The Effects of Probiotics on Performance and Immune Response of Broiler Chickens during Coccidiosis

By

Miranda M. Ritzi

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Animal and Poultry Sciences

Rami A. Dalloul, Chair

Audrey P. McElroy

F. William Pierson

March 17, 2015

Blacksburg, VA

Keywords: probiotic, coccidiosis, broiler, performance, *Eimeria*

Effects of Probiotics and Application Methods on Performance and Response of Broiler Chickens to an *Eimeria* Challenge

Miranda M. Ritzi

ABSTRACT

Coccidiosis is endemic in the commercial broiler industry and inflicts devastating economic losses to poultry operations. Probiotics may provide a potential alternative to the prophylactic use of anticoccidials in commercial production. This study evaluated the effects of probiotic applications (feed and water) on bird performance and resistance to a mixed *Eimeria* infection in commercial broilers. On day of hatch, 1008 commercial male broilers (Cobb 500) were assigned to one of 6 treatments (8 replicate floor pens; 21 birds/pen), including non-infected negative control (NEG), *Eimeria*-infected positive control (POS), anticoccidial control (0.01% salinomycin, SAL), intermittent high dose water-applied probiotic (WPI), continuous low dose water-applied probiotic (WPC), and feed-supplemented probiotic (FSP). On d15, all birds except those in NEG were challenged with a mixed inoculum of *E*imeria *acervulina*, *E. maxima*, and *E. tenella*. Measurements were taken on d7, 15, 21, 28, 35 and 42. Fecal samples were collected from d20-d24 for oocyst counts, and lesion scores were evaluated on d21. Data were analyzed using the Fit Model platform in JMP Pro 10.0 (SAS). Differences in experimental treatments were tested using Tukey HSD following ANOVA with significance reported at $P \leq 0.05$. Overall, NEG birds outperformed all other groups. For performance, the probiotic groups were comparable to the SAL treated birds, except during the 6 days immediately following the *Eimeria* species challenge, where the SAL birds exhibited better performance. WPC birds had lower duodenal and jejunal lesion scores, indicating a healthier intestine and enhanced resistance to *Eimeria* species compared to POS. Birds in the WPI treatment shed fewer oocysts in the

feces, though this was not a trend for all of the probiotic treatment groups. The results of this study suggest probiotic supplementation without anticoccidials can enhance performance and help alleviate the negative effects of a mixed *Eimeria* infection.

Keywords: probiotic, coccidiosis, broiler, performance, *Eimeria*

Acknowledgments

My Mom, Ena, and Lional. Thank you for always being a wonderful support system, even when my passions and interests led me away from the event planning business. I certainly wouldn't have accomplished everything, including this degree, without your unwavering support and love. Thank you for showing me how to be a strong, caring person who isn't afraid to reach for her dreams!

Dr. Dalloul. Thank you for extending the graduate student opportunity to me at a time when I wasn't sure of the next steps in my plan. I learned so much over the past two years (and change) about research, poultry, and immunology, as well as a whole lot about myself. Thank you for pushing me to succeed and always having faith in me!

Dr. McElroy and Dr. Pierson. Thank you for being on my committee and helping me finish up and defend, despite the insanity that is veterinary school. I appreciate the different perspectives and ideas, as well as the support during this journey.

Chasity and Sungwon. Thank you so much for teaching me the ropes! I know it wasn't easy, but I appreciate all the help, support, patience, and advice. You both took me under your wing even before my first day as a graduate student, and continued to be great mentors even after continuing on to new chapters in your own lives. I couldn't have done this without you!

Tiffany. Thanks for being my partner in crime through this journey! I know we had our rough days, but I couldn't have done this without you. You were a good support system within the lab, and I hope I was the same for you!

Nathaniel. My minion! Thank you for helping me so much, and keeping me calm. I'm glad I convinced you to be a graduate student!

My fellow graduate students. Thank you for being great friends who always seemed to understand the life of a graduate student. I appreciate your support more than you realize.

Undergraduate students. Thank you for all your help. You were a valuable part of the lab and I hope you enjoyed your taste of graduate school and research.

Dale. Thank you for your assistance with my trials and everything else you did for me. I appreciate you making me laugh and keeping my spirits up, even during those long sampling days.

Faculty and Staff at Virginia Tech. Thank you for your assistance, valuable expertise and advice.

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CHAPTER I

Introduction

The decades-old practice of supplying food animals with sub-therapeutic doses of antibiotics to protect against infections and improve general health has recently been under scrutiny. These practices are perceived to lead to microbial resistance to the drugs in use, as well as consumer concerns regarding residues in food products. The relatively recent ban of sub-therapeutic doses of certain antibiotics as feed additives in the European Union led to a general decline in animal health (Castanon, 2007). This outcome, as well as the threat of a domestic ban, has led researchers to explore the next promising alternatives including live oocyst vaccines, probiotics, and potential combinations of the two.

Although the primary function of the gastrointestinal tract is to digest and absorb nutrients, a well-balanced gut microbiota is crucial for optimal animal health and performance. The gastrointestinal tract also serves as a vital barrier preventing the entry of potentially harmful pathogens and other environmental antigens (Kogut and Swaggerty, 2012). As the gut microbiota begins to establish within hours after the chick hatches, the earlier the introduction of nonpathogenic microorganisms, the more effective their establishment in the digestive tract (Timmerman et al., 2006; Torok et al., 2007). Also known as direct-fed microbials, probiotics are classified as live nonpathogenic microorganisms that are capable of maintaining a normal gut microbial population (Patterson and Burkholder, 2003; Ohimain and Ofongo, 2012). Probiotics help maintain a healthy balance of microorganisms within the intestine, which is accomplished through multiple modes of action. Those mechanisms include competitive exclusion, pathogen antagonism, and stimulation of the immune system (Ohimain and Ofongo, 2012). The presence of probiotics reduces the colonization of pathogenic bacteria and attenuates enteric diseases,

which ultimately result in enhanced performance of poultry (Kabir et al., 2004). Probiotics may provide a potential alternative to the prophylactic use of drugs in food animals due to their studied abilities to reduce enteric diseases in poultry (Patterson and Burkholder, 2003; Eckert et al., 2010). Probiotics can be composed of one or many strains of microbial species, with the more common ones belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, and *Pediococcus* (Gaggia et al., 2010).

Coccidiosis is endemic in the commercial broiler industry and inflicts devastating economic losses to poultry operations. Caused by development and reproduction of multiple species of the *Eimeria* protozoa, coccidiosis is estimated to cost the industry about $3 billion annually worldwide (Dalloul and Lillehoj, 2006). *Eimeria* species are unlike other protozoan parasites in that the primary target tissue is the intestinal epithelium, which results in considerable impairment of growth and feed utilization in poultry. The route of infection of these protozoa is through the consumption of fecal droppings of infected birds, as well as contaminated feed and litter (Li et al., 2005). Coccidiostats have been used to control coccidiosis, but *Eimeria* species have developed varying levels of resistance to both chemical and ionophore drugs over time (Stringfellow et al., 2011). Due to drug resistance and consumer concerns regarding drug usage, the practice of live vaccines to control coccidiosis has greatly increased. Vaccines provide an alternative for disease protection, capable of limited efficacy as they induce specific protective immunity by exposing the chicken's immune system to *Eimeria* antigens (Williams, 2002; Dalloul and Lillehoj, 2005; Stringfellow et al., 2011). Immunity is subsequently boosted and maintained by multiple re-infections caused by cycling of oocysts present in the litter due to shedding and ingestion (Williams, 2002). One drawback to live vaccines is immunity to avian coccidia is strongly species-specific, therefore the bird will only

develop immunity to the species of *Eimeria* present in the vaccine (Williams, 2002; Dalloul and Lillehoj, 2006). This specificity mandates that the vaccine have the species of *Eimeria* known to be prevalent in that area, which are more likely to cause an outbreak (Dalloul and Lillehoj, 2005). The necessary early administration of vaccines (typically during the first 1 to 7 days of life) becomes a second disadvantage regarding live vaccines. Early exposure of the chick's immune system to antigen results in immunity developing at a younger age, minimizing risk of exposure while the chick is unprotected. However, administration of live oocysts in a vaccine at a young age can result in a low level infection, which can cause an early reduction in growth and may increase the chick's susceptibility to secondary infections, such as necrotic enteritis (Dalloul and Lillehoj, 2005; Li et al., 2005; Stringfellow et al., 2011). While the first drawback to use of live vaccines is unavoidable, the chick may be able to combat the potential consequences of vaccine administration at a young age with a healthy intestinal tract and presence of normal microbiota (Dalloul and Lillehoj, 2005; Stringfellow et al., 2011). Probiotics have potential to enhance host defenses and affect the digestive microbiota positively, while protecting against colonization by harmful bacteria and maintaining intestinal integrity (Dalloul et al., 2003; Dalloul and Lillehoj, 2005; Hume, 2011; Stringfellow et al., 2011). Based on these findings, probiotics may be able to attenuate the negative consequences of early vaccine administration.

The objective of this thesis project was to determine the effects of various administration methods of the probiotic product PoultryStar, as well as effects of the coccidia vaccine Immucox, on broiler performance and immune responses to *Eimeria* challenge as evaluated by oocyst shedding, intestinal lesion scores, histological alterations within the intestine, and immune-related gene expression.

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CHAPTER II

Literature Review

The information provided in this literature review is an introduction to avian immunology, the disease process investigated in this research, and the potential benefits of probiotic administration. All three components were integral in the studies done, yielding interesting results.

Avian Immune System

The structure of the avian immune system is similar to its mammalian counterpart regarding organization and mechanisms of action, but with few avian-specific differences. The avian immune system, like in all vertebrates, can be broken down into two key components: innate and adaptive.

Innate Immunity

Innate immunity is the primitive branch of the immune system and is characterized by non-specific defense mechanisms that are present and ready to be mobilized on the day the bird hatches. This system acts as the first line of defense providing immediate protection against an impending foreign challenge.

Physical and biological barriers

The epithelial layers, body secretions and mucous membranes of the body constitute physical barriers that are generally impermeable to most infectious agents. Most pathogens gain access to the host through mucosal surfaces of the respiratory and gastrointestinal tracts, which are in direct contact to the external environment. If a pathogen is inhaled, ciliated epithelium and mucus found within the respiratory tract act as filters to assist in keeping the airways clear (Juul-

Madsen et al., 2008). If a pathogen enters via the digestive tract, a thick mucus layer, produced by goblet cells, will block the pathogen from penetrating the host's cells. Additionally, the gastrointestinal tract is lined with a single layer of columnar intestinal epithelial cells (IECs), which regulate nutrient and water uptake and act as a physical barrier separating the potentially harmful contents of the intestinal lumen from the underlying tissues (Oswald, 2006). Due to their protective function, IECs have developed several mechanisms to reduce the risk of pathogen invasion in the surrounding tissues. Such mechanisms include the inhibition of bacterial passage and colonization along the luminal surface of the epithelial layer through production of secretory proteins, such as antimicrobial peptides, as well as the interaction with components of the underlying immune system (Pitman and Blumberg, 2000). The gut maintains a dense microbial flora capable of preventing colonization by invading organisms through competitive exclusion, as well as producing soluble factors capable of inhibiting the growth and development of pathogens in the gut. The lower pH found within the gastrointestinal tract also serves as a powerful chemical defense against ingested pathogens (Juul-Madsen et al., 2008). In addition, higher body temperature found in avian species is capable of prohibiting many infectious agents from causing disease (Butcher and Miles, 2001). Except in situations where these physical and chemical barriers are compromised, entry of the majority of pathogens is prohibited, preventing access to the host.

