85:932-938.

Jung, S.J., R. Houde, B. Baurhoo, X. Zhao, and B.H. Lee. 2008. Effects of galacto-oligosaccharides and a *Bifidobacteria lactis*-based probiotic strain on the growth performance and fecal microflora of broiler chickens. Poult. Sci. 87:1694-1699.

Keestra, A.M., M.R. de Zoete, L.I. Bouwman, M.M. Vaezirad, and J.P.M. van Putten. 2013. Unique features of the chicken Toll-like receptors. Dev. Comp. Immunol. 413:316-323.

Keestra, A.M., M.R. de Aoete, R.A.M.H. van Aubel, and J.P.M. van Putten. 2007. The central Leucine-rich repeat region of chicken TLR16 dictates unique ligand specificity and species-specific interaction with TLR2. J. Immunol. 178:7110-7119.

Leksrisompong, N., H. Romero-Sanchez, P.W. Plumstead, K.E. Brannan, and J. Brake. 2007. Broiler incubation. 1. Effect of elevated temperature during late incubation on body weight and organs of chicks. Poult. Sci. 86:2685-2691.

Li, L. 2018. Effect of *in ovo* injection of probiotic, prebiotic and synbiotic on growth performance and gut health parameters of broiler chickens. MS Thes. Univ. Hawaii. Manoa, Hawaii.

Livak, K.J. and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C(T)) Method. Methods 25:402-408.

Lourens, A., H. van den Brand, R. Meijerhof, and B. Kemp. 2005. Effect of eggshell temperature during incubation on embryo development, hatchability, and posthatch development. Poult. Sci. 84:914-920.

McDermott, A.J. and G.B. Huffnagle. 2014. The microbiome and regulation of mucosal immunity. Immunology 142:24-31.

Meslin, C., C. Desert, I. Callebaut, A. Djari, C. Klopp, F. Pitel, S. Leroux, P. Martin, P. Froment, and E. Guilbert. 2015. Expanding duplication of free fatty acid receptor-2 (GPR43) genes in the chicken genome. Genome Biol. Evol. 7:1332-1348.

Midilli, M., M. Alp, N. Kocabach, O.H. Muglah, N. Turan, H. Yilmaz, and S. Cakir. 2008. Effects of dietary probiotic and prebiotic supplementation on growth performance and serum IgG concentration of broilers. S. Afr. J. Anim. Sci. 38:21-27.

Mishra, A.K., S.S. Kumar, and A.R. Ghosh. 2019. Probiotic *Enterococcus faecalis* AG5 effectively assimilates cholesterol and produces fatty acids including propionate. FEMS Microbiol. Lett. 366:fnz039.

Mitchell, L.A. and M. Koval. 2010. Specificity of interaction between *Clostridium perfringens* enterotoxin and claudin-family tight junction proteins. Toxins 2:1595-1611.

Mookiah, S., C.C. Sieo, K. Ramasamy, N. Abdullah, and Y.W. Ho. 2014. Effects of dietary prebiotics, probiotic and synbiotics on performance, caecal bacterial populations and caecal fermentation concentrations of broiler chickens. J. Sci. Food Agric. 94:341-48.

Morrison, D.J., K. Cooper, S. Waldron, C. Slater, L.T. Weaver, and T. Preston. 2004. A streamlined approach to the analysis of volatile fatty acids and its application to the measurement of whole-body flux. Rapid Commun. Mass Spectrom. 18:2593-2600.

Parvez, S., K.A. Malik, S.A. Kang, and H.Y. Kim. 2006. Probiotics and their fermented food products are beneficial for health. J. Appl. Microbiol. 100:1171-1185.

Pender, C.M., S. Kim, T.D. Potter, M.M. Ritzi, M. Young, and R.A. Dalloul. Effects of *in ovo* supplementation of probiotics on performance and immunocompetence of broiler chicks to an *Eimeria* challenge. 2016. Ben. Microbes 7:699-705.

Pender, C.M., S. Kim, T.D. Potter, M.M. Ritzi, M. Young, and R.A. Dalloul. 2017. *In ovo* supplementation of probiotics and its effects on performance and immune-related gene expression in broiler chicks. Poult. Sci. 96:1052-1062.

Peng, Q., Z.F. Zeng, J.L. Zhu, S. Wang, X.T. Liu, C.L. Hou, P.A. Thacker, and S.Y. Qiao. 2016. Effects of dietary *Lactobacillus plantarum* B1 on growth performance, intestinal microbiota, and short chain fatty acid profiles in broiler chickens. Poult. Sci. 95:893-900.

Pessione, E. 2012. Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. Front. Cell Infect. Microbiol. 2:86.

Prescott, J.F., R. Sivendra, and D.A. Barnum. 1978. The use of bacitracin in the prevention and treatment of experimentally-induced necrotic enteritis in the chicken. Can. Vet. J. 19:181-183.

Richardson, A.J., A.G. Calder, C.S. Stewart., and A. Smith. 1989. Simultaneous determination of volatile and non-volatile acidic fermentation products of anaerobes by capillary gas chromatography. Lett. Appl. Microbiol. 9:5-8.

Robinson, K., Z. Deng, Y. Hou, and G. Zhang. 2015. Regulation of the intestinal barrier function by host defense peptides. Front. Vet. Sci. 2:57.

Sarra, P., L. Morelli, and V. Bottazzi. 1992. The lactic microflora of fowl. The Lactic Acid Bacteria, Volume 1, Springer, Boston, MA. Pp. 3-19.

Selsted, M.E. and A.J. Ouellette. 2005. Mammalian defensins in the antimicrobial immune system. Nat. Immunol. 6:551-57.

Sobolewska, A., G. Elminowska-Wenda, J. Bogucka, A. Dankowiakowska, A. Kulakowska, A. Szczerba, K. Stadnicka, M. Szpinda, and M. Bednarczyk. 2017. The influence of *in ovo* injection with the prebiotic DiNovo® on the development of histomorphological parameters of the duodenum, body mass and productivity in large-scale poultry production conditions. J. An. Sci. Biotech. 8:45.

Su, S., K.B. Miska, R.H. Fetterer, M.C. Jenkins, and E.A. Wong. 2015. Expression of digestive enzymes and nutrient transporters in Eimeria-challenged broilers. Exp. Parasitol. 150:13-21.

Su, S., D.M. Dwyer, K.B. Miska, R.H. Fetterer, M.C. Jenkins, and E.A. Wong. 2017. Expression of host defense peptides in the intestine of *Eimeria*-challenged chickens. Poult. Sci. 96:2421-2427.

Sumners, L.H., K.B. Miska, M.C. Jenkins, R.H. Fetterer, C.M. Cox, S. Kim, and R.A. Dalloul. 2011. Expression of Toll-like receptors and antimicrobial peptides during *Eimeria praecox* infection in chickens. Exp. Parasitol, 127:714-718.

Tian, X., Y. Shao, Z. Wang, and Y. Guo. 2016. Effects of dietary yeast B-glucans supplementation on growth performance, gut morphology, intestinal Clostridium perfringens population and immune response of broiler chickens challenged with necrotic enteritis. Anim. Feed Sci. Tech. 215:144-155.

To, H., T. Suzuki, F. Kawahara, K. Uetsuka, S. Nagai, and T. Nunoya. 2017. Experimental induction of necrotic enteritis in chickens by a netB-positive Japanese isolate of *Clostridium perfringens*. J. Vet. Med. Sci. 79:350-358.

Triplett, M.D., W. Zhai, E.D. Peebles, C.D. McDaniel, and A.S. Kiess. 2018. Investigating commercial *in ovo* technology as a strategy for introducing probiotic bacteria to broiler embryos. Poult. Sci. 97:658-666.

Van Immerseel, F., J.L. Rood, R.J. Moore, and R.W. Titball. 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends Microbiol. 17:32-36.

Venegas, D.P., M.K. De la Fuente, G. Landskron, M.J. Gonzalez, R. Quera, G. Dijkstra, H.J.M. Harmsen, K.N. Faber, and M.A. Hermoso. 2019. Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. Front. Immunol. 10:227.

Veterinary Feed Directive: A Rule by the Food and Drug Administration on 06/03/2015. 2015. Pages 31707-31735. Published by the Food and Drug Administration, HHS. https://www.federalregister.gov/documents/2015/06/03/2015-13393/veterinary-feed-directive.

Wade, B. and A. Keyburn. 2015. The true cost of necrotic enteritis. Poultry World – Meat. Accessed April 18, 2021.

Williams, R.B. 2005. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Av. Pathol. 34:159-180.

Willis, W., O. Isikhuemhen, and S. Ibrahim. 2007. Performance assessment of broiler chickens given mushroom extract alone or in combination with probiotics. Poult. Sci. 86:1856-1860.

Xiao, Y., A.L. Hughes, J. Ando, Y. Matsuda, J.F. Cheng, D. Skinner-Noble, and G. Zhang. 2004. A genome-wide screen identifies a single β-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. BMC Genomics 5:56.

Yalcin, S. and P.B. Siegel. 2003. Exposure to cold or heat during incubation on developmental stability of broiler embryos. Poult. Sci. 82:1388-1392.

Yalcinkaya, I., T. Gungor, and M. Basalan. 2008. Effects of mannanoligosaccharide (MOS) from *Saccharomyces cerevisiae* on some internal, gastrointestinal and carcass parameters in broilers. J. Anim. Vet. Adv. 7:789-792.

Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. Nature. 415:389-395.

Table 6.1. Treatment Groups.

[1]Trt	Diet	*In ovo* Application	NE Challenge	Reps	Animals/ Rep	Animals/ Trt
1. NC	Control = basal (standard corn/soy) diets	100µL water	-	6	17	102
2. AGP+	Control w/ virginiamycin	100µL water	+	6	17	102
3. NC+	Control	100µL water	+	6	17	102
4. PI+	Control	100µL probiotic	+	6	17	102
5. PD+	Control w/ probiotic	100µL water	+	6	17	102

[1]Treatments: NC = basal diet, sterile water injection, no NE challenge; AGP+ = basal diet with virginiamycin (0.5 kg/MT), sterile water injection, NE challenge; NC+ = basal diet, sterile water injection, NE challenge; PI+ = basal diet, probiotic *in ovo* injection, NE challenge; PD+ = basal diet with probiotic (1.3 kg/MT), sterile water injection, NE challenge.

Table 6.2. Feed Composition of broiler mash starter diet (0-14 d), grower mash diet (14-28 d), and finisher mash diet (28-42 d).