Cellular barriers

If pathogens are capable of breaching the physical and chemical barriers of the innate defense system, there are cellular checks in place to provide protection. The most widely known cells of the innate immune system are phagocytic cells that are capable of ingesting and destroying antigens. These cells include macrophages, heterophils (the avian equivalent to

mammalian neutrophils) and dendritic cells. Natural killer (NK) cells constitute another major cellular component of the innate immune system. Natural killer cells are cytotoxic lymphocytes that do not require activation in order to destroy cells deemed to be "non-self", and thus are not restricted by the major histocompatibility complex (MHC), unlike B and T lymphocytes. These cells of the innate system are derived from the lymphocyte lineage and are usually characterized by a large granular morphology in the cytoplasm. Natural killer cells function by releasing these granules from their cytoplasm, which contain proteins such as perforin and proteases in order to kill virus-infected and tumor cells (Juul-Madsen et al., 2008).

The cells of the innate immune system are usually triggered when conserved microbial motifs known as microbe-associated molecular patterns (MAMPs) are recognized by pattern recognition receptors (PRRs) located on the surface of the immune cell. Following activation and phagocytosis, the phagocyte will present a processed fragment of the pathogen to members of the adaptive immune system, mainly B and T lymphocytes, which will stimulate a response to the pathogen. As such, these innate immune cells also function as antigen-presenting cells (APCs). Recognition of pathogens by the innate immune system triggers both immediate innate defenses as well as the activation of the adaptive immune response. Dendritic cells and macrophages play a key role in detecting and processing antigens, then dictating the differentiation of naïve lymphocytes into appropriate effector cells in order to defeat specific types of pathogens (Lee and Iwasaki, 2007).

Initial encounter of pathogens with the innate system leads to destruction of pathogens, as well as initiation of a cascade of events which include recruitment of various immune components and induction and modulation of the adaptive immune system (Juul-Madsen et al., 2008).

Adaptive Immunity

For pathogens that cannot be controlled by the innate immune system, the adaptive immune system acts as a second line of defense and provides protection against re-offending pathogens. Adaptive immunity is more complex than innate immunity and offers antigen-specific protection to the host. After the antigen is processed by cells of the innate immune system, it is recognized by B and T lymphocytes, the key components of the adaptive immune system. Adaptive immunity also has a "memory" feature, which allows future responses against a specific antigen to be quicker and more robust.

Adaptive immunity is either humoral or cell mediated immunity (CMI). The immune system will utilize the humoral branch, CMI, or a combination of the two in order to clear the offending pathogen, depending on the pathogen characteristics. Primary lymphoid organs such as the thymus and bursa of Fabricius are responsible for lymphocyte production and development. Once developed, lymphocytes travel to the secondary lymphoid organs such as the spleen and mucosal-associated lymphoid tissues, where they come into contact with potential pathogens and other antigens and differentiate into effector cells (Dalloul and Lillehoj, 2006).

Humoral immune system

The humoral immune system is primarily mediated by B lymphocytes. B cells produce specific immunoglobulins (Ig), or antibodies, when stimulated by microbial exposure or other antigens. Antibodies defend the host against these invaders by three mechanisms: 1) Opsonization: antibodies will bind to receptors and coat the surface of the pathogen for it to be more readily and efficiently engulfed by phagocytes; 2) Neutralization: antibodies will react with epitopes on an infectious agent and inhibit its ability to infect the host; and 3) Complement

activation: the complement system is activated when antibodies bind to the surface of invading pathogens, which aid in phagocytosis (Juul-Madsen et al., 2008).

Unlike mammals who carry five main types of immunoglobulins, only three main classes are identified in birds: IgM, IgG (also known as IgY), and IgA. IgM is found on the surface of B cells and is the first antibody found in circulation during a primary immune response. IgG is the most abundant immunoglobulin found in avian as well as mammalian blood and is the primary antibody produced during a secondary immune response. IgA plays a critical role in mucosal immunity. Secretory IgA exists as a dimer and is most concentrated in mucosal surfaces, tears and saliva (Davison et al., 2008).

Cell-mediated immune system

Cell mediated immunity is characterized and controlled by T lymphocytes. As found in mammals, chicken T lymphocytes can be categorized into CD4+ (helper T cells or T_H cells) and CD8+ (cytotoxic T cells or T_C cells) subpopulations (Viertlboeck and Göbel, 2008). Helper T cells are activated by recognition of a class II MHC coupled with processed antigen on an APC. Once activated, T_H cells divide and produce a variety of cytokines in order to activate B and T lymphocytes as well as other immune cells. Proliferating T_H cells will differentiate into one of two major subtypes known as Type 1 and Type 2 helper T cells (T_H1 and T_H2, respectively), depending on the cytokine profile present in the environment. The T_H1 cells are primarily responsible for producing cytokines that encourage inflammation and activation of macrophages, and both B and T lymphocytes. The cytokines produced also inhibit the function of T_H2 cells, which is part of the response to generate immunity to intracellular pathogens. Cytokines secreted by T_H2 cells stimulate B lymphocyte proliferation and antibody production while inhibiting T_H1 cell function to enhance immunity to extracellular pathogens (Kaiser and Stäheli,

2008). A third and more recently discovered T_H cell lineage has been identified as T_H17 due to the production of its signature pro-inflammatory cytokine IL-17. Cytotoxic T (T_C) cells, on the other hand, are responsible for the recognition and lysis of cells infected with endogenous pathogens in association with a class I MHC molecule (Dalloul and Lillehoj, 2006).

Immune response genes

Numerous genes are involved in directing both innate and adaptive immunity by encoding for production of various elements of the immune system including antigen receptors, antimicrobial peptides, chemokines, cytokines and other factors. When exposed to antigens or chemotactic agents, macrophages will begin to produce inducible nitric oxide synthase (iNOS). This enzyme leads to the production of nitric oxide, which will subsequently react with superoxide anions to generate toxic derivatives, allowing macrophages to proficiently kill numerous types of pathogens (Kaspers et al., 2008). Trefoil factor (TFF)-2 is a stable secretory protein expressed in gastrointestinal mucosa responsible for protecting the epithelial layer from insults, stabilizing the mucus layer, and promoting the healing of the epithelium (Jiang et al., 2011).

Interferon (IFN)-γ is a vital pro-inflammatory cytokine that plays a central role in regulating the innate and adaptive immune responses, and is responsible for promoting T_H1 cell differentiation, suppressing T_H2 cell activity, and enhancing innate immune cell activation and function (Kaiser and Stäheli, 2008). Expression of lipopolysaccharide-induced tumor necrosis factor-α (LITAF) is principally in the spleen of chickens as well as in intestinal intraepithelial lymphocytes. LITAF is a transcription factor that mediates the expression of members of the tumor necrosis factor (TNF) ligand superfamily (Hong et al., 2006b).

Members of the interleukin family take part in many aspects of immunity. Interleukin (IL)-1β is produced by activated macrophages and plays a role in inflammatory reactions and host defense (Kaiser and Stäheli, 2008). Produced by macrophages and dendritic cells after activation, IL-12 is responsible for driving inflammatory T_H1 responses (Kaiser and Stäheli, 2008). IL-12 is also capable of inducing IFN-γ production from T_H1 and NK cells. IL-1β, TNF-α, and IL-12 facilitate innate immunity and associated inflammation (Elgert, 2009). IL-13 is a T_H2 cytokine that is predominant in the cytokine response initiated by an extracellular pathogen (Kaiser and Stäheli, 2008). IL-13 increases B cell proliferation and inhibits activation of macrophages through antagonizing IFN-γ mediated activities (Elgert, 2009). Transforming Growth Factor (TGF)-β plays an important role in immunoregulation (Kaiser and Stäheli, 2008), a cytokine with anti-inflammatory and immunosuppressive activities thought to prevent potential damage to the host from an excessive immune response (Elgert, 2009).

Gut-Associated Lymphoid Tissues (GALT)

The primary function of the gastrointestinal tract is to digest and absorb nutrients in order to meet metabolic demands for normal growth and development, but it also acts as a vital barrier preventing the entry of several antigens and potentially harmful pathogens from the external environment (Smith and Beal, 2008; Kogut and Swaggerty, 2012). The gut is a major site of development, residence, and portal of entry for pathogenic microorganisms. As such, any disruption of gut physiology is easily capable of resulting in substantial clinical consequences. Therefore, an effective immune capability in the gut is essential to cope with the plethora of potentially pathogenic microorganisms present (Smith and Beal, 2008). The GALT make up the largest component of the mucosa-associated lymphoid tissues (MALT) and are a significant source of immune cells that monitor and protect the mucosal layers of the intestine. The GALT

are continuously being exposed to food antigens, microflora and ingested pathogens (Yun et al., 2000). Protection of the gut is achieved through use of both the innate and adaptive immune systems.

The mucosal layer of the gut is comprised of the epithelium and the lamina propria. The epithelial layer is marked by the presence of mostly T lymphocytes, while the lamina propria is populated by a variety of immune cells including antibody-producing B lymphocytes (Lillehoj and Trout, 1996). Unlike the mammalian GALT, chickens do not possess lymph nodes; instead, they have scattered lymphoid aggregates as well as organized lymphoid structures, such as the bursa of Fabricius, cecal tonsils, Meckel's diverticulum and Peyer's patches (PP). The epithelium of the PP, as well as the other lymphoid structures of the GALT, contains areas occupied by phagocytic antigen sampling cells known as microfold (M) cells (Muir et al., 2000). These M cells are responsible for taking up antigens from the lumen and delivering them to APCs. Upon encountering the antigen in the PP, B and T cells will mount their specific immune response in order to combat the invading pathogen (Smith and Beal, 2008).

Coccidiosis

Coccidiosis is an economically devastating parasitic disease of the poultry industry caused by the development and reproduction of several species of the *Eimeria* protozoan within the intestine resulting in an estimated cost of $3 billion annually worldwide (Dalloul and Lillehoj, 2006). The chicken is host to seven species of *Eimeria*, including the most common *E. acervulina*, *E. maxima* and *E. tenella*, each of which infects a specific area of the gut and invades the intestinal epithelial cells resulting in varying levels of tissue damage and morbidity (McDonald and Shirley, 2009). Depending on the species, magnitude, and site of infection, coccidiosis can result in a limited enteritis resulting in fluid loss and malabsorption of nutrients

(typically due to *E. acervulina* and *E. mitis*), inflammation of the intestinal wall with pinpoint hemorrhages and sloughing of epithelia (as seen with *E. brunetti* and *E. maxima*), or complete villar destruction, leading to extensive hemorrhage and death (encountered with *E. necatrix* and *E. tenella*) (Chapman, 2014). The disruption of the intestinal epithelial layer naturally leads to the diminished ability of the intestine to absorb nutrients, resulting in reduced performance and higher susceptibility to other diseases, such as necrotic enteritis (Yegani and Korver, 2008).