Ingredient, g/kg	0-14 d	14-28 d	28-42 d
Corn	56.86	60.75	65.19
Soybean meal, CP 48%	35.63	32.62	27.55
Vegetable oil	2.18	2.40	3.60
Limestone	1.15	0.80	0.60
Dicalcium phosphate	2.25	1.90	1.80
DL-Methionine (98%)	0.45	0.25	0.20
L-Lysine HCl (78%)	0.40	0.34	0.12
L-Threonine	0.22	0.05	0.04
Salt	0.15	0.25	0.25
Sodium Bicarbonate	0.35	0.35	0.35
Vitamin-Mineral Premix (NB3000)*	0.36	0.30	0.30
Total	100.00	100.00	100.00
Chemical composition (Calculated)			
Dry Matter, %	87.11	87.14	87.16
Crude Protein, %	21.36	20.23	18.08
AME_n, kcal/kg	2962	3050	3190
Lysine, %	1.41	1.29	1.15
Dig. Lysine, %	1.25	1.15	0.99
Methionine + cysteine, %	1.09	0.97	0.91
Dig. Methionine + cysteine, %	0.96	0.86	0.80
Threonine, %	0.98	0.88	0.82
Dig. Threonine, %	0.87	0.78	0.72
Calcium, %	1.05	0.82	0.81
Available phosphorus, %	0.56	0.50	0.47

*Vitamins per kg diet: retinol 3.33 mg, cholecalciferol 0.1 mg, α-tocopherol acetate 23.4 mg, vitamin K3 1.2 mg, vitamin B1 1.6 mg, vitamin b2 9.5 mg, niacin 40 mg, pantothenic acid 9.5 mg, vitamin B6 2 mg, folic acid 1 mg, vitamin B12 0.016 mg, biotin 0.05 mg, choline 556 mg. Minerals per kg diet: Mn 144 mg, Fe 72 mg, Zn 144 mg, Cu 16.2 mg, I 2.1 mg, Se 0.22 mg.

Table 6.3. Primers used for qRT-PCR mRNA abundance. Forward primer sequence is listed before reverse primer sequence.

Gene[a]	Primer Sequence (5' – 3')	Accession Number
GAPDH	CCT AGG ATA CAC AGA GGA CCA GGT T GGT GGA GGA ATG GCT GTC A	NM_204305
TLR2	GCG AGC CCC CAC GAA GGA GTC GTT CTC ACT GTA GGA GAC A	NM_204278
TLR4	CCA CAC ACC TGC CTA CAT GAA GGA TGG CAA GAG GAC ATA TCA AA	NM_001030693
CLD1	GTG TTC AGA GGC ATC AGG TAT C CTC AGG TCA AAC AGA GGT ACA A	NM_001013611.2
MUC2	TTC ATG ATG CCT GCT CTT GTG CCT GAG CCT TGG TAC ATT CTT GT	XM_421035
TNF-α	CCC ATC CCT GGT CCG TAA C ATA CGA AGT AAA GGC CGT CCC	MF000729.1
LEAP2	TGT GCT TCC CTG CAC CAA GGC GTC ATC CGC TTC AGT	AY534899
AvBD8	ATG CGC GTA CCT AAC AAC GA TGC CCA AAG GCT CTG GTA TG	NM_001001781.1
AvBD10	CAG ACC CAC TTT TCC CTG ACA CCC AGC ACG GCA GAA ATT	NM_00100609.2
AvBD13	CAG CTG TGC AGG AAC AAC CA CAG CTC TCC ATG TGG AAG CA	NM_001001780.1
GPR43	GCC CCA TAG CAA ACT TCT GGG CAG CCA TAA AGA GAG	Meslin et al., 2015

[a] ***GAPDH*** *= Glyceraldehyde 3-phosphate dehydrogenase.* ***TLR*** *= Toll-like receptor.* ***CLD1*** *= Claudin-1.* ***MUC2*** *= Mucin 2.* ***TNF-α*** *= Tumor necrosis factor alpha.* ***LEAP2*** *= Liver-expressed antimicrobial peptide 2.* ***AvBD*** *= Avian β-defensin.* ***GPR43*** *= G-protein coupled receptor 43.*

Table 6.4. Effect of treatment on hatchability.

	No. Eggs Hatched/Eggs Placed	% Hatchability
[1]**Trt**	DOH	DOH
Water	1114/1289	86.42
Probiotic	112/515	22.00

[1]Treatments: Water = Sterile water *in ovo* treatment. Probiotic = Probiotic *in ovo* treatment. Data presented as number of eggs that hatched per number of eggs placed at start.

Table 6.5. Effect of treatment on mortality (%).

	Mortality (%)				
[1]**Trt**	DOH-7	D7-14	D14-28	D28-42	D0-42
1. NC	**0.4**[b]	0.0	0.5	0.0	**0.9**[b]
2. AGP+	**1.3**[b]	0.0	0.0	0.5	**1.7**[b]
3. NC+	**0.4**[b]	0.0	0.0	0.0	**0.4**[b]
4. PI+	**6.3**[a]	0.0	1.9	0.0	**7.4**[a]
5. PD+	**0.0**[b]	0.0	0.0	1.4	**1.0**[b]
	P ≤ 0.01	*N/A*	*P > 0.10*	*P > 0.10*	***P ≤ 0.01***
	[2]SEM = 1.0	*SEM = 0.0*	*SEM = 0.7*	*SEM = 0.6*	***SEM = 1.2***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.6. Effect of treatment on body weight (BW) per bird.

	Body Weight (g/bird)				
[1]**Trt**	DOH	D7	D14	D28	D42
1. NC	39.7^{b}	133.7^{a}	356.0^{a}	1215.8^{b}	2680.6^{b}
2. AGP+	39.6^{b}	112.9^{c}	344.6^{ab}	1321.8^{a}	2804.5^{a}
3. NC+	39.5^{b}	125.8^{b}	324.9^{b}	1151.0^{c}	2573.8^{c}
4. PI+	41.8^{a}	86.4^{d}	263.5^{c}	1067.6^{d}	2423.3^{d}
5. PD+	39.1^{b}	109.4^{c}	318.8^{b}	1202.1^{bc}	2527.0^{cd}
	P ≤ 0.01	*P ≤ 0.01*	*P ≤ 0.01*	*P ≤ 0.01*	*P ≤ 0.01*
	[2]SEM = 0.4	*SEM = 2.7*	*SEM = 9.8*	*SEM = 23.4*	*SEM =37.9*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.7. Effect of treatment on body weight gain (BWG) per bird.

	Body Weight Gain (g/bird)				
[1]**Trt**	DOH-7	D7-14	D14-28	D28-42	DOH-42
1. NC	94[a]	206[ab]	830[b]	1459[a]	2634[b]
2. AGP+	73[c]	215[a]	948[a]	1483[a]	2765[a]
3. NC+	86[b]	186[b]	798[bc]	1423[a]	2534[bc]
4. PI+	45[d]	146[c]	758[c]	1333[b]	2359[d]
5. PD+	70[c]	186[b]	830[b]	1325[b]	2488[c]
	P ≤ 0.01	*P ≤ 0.01*	*P ≤ 0.01*	*P ≤ 0.01*	*P ≤ 0.01*
	[2]SEM = 3	*SEM = 8*	*SEM = 15*	*SEM = 23*	*SEM = 38*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.8. Effect of treatment on feed intake (FI) per bird.

	Feed Intake (g/bird)				
[1]**Trt**	DOH-7	D7-14	D14-28	D28-42	DOH-42
1. NC	**130**[a]	**298**[c]	**1312**[ab]	**2397**[a]	4137
2. AGP+	**110**[cd]	**275**[c]	**1359**[a]	**2381**[a]	4125
3. NC+	**124**[ab]	**260**[c]	**1277**[b]	**2328**[a]	3988
4. PI+	**101**[d]	**786**[a]	**1175**[c]	**2148**[b]	4209
5. PD+	**116**[bc]	**688**[b]	**1244**[bc]	**2168**[b]	4215
	P ≤ 0.01	***P ≤ 0.01***	***P ≤ 0.01***	***P ≤ 0.01***	*P > 0.10*
	[2]SEM = 3	***SEM = 22***	***SEM = 24***	***SEM = 36***	*SEM = 69*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.9. Effect of treatment on feed conversion ratio (FCR) per bird.

	Feed Conversion Ratio (per bird)				
[1]**Trt**	DOH-7	D7-14	D14-28	D28-42	DOH-42
1. NC	**1.4^{c}**	**1.5^{c}**	**1.6^{ab}**	1.6	**1.6^{c}**
2. AGP+	**1.5^{bc}**	**1.3^{c}**	**1.4^{d}**	1.6	**1.5^{d}**
3. NC+	**1.4^{c}**	**1.4^{c}**	**1.6^{a}**	1.6	**1.6^{c}**
4. PI+	**2.3^{a}**	**5.5^{a}**	**1.6^{b}**	1.6	**1.8^{a}**
5. PD+	**1.7^{b}**	**3.7^{b}**	**1.5^{c}**	1.6	**1.7^{b}**
	P ≤ 0.01	***P ≤ 0.01***	***P ≤ 0.01***	*P > 0.10*	***P ≤ 0.01***
	[2]SEM = 0.07	***SEM = 0.18***	***SEM = 0.01***	*SEM = 0.02*	***SEM = 0.01***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.10. Effect of treatment on D7 NE lesion scores.

	D7 NE Lesion Scores (*n* = 18 birds/trt)		
[1]**Trt**	Duodenum	Jejunum	Ileum
1. NC	**0.5[b]**	**0.1[c]**	**0.0[c]**
2. AGP+	**1.0[a]**	**0.4[b]**	**0.1[bc]**
3. NC+	**1.0[a]**	**0.6[b]**	**0.1[bc]**
4. PI+	**1.2[a]**	**0.9[a]**	**0.3[a]**
5. PD+	**1.3[a]**	**0.9[a]**	**0.2[ab]**
	P ≤ 0.10	***P ≤ 0.01***	***P ≤ 0.01***
	[2]SEM = 0.13	***SEM = 0.09***	***SEM = 0.05***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.11. Effect of treatment on D42 body composition using DXA.

	D42 DXA Analysis (*n* = 18 birds/trt)		
[1]**Trt**	Bird Wt (g)	% Fat per Bird	% Lean per Bird
1. NC	**2658**[ab]	17	82
2. AGP+	**2799**[a]	17	82
3. NC+	**2599**[abc]	17	82
4. PI+	**2390**[c]	16	83
5. PD+	**2463**[bc]	16	83
	P ≤ 0.01	*P = 0.182*	*P = 0.192*
	[2]***SEM = 77***	*SEM = 0.4*	*SEM = 0.4*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.12a. Effect of probiotic application method on DOH mRNA levels in the ileum.

DOH Ileum (*n* = 8 birds/trt)										
[1]Trt	TLR2	TLR4	CLD1	MUC2	TNFa	LEAP2	AvBD8	AvBD10	AvBD13	GPR43
Water	**1.04**[a]	1.08	1.45	1.24	1.88	1.84	0.43	3.31	1.08	1.11
Prob.	**0.30**[b]	0.54	1.88	1.70	1.22	3.91	0.15	1.78	1.59	0.37
P-value	***≤0.05***	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*
[2]*SEM*	***0.12***	*0.27*	*0.56*	*0.43*	*1.05*	*1.05*	*0.11*	*6.06*	*0.55*	*0.22*

[1]Treatments: Water = *In ovo* injection with sterile water on E18; Prob. = *In ovo* injection with probiotic on E18. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.12b. Effect of probiotic application method on DOH mRNA levels in the cecal tonsils.