Eimeria Life Cycle

Birds become infected through the ingestion of sporulated *Eimeria* oocysts from contaminated feed, water or litter (Li et al., 2005). The oocyst wall is crushed by the gizzard releasing the sporocysts. Once the sporocysts are broken down with the aid of trypsin and bile in the duodenum, sporozoites are released. The sporozoites then invade the intestinal epithelial cells where they develop into schizonts containing many offspring called merozoites. This developmental stage is capable of breaking out of the epithelial cells, invading other cells and asexually replicating further. Due to this cyclic reproduction, many of the epithelial cells are destroyed. Once asexual reproduction ceases, the later generation merozoites develop into sexual male and female gametocytes, identified as microgametes and macrogametes, respectively. The microgamete subsequently fertilizes the macrogamete to produce a zygote. The zygote matures into an oocyst, ruptures the intestinal cell, and is passed with the feces (Blake and Tomley, 2014). Once outside the host, the oocysts will sporulate when environmental conditions such as temperature, humidity, and oxygen become conducive to growth and once again become infective (Williams, 2002). The entire lifecycle from ingestion to release may take 4-6 days to complete (McDougald, 1998; Allen and Fetterer, 2002).

Coccidiosis Prevention and Treatment

Currently, most poultry producers rely on prophylactic measures such as anticoccidial drugs and vaccines to prevent coccidiosis in their flocks. Anticoccidial drugs, commonly referred to as coccidiostats, are administered in the feed at low doses during the grow-out period, with a withdrawal period prior to market age and weight. There are two classes of coccidiostats; chemical and ionophore. Chemical anticoccidials have specific modes of action against parasite metabolism, while ionophore anticoccidials work by disrupting osmotic balance and altering ion transport (McDougald, 1998). However, *Eimeria* species have developed resistance to both chemical and ionophore drugs over time (Stringfellow et al., 2011). These means of control are considered effective still only because parasite growth is suppressed sufficiently to allow natural immunity to develop (Blake and Tomley, 2014). Due to drug resistance and consumer concerns regarding drug usage, the practice of live vaccines to control coccidiosis has greatly increased. Vaccines provide an alternative for disease protection, capable of limited efficacy as they induce specific protective immunity by exposing the chicken's immune system to *Eimeria* antigens (Williams, 2002; Dalloul and Lillehoj, 2005; Stringfellow et al., 2011). Immunity is subsequently boosted and maintained by multiple re-infections caused by oocysts present in the litter due to shedding and ingestion (Williams, 2002; Chapman et al., 2005). Li and colleagues (2005) found that birds receiving a coccidia vaccine without therapeutic levels of an ionophore anticoccidial in the feed had less severe lesion scores in the upper and middle intestinal segments when compared to un-vaccinated, medicated birds following challenge with three *Eimeria* species. A second study found that birds given one of three different coccidia vaccine doses had less severe lesion scores than the positive un-vaccinated control (Li et al., 2005). Fewer and less severe lesion scores are indicative of less damage to the intestinal epithelium, leading to infected

birds having a greater chance of recovery from disease. However, one drawback to live vaccines is that immunity to avian coccidia is strongly species-specific, therefore the bird will only develop immunity to the species of *Eimeria* present in the vaccine (Williams, 2002; Dalloul and Lillehoj, 2006). This specificity mandates that the vaccine have the species of *Eimeria* known to be prevalent in that area, therefore more likely to cause an outbreak (Dalloul and Lillehoj, 2005). The necessary early administration of vaccines (typically during the first 1 to 7 days of life) becomes a second disadvantage regarding live vaccines. Early exposure of the chick's immune system to antigens results in immunity developing at a younger age, minimizing risk of exposure while the chick is unprotected. However, administration of live oocysts in a vaccine at a young age can result in a low level infection, which can cause an early reduction in growth and may increase the chick's susceptibility to secondary infections, such as necrotic enteritis (Dalloul and Lillehoj, 2005; Li et al., 2005; Stringfellow et al., 2011). Even though these methods are generally considered to be successful, due to the issues related to the use of anticoccidial drugs and vaccines, as well as the impending ban on animal feed additives, research has recently focused on more 'natural' means of controlling and managing coccidiosis.

Host Immune Response to Eimeria

The complex life cycle of coccidia elicits a number of immunological responses involving both the innate and adaptive immune systems, with each response being specific to the *Eimeria* species involved. Prior to the activation of an adaptive immune response, the innate immune system of a naïve host will attempt to prohibit the *Eimeria* infection through various pathways such as competitive exclusion by commensal microflora, gastric secretions, phagocytosis, and complement components. The GALT play an invaluable role in protecting the host and bridging the innate and adaptive immune systems. The GALT provide protection to the

host by performing three main functions: processing and presenting antigens, producing intestinal antibodies by activating the humoral immune system, and activating cell mediated immunity (Yun et al., 2000; Dalloul and Lillehoj, 2005).

Humoral immune response

The intestine is considered to be the largest immunological organ containing approximately 70-80% of the total immunoglobulin-producing cells, with IgA and IgM being the predominant isotypes found within intestinal secretions (Yun et al., 2000). Chickens infected with *Eimeria* produce increased levels of parasite specific antibodies in response to the challenge confirming that *Eimeria* parasites promote activation of the humoral immune system (Lillehoj and Lillehoj, 2000). However, the role of the humoral immune response in protecting the bird is still not well understood. This is partly attributed to studies using hormonally and chemically bursectomized chickens that were resistant to reinfection, thus suggesting that antibodies play a lesser role in protecting the host by potentially reducing, not eliminating, the pathogen (Lillehoj and Trout, 1996; Dalloul and Lillehoj, 2006).

Cell-mediated immune response

Cell-mediated immune responses are thought to be the most effective against intracellular parasites such as *Eimeria*. Following an exposure to *Eimeria*, proliferation and infiltration of T lymphocytes, especially CD8+ T lymphocytes, are increased at the site of infection. Elimination of CD8+ T lymphocytes results in aggravated severity of coccidiosis and greater oocyst shedding demonstrating their importance in providing immunity to the disease. Suppression of T lymphocyte function also resulted in impaired immunity (Dalloul and Lillehoj, 2006). Higher levels of CD8+ T lymphocytes were also found to be related to reduced oocyst shedding (Bessay

et al., 1996). Furthermore, in chickens, splenocytes and peripheral blood lymphocytes from immune birds can transfer resistance to infection to naïve birds (Lillehoj, 1998). Though the role of the CD8+ T lymphocytes in controlling *Eimeria* infections is evident, it has been suggested that CD4+ T lymphocytes are important during primary infections while CD8+ cells are essential during secondary infections (Lillehoj, 1998). Chickens treated with anti-CD4 antibodies shed more oocysts following a primary infection of *E. tenella* (Trout and Lillehoj, 1996). Single infections of *E. maxima* or *E. tenella* have led to an increase of CD4+ T cells in the duodenum and ceca respectively (Cornelissen et al., 2009). Hong et al. (2006c) reported a rise in CD4+ and CD8+ cells following an initial exposure to *E. maxima*. These results suggest that the different subsets of T lymphocytes work together in order to clear initial infections of *Eimeria* and provide enhanced protection against future encounters.

The response of T lymphocytes against an *Eimeria* infection is predominantly controlled and regulated by cytokines. Following primary *E. maxima* infection, mRNA of the T_H1 cytokines IFN-γ, IL-1ß, IL-6, IL-12, IL-15, IL-17 and IL-18, are up-regulated as well as the T_H2 cytokines IL-4, IL-10, IL-13 and granulocyte macrophage colony stimulating factor (GM-CSF) (Hong et al., 2006c). In a similar study by the same group, an initial infection of *E. acervulina* resulted in the up-regulation of IFN-γ, IL-2, IL-12, IL-15, IL-16, IL-18, and GM-CSF and down-regulation of IL-4 and IL-13 mRNAs (Hong et al., 2006a). Similarly, Cornelissen et al. (2009) reported that *E. acervulina* or *E. tenella* infection up-regulated mRNA expression of the T_H1 cytokines IL-2, IL-18 and IFN-γ. However, they observed an increase in gene expression of T_H2 cytokines IL-4 and IL-10 as well as the chemokine IL-8 (Cornelissen et al., 2009). These data further confirm the importance of both the T_H1 and T_H2 subsets in controlling *Eimeria* infections.

Probiotics and Poultry

Prophylactic use of anticoccidial feed additives is currently the most common method of controlling coccidiosis. However, due to increasing concerns regarding drug use and high costs of vaccines, alternative control methods have taken the forefront in the research community. The use of an immunomodulator to manipulate the immune system is currently the most promising alternative. Probiotic supplementation is one option currently being explored as a means of reducing the amount and severity of enteric diseases in poultry and subsequent contamination of poultry products for human consumption (Rolfe, 2000; Patterson and Burkholder, 2003; Eckert et al., 2010). A probiotic is defined as "a live microbial feed supplement, which beneficially affects the host animal by improving intestinal balance" (Fuller, 1989). The use of probiotics is based on the understanding that a well-balanced gut microbiota is crucial for optimal animal health and performance. Alterations in the microbial profile can influence all aspects of the gastrointestinal tract, including development, physiology, immunology, and resistance to enteric infections, through interactions of the microorganisms with the intestinal lining and lymphoid tissues.

Modes of Action

Probiotics are used to help maintain a healthy microbial balance within the intestine to promote gut integrity and prevent enteric disease. As the gut microbiota begins to establish within hours after the chick hatches, the earlier the introduction of nonpathogenic microorganisms, the more effective their establishment in the digestive tract (Timmerman et al., 2006; Torok et al., 2007). This is accomplished through three main mechanisms: competitive exclusion, bacterial antagonism, and stimulation of the immune system (Ohimain and Ofongo, 2012). Competitive exclusion is the idea that probiotic strains have the ability to inhibit

establishment of pathogenic bacteria through competition for space, attachment sites, and available nutrients by colonizing the intestinal tract themselves. Probiotic bacteria may also produce antimicrobial substances such as volatile fatty acids, bacteriocins, and low pH that limit the growth and/or survival of pathogenic microbes (Hume, 2011). Bacteriocins are a small class of secreted peptides or proteins produced by bacteria that kill closely related bacterial strains by forming pores in their cellular membranes or hindering essential enzymes. Furthermore, many probiotics, predominantly those containing *Lactobacillus* species, lower the environmental pH through their production of lactic acid, which can also impede the growth of acid sensitive microbes (Travers et al., 2011). One of the most important functions of probiotics is to stimulate the immune system against invading microorganisms, which will be discussed further in this review.

Probiotics, Performance, and Intestinal Development

Due to the reduction in the sub-therapeutic use of antibiotics and chemotherapeutic agents, poultry producers and researchers have been actively searching for a viable alternative that offers the same growth promoting benefits while thwarting pathogenic threats. The status of the intestinal tract plays a major role in influencing the performance of poultry. The microflora present in the gut is an essential component of a healthy intestinal tract. The gut microbial profile can be manipulated through the use of probiotics in order to create conditions favorable to enhancing performance. Probiotics have been shown to result in improved body weight gain and feed conversion ratios in chickens (Kabir et al., 2004; Khaksefidi and Ghoorchi, 2006; Nayebpor et al., 2007; Talebi et al., 2008; Ignatova et al., 2009; Sen et al., 2012). Probiotic supplementation has also been shown to have growth-promoting effects comparable to those of avilamycin, further promoting it as a viable antibiotic alternative (Mountzouris et al., 2007).

Despite the plethora of data demonstrating the positive effects of probiotics on performance, several researchers have reported no significant enhancements due to probiotic supplementation (Rahimi et al., 2011; Wolfenden et al., 2011). These differences could be due to a variety of factors that can alter the efficacy of a probiotic such as strain(s) of bacteria utilized, composition and viability of the probiotic bacteria, and the preparation methods. Further, other factors may include probiotic dosage, method and/or frequency of application, overall diet, condition and age of the birds, potential drug interactions, as well as environmental stress factors such as temperature and stocking density (Patterson and Burkeholder, 2003; Mountzouris et al., 2007; Cox and Dalloul, 2015).