DOH Cecal Tonsils (*n* = 8 birds/trt)										
[1]Trt	TLR2	TLR4	CLD1	MUC2	TNFa	LEAP2	AvBD8	AvBD10	AvBD13	GPR43
Water	1.10	1.14	1.19	1.06	1.18	1.60	0.43	2.95	1.48	1.18
Prob.	0.63	0.84	1.56	2.57	0.69	0.70	0.25	4.98	0.71	0.71
P-value	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*	*>0.10*
[2]*SEM*	*0.22*	*0.26*	*0.74*	*0.94*	*0.31*	*0.47*	*0.08*	*3.26*	*0.60*	*0.60*

[1]Treatments: Water = *In ovo* injection with sterile water on E18; Prob. = *In ovo* injection with probiotic on E18. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.13a. Effect of probiotic application method on TLR2 mRNA levels in the ileum and cecal tonsils at three time periods.

TLR2 Fold Change (n = 6 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.02	1.54	**0.71^{c}**	1.27	1.11	**0.71^{b}**
NC+	0.94	2.00	**1.67^{ab}**	2.21	0.73	**0.82^{b}**
AGP+	0.75	0.90	**1.69^{a}**	4.80	0.77	**0.63^{b}**
PI+	0.76	1.42	**0.69^{bc}**	36.04	0.56	**1.47^{a}**
PD+	0.92	1.47	**1.54^{abc}**	1274.52	0.60	**0.74^{b}**
	P > 0.10	*P* > 0.10	***P* ≤ *0.01***	*P* > 0.10	*P* > 0.10	***P* ≤ 0.05**
	[2]SEM = 0.29	*SEM = 0.49*	***SEM = 0.33***	*SEM = 507.76*	*SEM = 0.16*	***SEM = 0.19***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.13b. Effect of probiotic application method on TLR4 mRNA levels in the ileum and cecal tonsils at three time periods.

TLR4 Fold Change (n = 6 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.29	1.49	0.43	0.98	**1.07^{b}**	0.70
NC+	4.06	1.63	0.97	16.96	**2.04^{b}**	0.82
AGP+	5.58	0.78	0.78	0.78	**0.82^{b}**	0.69
PI+	19.47	0.99	0.45	4.72	**3.25^{ab}**	0.91
PD+	6.52	1.37	0.87	12.95	**6.94^{a}**	0.96
	P > 0.10	*P* > 0.10	*P* > 0.10	*P* > 0.10	***P* ≤ 0.05**	*P* > 0.10
	[2]SEM = 4.89	*SEM = 0.41*	*SEM = 0.17*	*SEM = 6.87*	***SEM = 1.28***	*SEM = 0.17*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.13c. Effect of probiotic application method on CLD1 mRNA levels in the ileum and cecal tonsils at three time periods.

CLD1 Fold Change (n = 6 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.10	1.36	0.71	1.76	1.15	0.74
NC+	1.46	1.38	1.61	2.02	2.54	0.85
AGP+	1.93	0.68	0.87	1.22	3.27	0.99
PI+	1.85	1.34	1.13	15.00	1.16	0.68
PD+	0.77	1.16	1.79	11.45	1.85	0.78
	P > 0.10	*P* > 0.10	*P* > 0.10	*P* > 0.10	*P* > 0.10	*P* > 0.10
	[2]SEM = 0.58	*SEM = 0.37*	*SEM = 0.33*	*SEM = 5.20*	*SEM = 0.91*	*SEM = 0.21*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.13d. Effect of probiotic application method on MUC2 mRNA levels in the ileum and cecal tonsils at three time periods.

MUC2 Fold Change (n = 6 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	**1.15^{b}**	1.02	**0.75^{ab}**	1.08	1.39	**0.57^{b}**
NC+	**1.14^{b}**	1.07	**0.46^{bc}**	1.56	1.41	**1.52^{a}**
AGP+	**1.06^{b}**	0.89	**0.34^{c}**	4.66	0.57	**0.53^{b}**
PI+	**10.12^{a}**	1.53	**0.95^{a}**	5.67	0.86	**1.50^{a}**
PD+	**3.98^{b}**	1.54	**0.57^{bc}**	2.77	1.10	**0.98^{ab}**
	***P* ≤ 0.01**	*P* > 0.10	***P* ≤ 0.01**	*P* > 0.10	*P* > 0.10	***P* ≤ 0.05**
	[2]SEM = 1.70	*SEM = 0.25*	***SEM = 0.12***	*SEM = 2.39*	*SEM = 0.33*	***SEM = 0.28***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.13e. Effect of probiotic application method on TNF-α mRNA levels in the ileum and cecal tonsils at three time periods.

TNF-α Fold Change (*n* = 6 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.33	**1.48^{a}**	**0.72^{b}**	1.61	**1.29^{b}**	**0.77^{b}**
NC+	4.80	**0.36^{b}**	**0.99^{b}**	7.78	**1.07^{b}**	**0.88^{b}**
AGP+	1.06	**0.20^{b}**	**0.94^{b}**	9.13	**0.81^{b}**	**0.79^{b}**
PI+	9.66	**0.22^{b}**	**1.85^{a}**	1.43	**2.57^{ab}**	**2.29^{a}**
PD+	3.61	**0.28^{b}**	**1.57^{a}**	5.70	**3.65^{a}**	**1.01^{b}**
	P > 0.10	***P* ≤ *0.01***	***P* ≤ *0.01***	*P* > 0.10	***P* ≤ *0.10***	***P* ≤ 0.05**
	[2]SEM = 2.40	***SEM = 0.21***	***SEM = 0.19***	*SEM = 3.95*	***SEM = 0.76***	***SEM = 0.33***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.13f. Effect of probiotic application method on LEAP2 mRNA levels in the ileum and cecal tonsils at three time periods.