Augmented performance due to probiotic supplementation could be attributed to enhanced gut development. The use of probiotics has been shown to increase the length of intestinal villi and decrease the depth of crypts in poultry (Samanya and Yamauchi, 2002; Marković et al., 2009). The increase in villus length suggests a greater surface area with increased absorptive capacity. The intestinal crypt is the site of continuous proliferation of enterocytes in order to replace cells lost at the villus tip due to normal sloughing or inflammation from insults (Awad et al., 2009). The depth of the intestinal crypt is directly correlated with epithelial cell turnover. Increased cellular turnover requires a substantial amount of energy that might otherwise be utilized towards growth. Thus, longer villi and shallower crypts are related to decreased cell replacement, longer enterocyte lifespan, and improved performance (Marković et al., 2009).

Probiotics may also offer protection against damage to intestinal morphology caused by deleterious agents. For example, Awad and colleagues (2006) evaluated the effects of probiotics in broiler chicks fed diets contaminated with deoxynivalenol (DON), which altered the small

intestine morphology by decreasing villus height and width in the duodenum and jejunum. Probiotic supplementation ameliorated the negative effects caused by the mycotoxins by diminishing villus atrophy (Awad et al., 2006).

Probiotics and Innate Immunity

Characterized by non-specific defense mechanisms, the innate immune system acts as the first line of defense to provide immediate protection against an impending foreign challenge. Several studies have provided evidence that specific strains of probiotics are able to stimulate various aspects of innate immunity. The avian heterophil is considered to be equivalent to the mammalian neutrophil, and is therefore the first line of cellular defense against a broad spectrum of microbial pathogens. These innate immune cells are highly phagocytic and utilize bactericidal mechanisms such as oxidative burst and degranulation to destroy phagocytized pathogens (Harmon, 1998). Farnell and colleagues (2006) found that heterophils isolated from broiler chicks fed the probiotic bacteria *Bacillus subtilis*, *Lactococcus lactis lactis*, or *Lactobacillus acidophilus* elicited an increase in oxidative burst and degranulation. Similar results were seen in a later study where birds treated with the probiotic product PoultryStar® had greater levels of heterophil oxidative burst (Stringfellow et al., 2011). Macrophages are phagocytic immune cells that play a key role in detecting and processing antigens, as well as dictating the differentiation of naïve lymphocytes into appropriate effector cells in order to defeat specific types of pathogens (Qureshi et al., 2000). Unchallenged broiler chicks given the commercially available *Lactobacillus*-based probiotic known as FM-B11 had an increased number of macrophages in the ileum and ceca, but those birds exposed to *Salmonella* Enteritidis had decreased numbers of macrophages in the ileum when given probiotics one hour after challenge. The reduction of macrophage numbers within the infected birds could be due to a decrease in the bacterial load

caused by the addition of probiotics, potentially caused by competitive exclusion. Furthermore, abdominal exudate cells from *Salmonella*-challenged birds had greater phagocytic capacity when treated with probiotics (Higgins et al., 2007a). The up-regulation of host innate immunity, including enhanced antimicrobial defenses and phagocytosis, may result in the reduction and possible elimination of pathogenic invaders.

Probiotics and Adaptive Immunity

The adaptive immune system acts as a second line of defense and provides protection against re-offending pathogens. Recent reports demonstrate the importance of probiotics in potentiating the adaptive immune response in chickens. Probiotic supplementation increases antibody titers to sheep red blood cells, as well as many important disease agents such as Newcastle disease virus and infectious bursal disease virus (Kabir et al., 2004; Haghighi et al., 2005; Khaksefidi and Ghoorchi, 2006; Nayebpor et al., 2007; Apata, 2008; Karimi Torshizi et al., 2010). Furthermore, Haghighi et al. (2006) determined that female broilers orally gavaged with probiotics had enhanced production of natural antibodies. Probiotic-treated birds had increased levels of IgA reactive to tetanus toxoid, alpha-toxin, and bovine serum albumin, as well as increased levels of IgG against tetanus toxoid in the intestine. The administration of probiotics also augmented serum levels of IgG and IgM antibodies reactive to tetanus toxoid and alpha-toxin (Haghighi et al., 2006). In a study to compare the efficacy of the antibiotic avilamycin and probiotics on the humoral immune response, Mountzouris et al. (2009) found that both treatments resulted in reduced levels of plasma IgA and IgG and intestinal IgA against *Salmonella* Enteritidis when compared to the challenged control. In addition, the treated levels were not different from those of the negative controls. The reduction in antibody levels could be a result of enhanced clearance and accelerated recovery caused by their experimental treatments.

It has also been shown that *Eimeria acervulina* infected birds fed probiotics produced more *Eimeria*-specific antibodies (Lee et al., 2007a; 2007b).

Probiotics magnified the numbers of intestinal intraepithelial lymphocytes expressing the cell surface markers CD3, CD4 and CD8 (Dalloul et al., 2003; Noujaim et al., 2008). Supplementation of probiotics through drinking water enhanced cellular immune response to a DNCB (1-chloro-2, 4-dinitrobenzene) challenge, while administration in feed or water improved cellular immunity to a PHA-M (phytohemagglutinin-M) injection, demonstrated through increased skin thickness (Karimi Torshizi et al., 2010). *Lactobacillus* and *Bacillus*-based probiotics can modulate the levels of several cytokines including pro-inflammatory cytokines (IL-1ß, IL-6, IL-17a, IL-18), Th1 cytokines (IFN-γ, IL-2, IL-12), and Th2 cytokines (IL-4, IL-10, IL-13). However, discrepancies have been noted due to differences in the probiotic strains used (Dalloul et al., 2005; Brisbin et al., 2010; Lee et al., 2010a).

Probiotics and Host Defense against Pathogens

The use of probiotics is based on the understanding that microflora in the gut are an essential component involved in resistance to enteric infections. Probiotics aid in protection against a variety of economically important enteric diseases (Dalloul and Lillehoj, 2005). Chicks fed diets supplemented with probiotics and subsequently infected with *E. acervulina* or *E. tenella* had significantly reduced oocyst shedding (Dalloul et al., 2003; Dalloul et al., 2005; Lee et al., 2007a; 2007b), suggesting reduced susceptibility to infection. Moreover, *Bacillus*-based probiotics reduced lesion severity in *E. maxima* challenged broilers (Lee et al., 2010b). The addition of probiotics was also efficacious in reducing *Salmonella* colonization in the ceca, liver and spleen of broiler chicks (Revolledo et al., 2009). Furthermore, researchers have observed a reduction in lesion score severity, mortality, and numbers of *Clostridium perfringens* due to

probiotic treatment during experimentally induced cases of necrotic enteritis (McReynolds et al., 2009). Numerous studies have found that probiotic supplementation leads to significant reductions in numbers of other intracellular pathogens present in the intestine, such as *Salmonella* Enteritidis and *Campylobacter jejuni* (Higgins et al., 2007b; Ghareeb et al., 2012). Such effect of probiotic supplementation could prove to be exceptionally beneficial to the bird, as some microorganisms such as *Clostridium* and *Salmonella* may exacerbate *Eimeria* infections and vice versa (Chapman et al., 2002). Ultimately, the reduction in the presence of intracellular pathogens is indicative of a healthier intestine, with minimal damage done to the epithelium. An intact intestinal epithelium serves as the vital barrier preventing entry of potential pathogens and results in proper nutrient absorption and utilization, leading to optimal health and performance of the bird. These results suggest that maintaining a proper balance of intestinal microflora through the use of probiotics could prove to be effective in preventing and treating enteric infections and a suitable alternative to the use of chemotherapeutic agents.

The information in the literature review provides a background to better understand the studies performed investigating immunological interactions in the face of coccidiosis.

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CHAPTER III

Effects of Probiotics and Application Methods on Performance and Response of Broiler Chickens to an *Eimeria* Challenge

Abstract: Coccidiosis is endemic in the commercial broiler industry and inflicts devastating economic losses to poultry operations. Probiotics may provide a potential alternative to the prophylactic use of anticoccidials in commercial production. This study evaluated the effects of probiotic applications (feed and water) on bird performance and resistance to a mixed *Eimeria* infection in commercial broilers. On day of hatch, 1008 commercial male broilers (Cobb 500) were assigned to one of 6 treatments (8 replicate floor pens; 21 birds/pen), including non-infected negative control (NEG), *Eimeria*-infected positive control (POS), anticoccidial control (0.01% salinomycin, SAL), intermittent high dose water-applied probiotic (WPI), continuous low dose water-applied probiotic (WPC), and feed-supplemented probiotic (FSP). On d15, all birds except those in NEG were challenged with a mixed inoculum of *E*imeria *acervulina, E. maxima*, and *E. tenella*. Measurements were taken on d7, 15, 21, 28, 35 and 42. Fecal samples were collected from d20-d24 for oocyst counts, and lesion scores were evaluated on d21. Data were analyzed using the Fit Model platform in JMP Pro 10.0 (SAS). Differences in experimental treatments were tested using Tukey HSD following ANOVA with significance reported at $P \leq 0.05$. Overall, NEG birds outperformed all other groups. For performance, the probiotic groups were comparable to the SAL treated birds, except during the 6 days immediately following the *Eimeria* species challenge, where the SAL birds exhibited better performance. WPC birds had lower duodenal and jejunal lesion scores, indicating a healthier intestine and enhanced resistance to *Eimeria* species compared to POS. Birds in the WPI treatment shed fewer oocysts in the feces, though this was not a trend for all of the probiotic treatment groups. The results of this

study suggest probiotic supplementation without anticoccidials can enhance performance and help alleviate the negative effects of a mixed *Eimeria* infection.

Introduction

The decades-old practice of supplying food animals with sub-therapeutic doses of antibiotics to protect against infections and improve general health has recently been under scrutiny. These practices are perceived to lead to microbial resistance to the drugs in use, resulting in consumer concerns regarding residues in food products. The relatively recent ban of sub-therapeutic doses of certain antibiotics as feed additives in the European Union led to a general decline in animal health (Castanon, 2007). This outcome, as well as the threat of a domestic ban, has led researchers to explore the next promising alternatives.

Probiotics present a potential alternative to the prophylactic use of antibiotics in feed animals. Also known as direct-fed microbials, probiotics are classified as live nonpathogenic microorganisms that are capable of maintaining a normal gastrointestinal microbiota (Patterson and Burkholder, 2003; Ohimain and Ofongo, 2012). Probiotic, meaning "for life" in Greek, has been defined as "a live microbial feed supplement, which beneficially affects the host animal by improving intestinal balance" (Fuller, 1989). Probiotics can be composed of one or many strains of microbial species, with the more common ones belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, and *Pediococcus* (Gaggia et al., 2010).

Although the primary function of the gastrointestinal tract is to digest and absorb nutrients, a well-balanced gastrointestinal microbiota is crucial for optimal animal health and performance. The gastrointestinal tract also serves as a vital barrier preventing the entry of potentially harmful pathogens and other environmental antigens (Kogut and Swaggerty, 2012). As the gastrointestinal tract begins to be colonized within hours after the chick hatches, the

earlier the introduction of non-pathogenic microorganisms, the more effective their establishment in the digestive tract (Timmerman et al., 2006; Torok et al., 2007). Probiotics help maintain a healthy balance of microorganisms within the intestine, which is accomplished through multiple modes of action. Those mechanisms include competitive exclusion, pathogen antagonism, and stimulation of the immune system (Ohimain and Ofongo, 2012). The presence of probiotics reduces colonization of the gastrointestinal tract by pathogenic bacteria and attenuates enteric diseases, which ultimately result in enhanced performance of poultry (Kabir et al., 2004).