LEAP2 Fold Change (*n* = 6 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	**1.49^{c}**	1.18	**0.72^{a}**	1.36	**1.20^{ab}**	**0.57^{bc}**
NC+	**1.44^{c}**	1.12	**0.71^{a}**	1.28	**1.56^{a}**	**0.57^{c}**
AGP+	**1.73^{bc}**	0.62	**0.75^{a}**	0.49	**0.63^{b}**	**0.50^{c}**
PI+	**5.75^{a}**	1.05	**0.35^{b}**	3.98	**0.67^{b}**	**1.54^{ab}**
PD+	**5.52^{ab}**	0.97	**0.34^{b}**	7.55	**1.37^{ab}**	**1.61^{a}**
	***P* ≤ 0.05**	*P* > 0.10	***P* ≤ 0.05**	*P* > 0.10	***P* ≤ *0.10***	***P* ≤ 0.05**
	[2]SEM = 1.27	*SEM = 0.18*	***SEM = 0.11***	*SEM = 2.15*	***SEM = 0.29***	***SEM = 0.32***

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.13g. Effect of probiotic application method on AvBD8 mRNA levels in the ileum and cecal tonsils at three time periods.

AvBD8 Fold Change (n = 6 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.38	1.63	0.76	3.76	1.04	1.02
NC+	1.55	2.45	1.50	3647.56	6.50	1.36
AGP+	1.36	1.01	1.46	4360.88	3.19	1.93
PI+	1.06	1.41	0.73	1443.18	23.55	0.40
PD+	1.45	2.79	1.26	1.72	7.44	1.21
	$P > 0.10$	$P > 0.10$	$P > 0.10$	$P > 0.10$	$P > 0.10$	$P > 0.10$
	[2]SEM = 0.40	*SEM = 0.67*	*SEM = 0.28*	*SEM = 2435.19*	*SEM = 10.54*	*SEM = 0.85*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.13h. Effect of probiotic application method on AvBD10 mRNA levels in the ileum and cecal tonsils at three time periods.

AvBD10 Fold Change (n = 6 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.61	1.92	0.92	1.07	1.40	1.32
NC+	3.14	2.02	2.49	24.04	5.49	0.67
AGP+	1.74	1.00	1.42	5.04	3.37	0.27
PI+	3.30	1.38	0.54	7.67	11.81	0.27
PD+	3.06	1.91	2.16	24.24	5.10	1.61
	$P > 0.10$	$P > 0.10$	$P > 0.10$	$P > 0.10$	$P > 0.10$	$P > 0.10$
	[2]SEM = 0.85	*SEM = 0.67*	*SEM = 0.58*	*SEM = 10.92*	*SEM = 3.30*	*SEM = 0.46*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.13i. Effect of probiotic application method on AvBD13 mRNA levels in the ileum and cecal tonsils at three time periods.

AvBD13 Fold Change (n = 6 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.43	2.31	0.85	1.40	1.14	1.47
NC+	1.83	1.79	2.34	0.61	5.33	1.54
AGP+	0.65	0.09	1.24	124.81	5.61	1.92
PI+	3.18	1.24	1.55	553.59	2.66	0.27
PD+	1.78	2.03	1.86	578.60	3.97	1.35
	P > 0.10	*P* > 0.10	*P* > 0.10	*P* > 0.10	*P* > 0.10	*P* > 0.10
	[2]SEM = 0.83	*SEM = 0.76*	*SEM = 0.51*	*SEM = 297.89*	*SEM = 1.64*	*SEM = 0.53*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.13j. Effect of probiotic application method on GPR43 mRNA levels in the ileum and cecal tonsils at three time periods.

GPR43 Fold Change (n = 6 birds/trt)						
	Ileum			Cecal Tonsils		
[1]Trt	D7	D14	D42	D7	D14	D42
NC	1.25	1.62	**0.55^{b}**	1.67	**1.19^{abc}**	0.67
NC+	1.73	1.93	**3.62^{a}**	0.98	**2.57^{ab}**	1.03
AGP+	2.28	0.44	**1.10^{b}**	12.53	**3.14^{a}**	0.71
PI+	1.59	2.60	**1.44^{b}**	204.45	**0.71^{bc}**	0.77
PD+	2.92	0.91	**0.32^{b}**	174.46	**0.33^{c}**	0.75
	P > 0.10	*P* > 0.10	***P* ≤ 0.05**	*P* > 0.10	***P* ≤ 0.10**	*P* > 0.10
	SEM = 1.00	*SEM = 0.74*	***SEM = 0.74***	*SEM = 86.38*	***SEM = 0.75***	*SEM = 0.12*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.14a. Effect of probiotic application method on cecal fatty acid measurements on D7.

D7 Cecal Content: Fatty Acids (mmol/kg sample) (*n* = 6 birds/trt)						
[1]Trt	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate
NC	82	2	6	16	5	2
NC+	63	2	4	14	3	1
AGP+	68	1	5	14	4	2
PI+	101	2	5	18	6	4
PD+	98	2	4	12	5	3
	P > 0.10 [2] *SEM = 21*	*P* > 0.10 *SEM = 0.4*	*P* > 0.10 *SEM = 1*	*P* > 0.10 *SEM = 4*	*P* > 0.10 *SEM = 1*	*P* > 0.10 *SEM = 0.9*

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.14b. Effect of probiotic application method on cecal fatty acid measurements on D14.