Coccidiosis is endemic in the commercial broiler industry and inflicts devastating economic losses to poultry operations. Caused by development and reproduction of multiple species of the *Eimeria* protozoa, coccidiosis is estimated to result in a loss of US $3 billion annually to the industry worldwide (Dalloul and Lillehoj, 2006). *Eimeria* species are unlike other protozoan parasites in that the primary target tissue is the intestinal epithelium, which results in considerable impairment of growth and feed utilization in poultry. Probiotics may provide a potential alternative to the prophylactic use of drugs in food animals due to their studied abilities to reduce enteric diseases in poultry (Patterson and Burkholder, 2003; Eckert et al., 2010). This study aimed to evaluate the effects of a multi-species, host specific probiotic product containing *Enterococcus*, *Bifidobacterium*, and *Lactobacillus* species, in the feed and water on performance parameters and resistance to coccidiosis (*Eimeria* infection) in commercial broiler chickens.

Materials and Methods

Birds and Experimental Treatments

The study performed was a 42-day trial with broilers housed on floor pens, with 8 replicate pens per treatment and 21 birds per pen. On day of hatch (DOH), 1,008 commercial male broilers (Cobb 500) were assigned to one of 6 treatments, including non-infected negative control (NEG), *Eimeria*-infected positive control (POS), anticoccidial control (salinomycin as Sacox-60® at the rate of 0.01%, SAL), continuous low dose water-applied probiotic (WPC), intermittent high dose water-applied probiotic (WPI), and feed-supplemented probiotic (FSP). Birds in the low dose WPC group were given probiotics at the rate of 2 mg/bird/day every day. Birds in the high dose WPI treatment were given probiotics at the rate of 20 mg/bird/day intermittently. WPI birds received probiotics on the first three days of life, once a week, during the week of *Eimeria* species challenge starting one day before inoculation, and one day before, the day of, and one day after feed changes. The diet for birds receiving probiotics in the feed was mixed and pelleted weekly. Probiotics for the feed application were microencapsulated to protect the probiotic bacteria from high pelleting temperature. All birds were fed a basal diet *ad libitum* (Table 3.1). All animal protocols were approved and conducted under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee.

Probiotics Mixture Preparation

The probiotic product used in the experiment was a multi-species, host specific probiotic (PoultryStar, BIOMIN GmbH, Austria) containing *Bifidobacterium animalis* subspecies *animalis* DSM 16284, *Lactobacillus salivarius* subspecies *salivarius* DSM 16351 and *Enterococcus faecium* DSM 21913. For WPC and WPI groups, the CFU content of the product was $5x10^{12}$per

kg. For FSP group, the CFU content of the product was 10^{11} CFU per kg and was mixed into the finished feed at an inclusion rate of 1g per kg feed, giving a final concentration of 10^{8}CFU per kg of feed. This dosage was determined based on recovery rate of probiotic bacterial counts in pelleted feed samples from previous studies.

Eimeria Challenge

On d15 of age, all birds except those in the NEG group were challenged via oral gavage with a mixed inoculum of *Eimeria acervulina* (USDA isolate #12), *E. maxima* (Tysons isolate), and *E. tenella* (Wampler isolate). The dose was 1 mL per bird containing 50,000 *E. acervulina* oocysts, 10,000 *E. maxima* oocysts and 2,500 *E. tenella* oocysts. The inoculation rates of salinomycin-sensitive *Eimeria* species were based on previous studies in our laboratories. On d21 (6 d post infection), 24 birds per treatment (3 birds from each replicate pen) were randomly selected and euthanized for scoring of intestinal lesions caused by *Eimeria* infection. Lesions in the duodenum, jejunum and ceca were scored according to the method of Johnson and Reid (1970) by personnel blinded to treatment based on scores ranging from 0 (no gross lesion) to 4 (most severe lesion). Fecal samples were collected from each pen on d20-d24 (d5-d9 post infection) and kept in separate airtight plastic bags. For each pen, fresh excreta samples were collected from either side of the feeder, either side of the water supply, and from the center of the pen on each collection day. Starting excreta weights were recorded for each sample for later calculations of oocysts per gram of excreta as described by Dalloul and colleagues (2003). The weighed excreta samples for each day of collection were pooled according to pen, then homogenized and stored at 4°C until oocysts were counted microscopically using a McMaster counting chamber. Results were expressed as oocysts per gram of excreta.

Performance Measurements

Pen and feed weights were taken on DOH, d15, d21, d35 and d42. From these data, body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) were determined on a pen basis, and then averaged by treatment.

Statistical Analysis

Data were analyzed using the Fit Model platform in JMP Pro 10.0 (SAS Institute Inc., Cary, NC). Values were considered statistically different at $P \leq 0.05$. Results are reported as least square means (LS means) with standard errors of the mean (SEM).

Results

Eimeria Challenge

Lesion scores. On d21, lesion scores in the duodenum caused by *E. acervulina* infection showed a significant effect of treatment ($P < 0.0001$), as presented in Figure 3.1-A. The NEG treatment group had significantly lower lesion scores than all challenged treatment groups. The POS treatment group had higher lesion scores than the SAL group and WPC group. WPC had lower lesion scores than the FSP treatment. Similar to the duodenum, lesion scores caused by *E. maxima* in the jejunum on d21 (Figure 3.1-B) showed a significant effect of treatment ($P < 0.0001$). The NEG treatment group had lower lesion scores when compared to all challenged treatments. The POS treatment group had higher lesion scores than WPC and WPI, the two water administered probiotics. No significant lesions were observed in the ceca (Figure 3.1-C), which is the site of *E. tenella* infection ($P = 0.0473$).

Oocyst shedding. Fecal samples were collected on d20-d24 from each pen for evaluating oocyst shedding in the feces as presented in Figure 3.2. There was a significant effect of treatment on number of oocysts shed ($P = 0.0014$). The NEG control birds shed significantly fewer oocysts than those in the POS and SAL treatments. The POS group shed significantly more oocysts than the NEG, WPI, and FSP treatments. Numerically, all probiotic treated groups shed less oocysts than POS and SAL treatment groups.

Performance

Body weight (BW). Presented in Figure 3.3-A, a significant effect of treatment on BW was noted on d21 ($P < 0.0001$) (d6 after *Eimeria* infection). Birds in the NEG treatment had significantly higher BW than all other treatments, while the SAL birds exhibited higher BW than all three probiotic treatments and the POS group. A significant effect of treatment on BW was also noted on d35 ($P < 0.0001$), shown in Figure 3.3-B, and d42 ($P < 0.0001$), presented in Figure 3.3-C, with the NEG group showing heavier BW.

Body weight gain (BWG). A significant effect of treatment was evident from d15-d21, with the NEG group showing a greater BWG when compared to all other treatments ($P < 0.0001$), as presented in Figure 3.4-A. The SAL group showed greater BWG when compared to the POS and all probiotic product treatments, though still less than the NEG birds. From d21-d35, a significant effect of treatment was observed ($P < 0.0001$) where the NEG treatment had greater BWG than all other treatment groups (Figure 3.4-B). When comparing total BWG through the course of the study (DOH-d42), there was also a significant effect of treatment (Figure 3.4-C). The NEG birds gained significantly more weight when compared to all of the challenged treatment groups, while the probiotic treatments gained a comparable weight to the SAL birds ($P < 0.0001$).

Feed intake (FI). As shown in Figure 3.5-A, there was a significant treatment effect on FI from DOH-d15 ($P = 0.0050$), where SAL birds consumed more feed per bird per day than POS, WPC and WPI birds. From d15-d21, the NEG group consumed significantly more feed when compared to all other treatments (Figure 3.5-B). Also, the SAL birds consumed more feed than the WPC birds ($P < 0.0001$).

Feed conversion ratio (FCR). There was a significant effect of treatment on FCR during DOH-d15 (Figure 3.6-A). Treatment groups that received probiotics in drinking water had significantly lower FCR than SAL group ($P = 0.0017$). Figure 3.6-B presents the significant effect of treatment noted from d15-d21, with the NEG group exhibiting a significantly lower FCR when compared to all other treatment groups ($P < 0.0001$). The POS group had a higher FCR when compared to the SAL and NEG groups ($P < 0.0001$). The WPI treatment had a significantly higher FCR than the SAL group and the negative control ($P < 0.0001$), while treatment groups that received low dose of probiotics continuously in drinking water and in feed did not differ significantly from SAL treated group.

Discussion

In this study, the effects of probiotic products in the feed and water of broilers and their resistance to an *Eimeria* infection were investigated. Birds in the WPC treatment group had less severe duodenal and jejunal lesion scores, indicating a healthier intestine. While present in the challenge inoculum, the dose of *E. tenella* may not have been sufficient to cause extensive damage to the site of infection, resulting in the absence of lesions in the ceca. Similar to present results, Lee and colleagues (2010) reported that birds given a strain of a *Bacillus*-based direct-fed microbial had significantly lower lesions scores in the gastrointestinal tract than birds given the

non-supplemented diet following an *E. maxima* challenge. Studies investigating necrotic enteritis (NE) in broilers found birds given two different blends of direct-fed microbials had significantly reduced intestinal lesions due to NE than birds in the positive control (McReynolds et al., 2009). Fewer and less severe lesion scores are indicative of less damage to the epithelium of the intestine, leading to infected birds having a greater chance of recovery from disease. Numerous studies have found probiotic supplementation leads to significant reductions in numbers of other intracellular pathogens present in the intestine, such as *Salmonella* Enteritidis and *Campylobacter jejuni* (Higgins et al., 2007; Ghareeb et al., 2012). The pathogen load reductions could be due to multiple mechanisms of action employed by direct-fed microbials, depending on strains present in various products employed in those studies. Ultimately, the reduction in the presence of intracellular pathogens is indicative of a healthier intestine, with minimal damage done to the epithelium. An intact intestinal epithelium serves as the vital barrier preventing entry of potential pathogens and results in proper nutrient absorption and utilization, leading to optimal health and performance of the bird.

Birds receiving a high probiotic dose in the water supply on intermittent days (WPI) and in the feed (FSP) shed fewer oocysts in the feces than the positive control. Corroborating our findings, Dalloul and colleagues (2003, 2005) found that broilers provided a *Lactobacillus*-based probiotic in the feed shed significantly fewer *E. acervulina* oocysts when compared to the challenged control. A reduction in oocysts shed in the feces indicates improved resistance of the bird to *Eimeria* species infection. Not surprisingly, the birds receiving the anticoccidial in the feed handled the challenge during the peak infection period better than probiotic-treated birds with regard to performance. However, as the study progressed, the probiotics helped the birds to perform as well as the birds receiving anticoccidial, and better than birds in the positive control.

The reductions in BW and BWG due to the *Eimeria* challenge were not surprising, as coccidial infections are known to cause significant damage to the intestinal mucosa and enterocytes during the progression of their lifecycle. This extensive damage causes nutrient malabsorption and subsequent reduced performance. Furthermore, parasitic infections result in nutrient resource allocation shifting from growth to immune response, which can also lead to noticeable differences in growth (Allen and Fetterer, 2002; Dalloul and Lillehoj, 2005). Supporting the current findings, Mountzouris and colleagues (2007) found that broilers receiving probiotic in the feed performed as well as broilers receiving the coccidiostat in terms of BWG and FCR over the duration of the trial. Numerous studies investigating probiotics as dietary additives in poultry have resulted in varying effects of probiotics on performance. Some studies reported that probiotic supplementation in the diet can improve BWG and FCR in chickens (Kabir et al., 2004; Nayebpor et al., 2007; Apata, 2008; Talebi et al., 2008; Ignatova et al., 2009; Sen et al., 2012), while others found no significant benefit to probiotic addition (Rahimi et al., 2011; Wolfenden et al., 2011). These differences could be due to a variety of factors that can alter the efficacy of a probiotic such as strain(s) of bacteria utilized, composition and viability of the probiotic bacteria, and the preparation methods. Further, other factors may include probiotic dosage, method and/or frequency of application, overall diet, condition and age of the birds, potential drug interactions, as well as environmental stress factors such as temperature and stocking density (Patterson and Burkeholder, 2003; Mountzouris et al., 2007).

Consistent with previous studies (Mountzouris et al., 2007; Karimi Torshizi et al., 2010), our data suggest that water administration of the probiotic product would be the method of choice, especially in a coccidiosis challenge situation. While not examined in the current study, Karimi Torshizi et al. (2010) speculated that probiotics in water survive the demanding

conditions in the upper gastrointestinal tract for a few reasons. The first possibility is a shorter transit time when compared to solid feed. The second potential explanation is the water may limit the negative effects of gastric acid and digestive secretions on the microorganisms.

In conclusion, our data suggest that probiotics treatment (PoultryStar) helped alleviate the negative effects of the *Eimeria* species infection and may be utilized as a promising and beneficial 'anticoccidial.' Poultry researchers are investigating the latest alternatives that will protect flocks from disease while not hindering performance or negatively impacting profit margins, in order to remove feed grade medications. Early establishment of beneficial microbiota by probiotics in poultry may lead to increased overall health and wellbeing while decreasing the need for prophylactic antibiotic use. Numerous studies have demonstrated that commensal intestinal microbiota inhibit pathogens and that probiotics can increase resistance to infection (Rolfe, 2000; Patterson and Burkeholder, 2003). As PoultryStar is a product that contains multiple probiotic species of bacteria, there is a greater promise that such probiotics will be active in a wider range of conditions, similar to other multi-strain probiotics, resulting in greater efficacy (Fuller, 1989; Dalloul et al., 2003, 2005; Timmerman et al., 2006). Future research evaluating pertinent gene expression within the intestinal and immune tissues, microbial profiles, histological changes and other measurable parameters will provide further understanding of the probiotic effects and their mechanisms of action.

Acknowledgements

The authors wish to acknowledge Dr. Ray Fetterer (USDA-ARS, Beltsville) for providing *Eimeria* oocysts and Dr. Diego Paiva for technical assistance.

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Table 3.1. Composition of broiler diets during 3 growing phases.

Item	Starter (DOH to d15)	Grower (d15 to d35)	Finisher (d35 to d42)
Ingredient, %			
Corn	60.55	65.63	69.90
Soybean meal	22.42	16.43	10.76
Distiller's grain	7.00	8.00	9.00
Poultry by-product meal	5.00	5.00	4.00
Grease (yellow)	1.91	2.12	2.79
Dicalcium phosphate	1.15	0.90	0.78
L-Lysine	0.63	0.60	0.80
Limestone	0.58	0.54	0.70
DL-Methionine	0.18	0.30	0.80
Salt	0.27	0.17	0.16
L-Threonine	0.10	0.10	0.10
Southern States vitamin premix	0.10	0.10	0.10
Southern States trace mineral premix	0.10	0.10	0.10
Optiphos	0.01	0.01	0.01
Total	100.00	100.00	100.00
Calculated nutrient level			
ME, kcal/kg	3,036.00	3,102.00	3,157.00
CP, %	21.00	19.00	17.00
Ca, %	0.90	0.80	0.76
Available P, %	0.45	0.40	0.35
Total P, %	0.71	0.64	0.57
Digestible Lys, %	1.50	1.33	1.32
Digestible Meth, %	0.50	0.60	1.06
Digestible Thr, %	0.89	0.81	0.71
Digestible Trp, %	0.22	0.19	0.16

Figure Captions

Figure 3.1. Effect of administration of probiotics (PoultryStar) on d21 lesion scores of Cobb 500 male broiler challenged with Eimeria species on d15. Data are presented as Least Square Means ± SEM; bars lacking a common letter differ significantly. There was a significant effect of treatment on lesion scores in the duodenum (Fig. 3.1-A), the site of *E. acervulina* infection ($P < 0.0001$), in the jejunum (Fig. 3.1-B), the site of *E. maxima* infection ($P < 0.0001$), but no significant lesions were observed in the ceca (Fig. 3.1-C), the site of *E. tenella* infection ($P = 0.0473$). NEG = negative control; POS = positive control; SAL = Salinomycin continuous in feed; WPC = probiotic at low dose continuous water administration; WPI = probiotic at high dose intermittent water administration; FSP = probiotic continuous in feed.

Figure 3.1-A

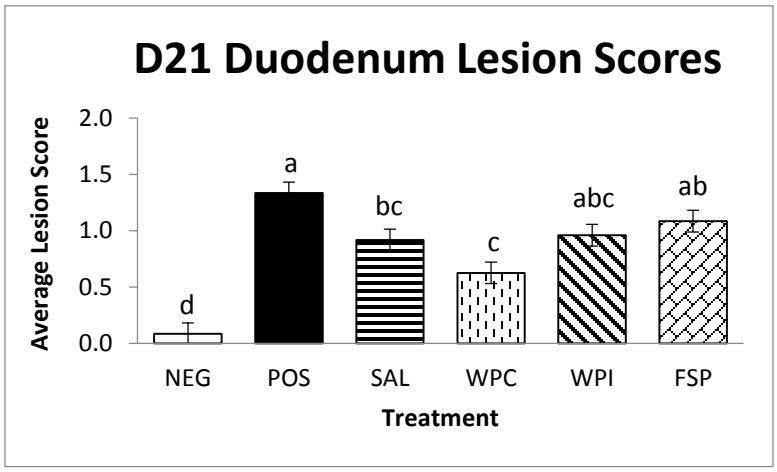


Figure 3.1-B

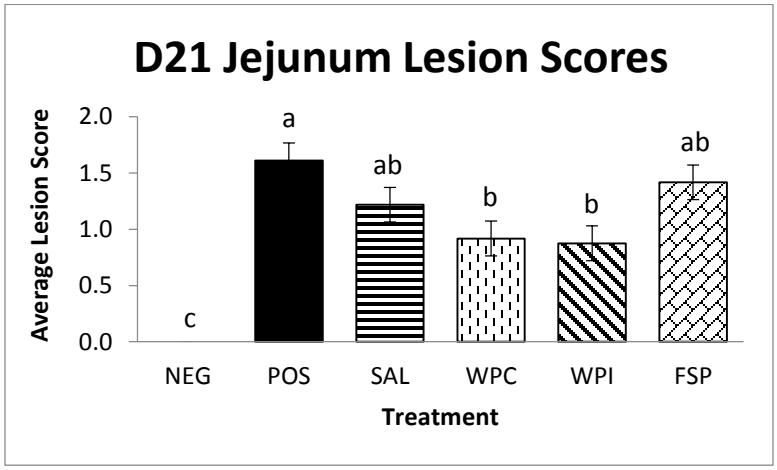


Figure 3.1-C

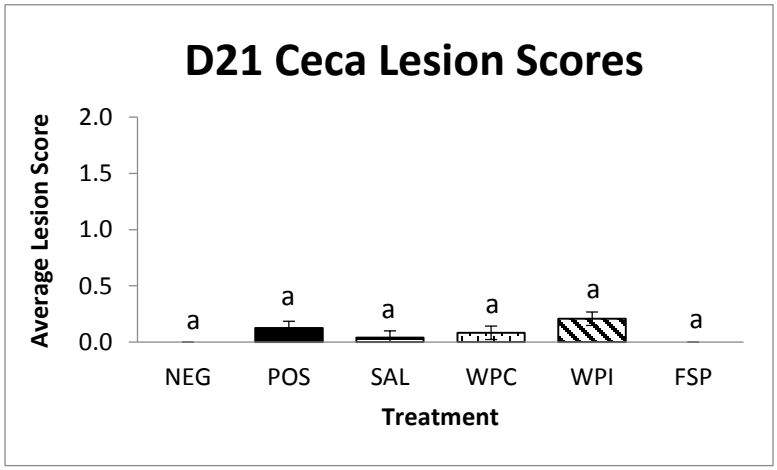


Figure 3.2. Effect of administration of probiotics (PoultryStar) on d20-d24 oocyst shedding in feces of Cobb 500 male broiler chicks challenged with Eimeria species on d15. Data are presented as Means ± SEM; bars lacking a common letter differ significantly. There was a significant effect of treatment ($P = 0.0014$). NEG = negative control; POS = positive control; SAL = Salinomycin continuous in feed; WPC = probiotic at low dose continuous water administration; WPI = probiotic at high dose intermittent water administration; FSP = probiotic continuous in feed.

Figure 3.2

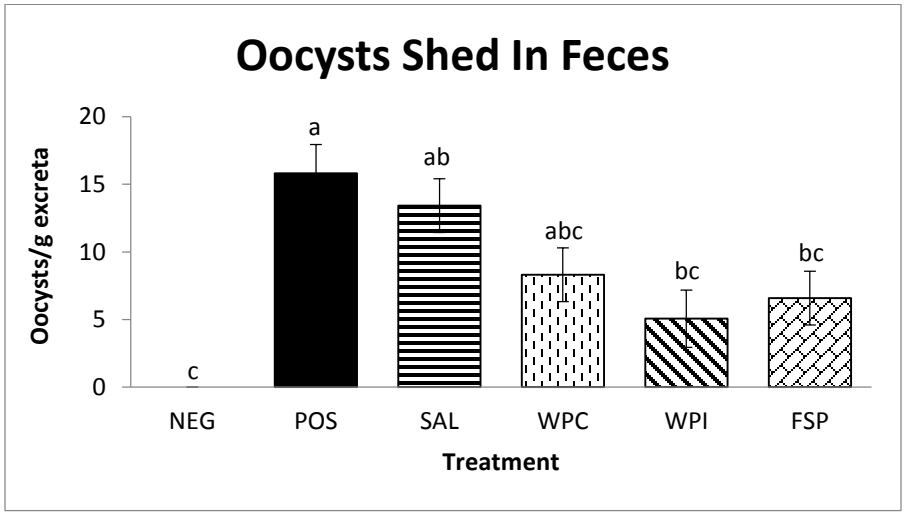

Oocysts Shed In Feces
Oocysts/g excreta
20
15
10
5
0
a
ab
abc
bc
bc
c
NEG
POS
SAL
WPC
WPI
FSP
Treatment

Figure 3.3. Effect of administration of probiotics (PoultryStar) on body weight of Cobb 500 male broiler chicks. Data are presented as Least Square Means ± SEM; bars lacking a common letter differ significantly. There was a significant effect of treatment ($P < 0.0001$) on d21 (Fig. 3.3-A), on d35 (Fig. 3.3-B), and on d42 (Fig. 3.3-C). NEG = negative control; POS = positive control; SAL = Salinomycin continuous in feed; WPC = probiotic at low dose continuous water administration; WPI = probiotic at high dose intermittent water administration; FSP = probiotic continuous in feed.