D14 Cecal Content: Fatty Acids (mmol/kg sample) (*n* = 6 birds/trt)						
[1]Trt	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate
NC	68	3	**6[b]**	32	**4[b]**	**9[b]**
NC+	73	3	**5[b]**	43	**4[b]**	**11[b]**
AGP+	81	4	**16[a]**	48	**17[a]**	**18[a]**
PI+	58	3	**5[b]**	25	**5[b]**	**6[b]**
PD+	80	3	**5[b]**	43	**3[b]**	**10[b]**
	P > 0.10 [2] *SEM* = 8	*P* > 0.10 *SEM* = 1	***P ≤ 0.01*** ***SEM* = 2**	*P* > 0.10 *SEM* = 7	***P ≤ 0.01*** ***SEM* = 3**	***P ≤ 0.01*** ***SEM* = 2**

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

Table 6.14c. Effect of probiotic application method on cecal fatty acid measurements on D42.

D42 Cecal Content: Fatty Acids (mmol/kg sample) (*n* = 6 birds/trt)						
[1]Trt	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate
NC	**236**[a]	**14**[a]	26.38	**197**[a]	15	**45**[a]
NC+	**246**[a]	**9**[ab]	16.55	**172**[ab]	11	**32**[ab]
AGP+	**210**[ab]	**13**[a]	30.89	**178**[a]	26	**45**[a]
PI+	**139**[b]	**8**[b]	22.01	**93**[c]	22	**26**[b]
PD+	**148**[b]	**8**[b]	16.80	**94**[bc]	15	**27**[ab]
	***P* ≤ 0.05** [2] ***SEM* = 29**	***P* ≤ 0.05** ***SEM* = 2**	*P* > 0.10 *SEM* = 4	***P* ≤ 0.05** ***SEM* = 27**	*P* > 0.10 *SEM* = 5	***P* ≤ 0.10** ***SEM* = 6**

[1]Treatments: NC = basal diet and sterile water injection only; AGP+ = basal diet with antibiotic growth promoter, sterile water injection, and NE challenge; NC+ = basal diet, sterile water injection, and NE challenge; PI+ = basal diet, probiotic injection, and NE challenge; PD+ = basal diet with probiotic, sterile water injection, and NE challenge. Data presented as least square means (LSM). Differing letters in the columns represent treatments that are significantly different from one another. [2]SEM: standard error means.

CHAPTER VII

Epilogue

Over the past century, improvements in nutrition, housing and management, advances in technology, vertical integration, and disease management have allowed the poultry industry to expand more rapidly than other livestock industries. Additionally, the sub-therapeutic use of antibiotics has played an integral role in growth of the poultry industry, allowing poultry growers to raise more birds in closer quarters while maintaining health. These medications enhance growth rates and feed efficiency, while reducing incidence of disease, even at low levels of inclusion. Soon however, concern grew over antibiotic resistant strains of bacteria. Although agriculture was not the only party responsible for the injudicious use of antibiotics, much of the blame fell on this sector. Parts of the world, including the E.U. and U.S., have reduced or even eliminated antibiotic use and increased its regulation over the past couple of decades. Diseases that had been previously controlled by sub-therapeutic use of antibiotics for decades started to emerge as some of the largest challenges. The control of costly enteric diseases, including necrotic enteritis and coccidiosis, continues to justify further research for alternative methods to maintain bird health and productivity. These diseases can infect the gut and cause a range of symptoms, from mild to severe. Enteric diseases delay growth, reduce feed efficiency, increase mortality, and pose both welfare and economic issues. Current research suggests that enteric diseases may be reduced through the use of natural alternatives, including live probiotics and the non-living prebiotic substances that support. These have demonstrated the competitive exclusion of pathogens, improved intestinal integrity, and ability to influence immune responses.

Numerous studies support the use of probiotics and synbiotics in poultry. The majority involve the dietary application of these products. Before major commercialization of poultry, precocial

chicks immediately began consuming feed and taking in healthy microbes from the hen after hatch. Currently, chicks in commercial hatcheries thrive on the internal yolk for 48-72 hours before receiving their first feed, but are still exposed to the external environment. The intestines are rapidly colonized by existing microbes in the surroundings, and there is great interest in controlling the types of microbes that the chick is exposed to. The early application of probiotics containing similar microbes that make up the intestinal composition of healthy chickens is a logical method of attempting to introduce desirable microbes that may out-compete pathogenic ones.

In the first study of this dissertation, the objective was to apply synbiotics at the hatchery and investigate any perceived benefits of early application. Although chicks feed off of the internalized yolk sac for up to 3 days, applying synbiotics may help establish a healthy gut microbiota and trigger intestinal growth before birds reach exogenous feed at the growout facility. Rapid intestinal growth may translate to better nutrient absorption, feed efficiency, and overall bird growth. For the first two weeks, birds that received synbiotics at the hatchery or in the feed grew less than the birds without the synbiotic. These birds caught up by the end of the experiment, ultimately having improved feed efficiency. Birds that received hatchery and dietary synbiotics showed a synergistic effect through greater mRNA levels of the nutrient transporter PepT1 and the anti-inflammatory cytokine IL-10 in the ileum. This study showed some positive effects of early application of synbiotics, and it would be beneficial to carry out a longer study to evaluate the viability of hatchery-applied synbiotics. A hatchery has numerous control points to reduce contamination in the air and on surfaces, but is far from sterile. Introducing synbiotics at the hatchery and combining it with dietary use may contribute to early establishment of desirable microbiota in antibiotic-free production.