Figure 3.3-A

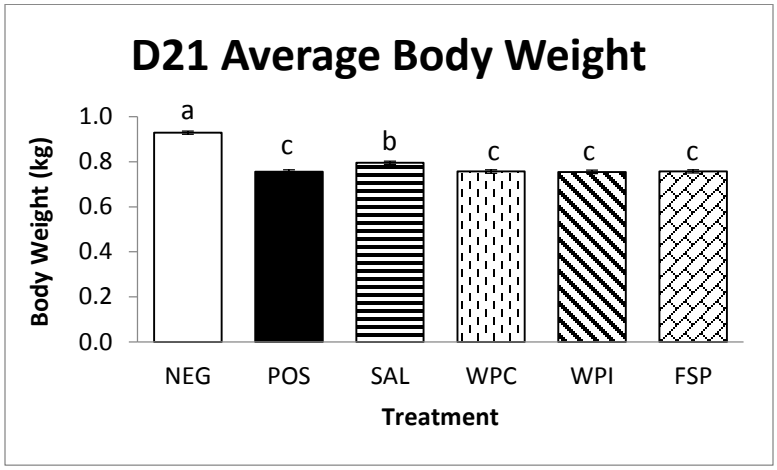


Figure 3.3-B

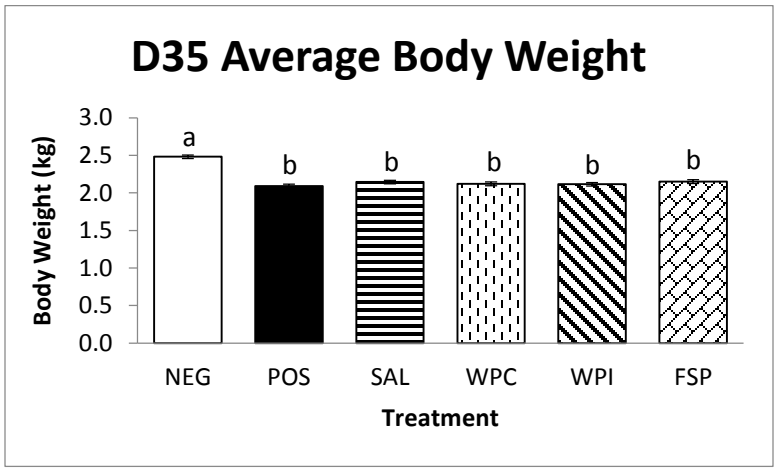


Figure 3.3-C

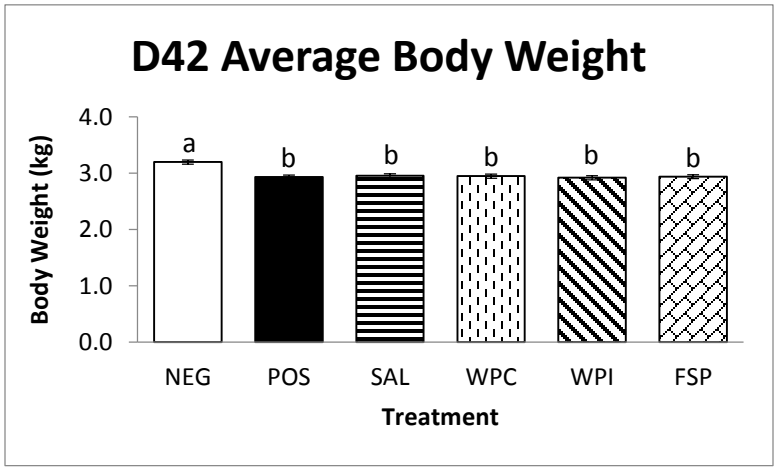


Figure 3.4. Effect of administration of probiotics (PoultryStar) on body weight gain (BWG) of Cobb 500 male broiler chicks. Data are presented as Least Square Means ± SEM, bars lacking a common letter differ significantly. There was a significant effect of treatment ($P < 0.0001$) on BWG from d15-d21, the 6 days immediately following challenge (Fig. 3.4-A), from d21-d35 (Fig. 3.4-B), and through the course of the trial from DOH-d42 (Fig. 3.4-C). NEG = negative control; POS = positive control; SAL = Salinomycin continuous in feed; WPC = probiotic at low dose continuous water administration; WPI = probiotic at high dose intermittent water administration; FSP = probiotic continuous in feed.

Figure 3.4-A

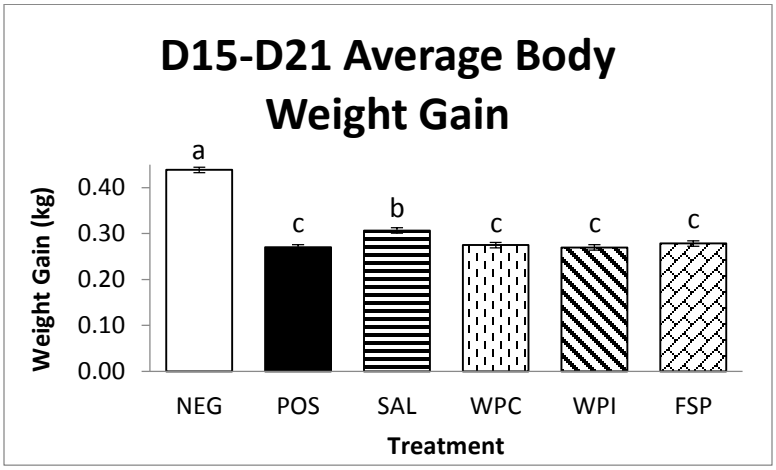


Figure 3.4-B

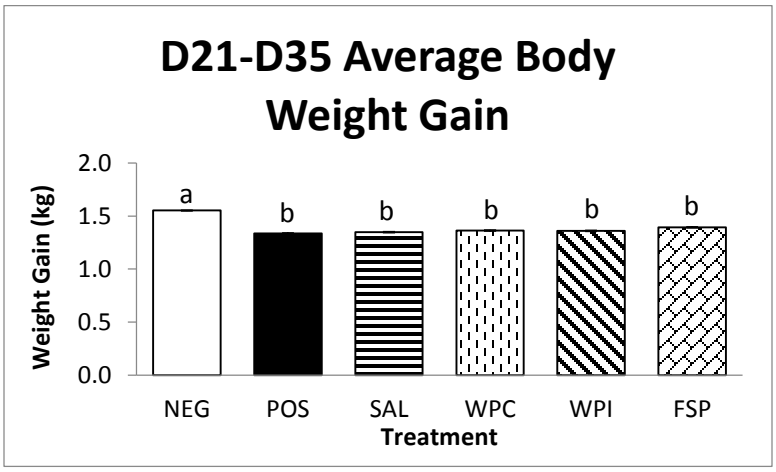


Figure 3.4-C

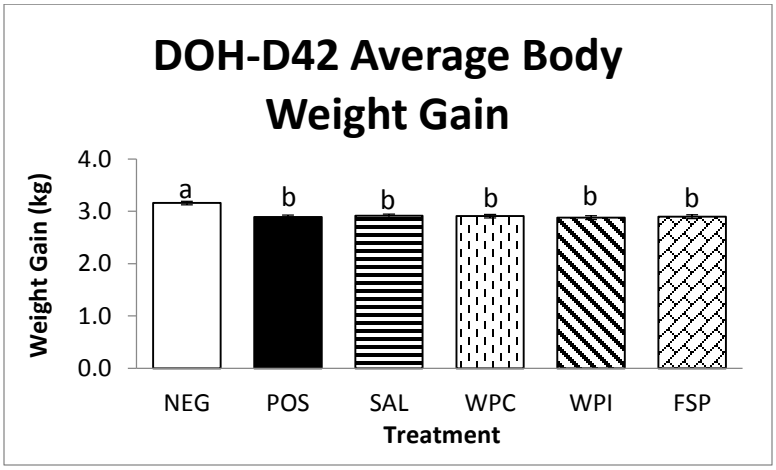


Figure 3.5. Effect of administration of probiotics (PoultryStar) on feed intake (FI) per bird per day of Cobb 500 male broiler chicks. Data are presented as Least Square Means ± SEM. Bars lacking a common letter differ significantly. There was a significant effect of treatment on FI from DOH-d15 (Fig. 3.5-A, $P = 0.0050$) and from d15-d21 (Fig. 3.5-B, $P < 0.0001$). NEG = negative control; POS = positive control; SAL = Salinomycin continuous in feed; WPC = probiotic at low dose continuous water administration; WPI = probiotic at high dose intermittent water administration; FSP = probiotic continuous in feed.

Figure 3.5-A

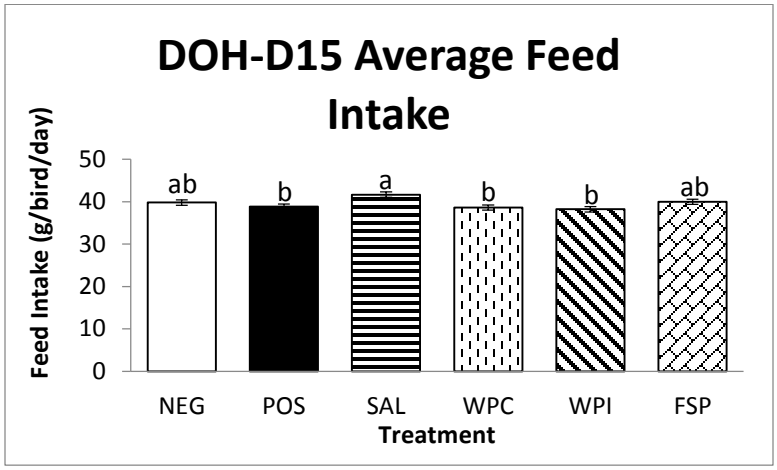


Figure 3.5-B

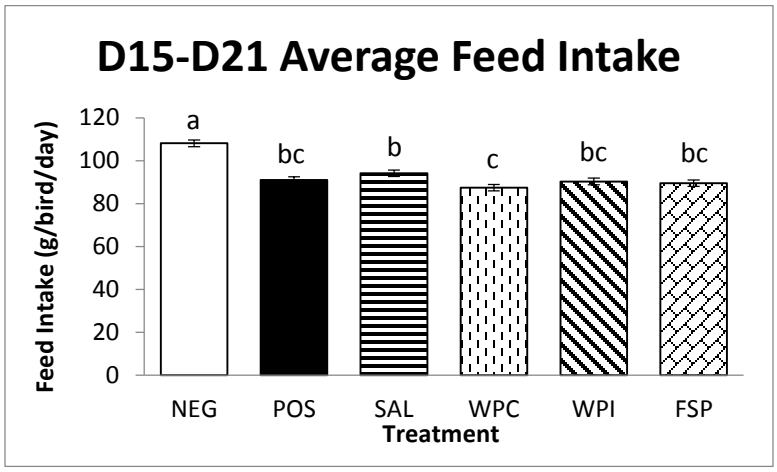


Figure 3.6. Effect of administration of probiotics (PoultryStar) on feed conversion ratio (FCR) of Cobb 500 male broiler chicks. Data are presented as Least Square Means ± SEM; bars lacking a common letter differ significantly. There was a significant effect of treatment on FCR from DOH-d15 (Fig 3.6-A, P =0.0017) and from d15-d21 (Fig 3.6-B, $P < 0.0001$). NEG = negative control; POS = positive control; SAL = Salinomycin continuous in feed; WPC = probiotic at low dose continuous water administration; WPI = probiotic at high dose intermittent water administration; FSP = probiotic continuous in feed.