The second, third, and fourth studies investigated the effects and possible advantages of applying synbiotics or probiotics even earlier than post-hatch, but via *in ovo* at embryonic day 18. Establishing a healthy microbiome is important to any production setting. However, it is increasingly important to evaluate the effectiveness during subsequent disease challenges with impactful enteric diseases to commercial poultry production – coccidiosis-induced necrotic enteritis. To combat illness, traditional antibiotic use increases during disease outbreak. Yet with progressively stricter restrictions, it becomes more relevant to investigate probiotics and synbiotics as a preventative to disease and an alternative to antibiotics during actual disease challenges. In this dissertation, the pilot study investigated low, medium, and high doses of probiotic or synbiotic. This small study yielded few significant differences in performance, lesion scores, or mRNA abundance of various genes in the ileum. However, the low number of replicates may have impacted the ability to detect major differences, which was addressed by the two larger, subsequent studies.

The third study demonstrated the ability of synbiotics to reduce FI and improve FCR compared to challenged control birds during a disease challenge, and improve the lean-to-fat ratio of market age birds compared to challenged birds on the AGP (Virginiamycin). Here, there were often additive effects of synbiotic application *in ovo* and in the feed, including greater D42 ileal TLR2, TLR4, TNF-α, and AvBD10 than application of synbiotic in the feed or synbiotic *in ovo* alone. There was also greater ileal GPR43 compared to SI+ alone. Synbiotic in the diet or *in ovo* alone was not enough to alter VFAs during peak NE challenge on D7, but the combination increased four VFAs compared to the challenged control and AGP treatments. Although the antibiotic treatment performed best, the combination led to improvements in bird performance, which would translate to an economic benefit in feed savings.

The fourth treatment reports data from the *in ovo* study that incurred an accidental heat stress challenge in the hatchers immediately before hatch. Although it was hard to draw conclusions because of this large unintended variable, the data still could be re-evaluated after eliminating the single treatment that was briefly heat-stressed. Since other treatments were not exposed, it would be relevant to evaluate the probiotic against the other control treatments.

Importantly, the *in ovo* injections were found to be safe in each study. They did not reduce hatchability or negatively impact growth. The first and third studies resulted in some positive results after use of synbiotics, and led to stronger effects when the application methods were combined. A possible explanation for the lower body weights and body weight gains in the synbiotic treatments at the beginning of many experiments is the energy demands of the microbes themselves. Establishing a microbiome engages the immune system, and the microbes use up nutrients from the feed. These treatments often caught up to the birds that did not receive synbiotics or probiotics. This shift could indicate the point when the microbiome may have begun to benefit the bird through production of VFAs useful to the host and or balanced inflammation in the gut. This could explain why feed intake and feed conversion ratio were improved in some of these treatments, which would lead to a direct economic benefit.

As expected, the birds in the AGP treatments had the best performance. Replacing antibiotics is a challenge, and the synbiotics and probiotics were not as effective against the negative side-effects of disease. The microbiome is extremely complex, and it is difficult to study the effects of a single microbial species inside a host. It behaves differently in response to other microbes, the diet, the species of bird, and the environment. A diet consisting of more non-starch polysaccharides would mean more prebiotic substances in the diet to promote the growth of probiotic bacteria in the gut. On the other hand, the same non-starch polysaccharides in the feed

may also contribute to outbreaks of NE by slowing passage of feed, increasing digesta viscosity, and promoting growth of CP. To understand the composition of the microbiome of these birds throughout the experiment, it would be beneficial to analyze and characterize the microbial profiles.

In ovo injection of probiotics and prebiotics at a hatchery may be convenient on embryonic day 18, when eggs are already being vaccinated for Marek's disease and transferred to hatch trays. Still, anytime something is introduced into an egg, there is a chance of contamination. Caution must be taken if *in ovo* probiotic application became common in hatcheries. If a probiotic became contaminated with something harmful, thousands of chicks could be impacted before the mistake was corrected. During the second study investigating low, medium, or high doses of probiotics or synbiotics, the low and medium levels often reduced performance. The expense of using these additives may not always be beneficial, particularly if the chances of disease outbreak are unknown. Since the effectiveness is hard to predict, further research is warranted using studies without disease challenges.

By evaluating the mRNA abundance of various genes in areas of the gut close to the microbe-rich ceca, it has been shown that synbiotics can affect mRNA levels of various immune response genes and nutrient transporters even as early as day of hatch. In addition to the ileum, it would be relevant to investigate the mRNA abundance in the duodenum and jejunum, areas where NE has higher disease impact. Due to the nature of qRT-PCR of a homogenized tissue sample, measuring mRNA abundance alone is not always a good indicator of protein function, nor is it useful in determining the exact area along villi and microvilli where certain genes are being expressed in the animal. It would be useful to evaluate subsequent protein levels and compare these mRNA results to similar immune markers in blood samples. Performing in situ

hybridization in the gut could also give a more precise understanding of how and where these genes are being expressed in response to synbiotics during disease, and how they impact different areas of the bird.

As observed in these studies, AGP resulted in the best overall performance and finding effective replacements will be demanding. As they continue to be phased out, researching for alternatives is increasingly necessary. Predicting the effectiveness of probiotics and synbiotics can be challenging largely due to the complex nature of host-microbe interactions, but data presented here contribute valuable knowledge to the current understanding of their function in poultry during enteric disease outbreaks.