Figure 3.6-A

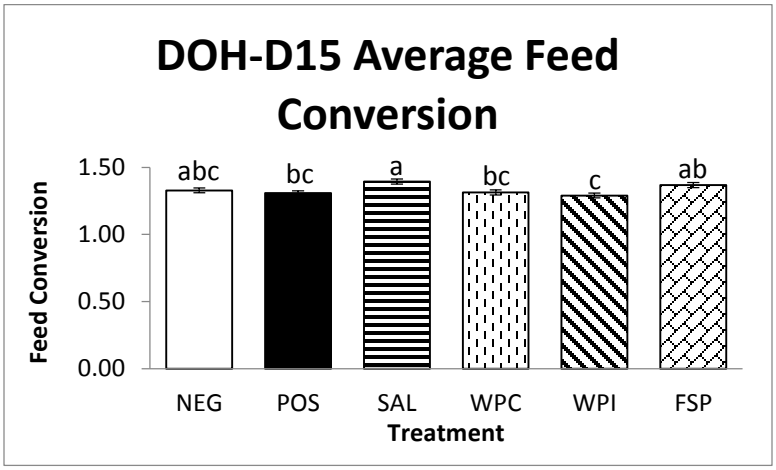
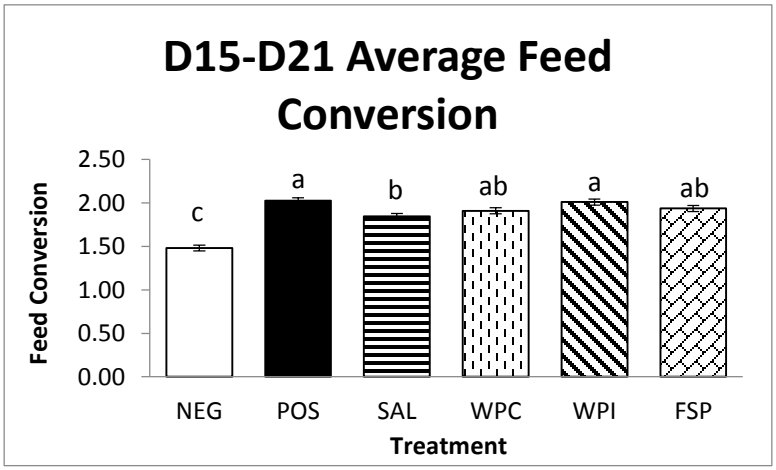


Figure 3.6-B



CHAPTER IV

Evaluation of the Combined Protective Effects of Probiotics and Coccidia Vaccine in *Eimeria*-challenged Birds

Abstract: Coccidiosis is endemic in the commercial broiler industry capable of inflicting devastating economic losses to poultry operations. While vaccines are relatively effective in controlling the disease, efficacy could potentially be improved with concurrent use of probiotics. This study evaluated the combined protective effect of Biomin PoultryStar probiotic and Ceva Immucox coccidia vaccine in response to a mixed *Eimeria* species challenge in commercial broilers. On day of hatch, 400 commercial male broilers (Cobb-500) were assigned to one of 4 treatment groups, including a control (CON), a vaccine-only gel application (VNC), a probiotic-only gel application (NPC), and a vaccine and probiotic gel application (VPC). Birds were placed in floor pens with 6 replicate pens per treatment (16-17 birds per pen). NPC and VPC birds received PoultryStar in the water supply on days 2-4, 8, 14-20, 22, 29, and 34-36. On d15, birds were orally gavaged with 0.5 mL of a mixed inoculum of *Eimeria acervulina*, *E. maxima*, *E. necatrix* and *E. tenella*, prepared with Immucox vaccine at 10 X the normal dose. Performance measurements were recorded on first day and weekly afterwards, and lesion scores were evaluated on d21 (6 d post-challenge). Data were analyzed using the Fit Model platform in JMP Pro 10.0 (SAS) where differences in experimental treatments were tested using Tukey HSD following ANOVA with significance reported at $P \leq 0.05$. Overall, PoultryStar and Immucox resulted in an enhanced protective effect against the coccidia challenge, with VPC birds demonstrating significantly lower lesion scores in the duodenum than VNC or NPC birds. Birds in the VPC treatment also demonstrated significantly higher body weight gains during d1-d15, d7-d15, and d21-d28 when compared to the VNC birds. These results suggest that the

combination of PoultryStar and Immucox vaccine can enhance performance and provide an additional protective effect against a mixed *Eimeria* challenge.

Introduction

The decades-old practice of supplying food animals with sub-therapeutic doses of antibiotics to protect against infections and improve general health has recently been under scrutiny. These practices lead to development of resistance by the *Eimeria* parasites to the drugs in use, as well as consumer concerns regarding residues in food products. The relatively recent ban of sub-therapeutic doses of certain antibiotics as feed additives in the European Union led to a general decline in animal health with increased incidences of enteric conditions (Castanon, 2007), known as dysbiosis. This outcome, as well as the threat of a domestic ban, has led researchers to explore the next promising alternatives including live oocyst vaccines, probiotics, and potential combinations of the two.

Although the primary function of the gastrointestinal tract is to digest and absorb nutrients, a well-balanced gut microbiota is crucial for optimal animal health and performance. The gastrointestinal tract also serves as a vital barrier preventing the entry of potentially harmful pathogens and other environmental antigens (Kogut and Swaggerty, 2012). As the gut microbiota begins to establish within hours after the chick hatches, the earlier the introduction of nonpathogenic microorganisms, the more effective their establishment in the digestive tract (Timmerman et al., 2006; Torok et al., 2007). Also known as direct-fed microbials, probiotics are classified as live nonpathogenic microorganisms that are capable of maintaining a normal gut microbial population (Patterson and Burkholder, 2003; Ohimain and Ofongo, 2012). Probiotics help maintain a healthy balance of microorganisms within the intestine, which is accomplished through multiple modes of action. Those mechanisms include competitive exclusion, pathogen

antagonism, and stimulation of the immune system (Ohimain and Ofongo, 2012). The presence of probiotics prevents the colonization of pathogenic bacteria and attenuates enteric diseases, which ultimately result in enhanced performance of poultry (Kabir et al., 2004). Probiotics may provide a potential alternative to the prophylactic use of drugs in food animals due to their studied abilities to reduce enteric diseases in poultry (Patterson and Burkholder, 2003; Eckert et al., 2010). Probiotics can be composed of one or many strains of microbial species, with the more common ones belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, and *Pediococcus* (Gaggia et al., 2010).

Coccidiosis is endemic in the commercial broiler industry and inflicts devastating economic losses to poultry operations. Caused by development and reproduction of multiple species of the *Eimeria* protozoa, coccidiosis is estimated to cost the industry about US $3 billion annually worldwide (Dalloul and Lillehoj, 2006). *Eimeria* species are unlike other protozoan parasites in that the primary target tissue is the intestinal epithelium, which results in considerable impairment of growth and feed utilization in poultry. The route of infection of these protozoa is through the consumption of fecal droppings of infected birds (Li et al., 2005). Coccidiostats have been used to control coccidiosis, but *Eimeria* species have developed resistance to both chemical and ionophore drugs over time (Stringfellow et al., 2011). Due to drug resistance and consumer concerns regarding drug usage, the practice of live vaccines to control coccidiosis has greatly increased. Vaccines provide an alternative for disease protection, and they ultimately help in reducing *Eimeria* resistance as they systematically replace resistant field strains and induce specific protective immunity by exposing the chicken's immune system to *Eimeria* antigens (Williams, 2002; Dalloul and Lillehoj, 2005; Stringfellow et al., 2011). Immunity is subsequently boosted and maintained by multiple re-infections caused by oocysts

present in the litter due to shedding and ingestion (Williams, 2002). One characteristic of live vaccines is immunity to avian coccidia is strongly species-specific, therefore the bird will only develop immunity to the species of *Eimeria* present in the vaccine (Williams, 2002; Dalloul and Lillehoj, 2006). This specificity mandates that the vaccine have the species of *Eimeria* known to be prevalent in that area, which are more likely to cause an outbreak (Dalloul and Lillehoj, 2005). Early and uniform administration of coccidiosis vaccines (typically during the first 1 to 7 days of life) is extremely important for the success of these live vaccines. Early exposure of the chick's immune system to antigen results in immunity developing at a younger age, minimizing risk of exposure while the chick is unprotected. Administration of live oocysts of the vaccine results in a low level infection, necessary for immunity development. However, it can cause an early reduction in growth and may increase the chick's susceptibility to secondary infections, such as necrotic enteritis (Dalloul and Lillehoj, 2005; Li et al., 2005; Stringfellow et al., 2011). The potential consequences of coccidiosis vaccine administration at a young age could be overcome by proper and uniform delivery of the vaccine, as well as the chick having a healthy intestinal tract colonized by a normal microbiota (Dalloul and Lillehoj, 2005; Stringfellow et al., 2011). Probiotics have potential to enhance host defenses and affect the digestive microbiota positively, while protecting against colonization by harmful bacteria and maintaining intestinal integrity (Dalloul et al., 2003; Dalloul and Lillehoj, 2005; Hume, 2011; Stringfellow et al., 2011; Ritzi et al., 2014). Based on these findings, probiotics may be able to attenuate the negative consequences of early vaccine administration. This study aimed to evaluate the combined protective effects of a probiotic product (PoultryStar, BIOMIN GmbH, Austria) containing *Enterococcus*, *Bifidobacterium*, *Pediococcus* and *Lactobacillus* species and a coccidiosis vaccine

(Immucox, CEVA Santé Animale, Canada) containing *Eimeria acervulina*, *E. maxima*, *E. necatrix*, and *E. tenella* oocysts, against a coccidiosis challenge in commercial broiler chickens.

Materials and Methods

Birds and Experimental Treatments

The study performed was a 42-day grow-out with 400 Cobb-500 commercial male broilers housed in floor pens, with 6 replicate pens per treatment and 16 or 17 birds per pen. On day of hatch (DOH), 100 birds were treated for each of the following four treatments at the hatchery: 1) control (CON), 2) vaccinated-only (VNC), 3) water-applied probiotic only (NPC), and 4) vaccinated and water-applied probiotic (VPC). VNC and VPC birds received Immucox vaccine through gel droplet administration at the hatchery. Birds in NPC and VPC received PoultryStar via gel droplet application at the hatchery, as well as in the water intermittently through the course of the trial. The probiotic was administered in the water at 20 mg/bird per day on the first three days after placement, once a week, the week of *Eimeria* species challenge starting one day prior to inoculation, and one day before, the day of, and one day after a feed change. Probiotics were administered a total of 17 time points, including days 2-4, 8, 14-20, 22, 29, and 34-36. All birds received a basal diet *ad libitum*, shown in Table 4.1. All animal protocols were approved and conducted under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee.

Eimeria Challenge

On d15 of age, birds were challenged via oral gavage with 0.5 mL of Immucox vaccine at 10 X the vaccine dose given at hatch, providing a mixed *Eimeria* challenge. On d21 (6 d post infection), 18 birds per treatment were randomly selected and euthanized for scoring of lesions

from intestinal *Eimeria* infection. Lesions in the duodenum, jejunum, ileum and ceca were scored by the method of Johnson and Reid (1970) by personnel blinded to treatments based on scores ranging from 0 (no gross lesion) to 4 (most severe lesion). Excreta samples were collected from each pen on days 6-8 and day 14 after challenge. For each pen, fresh excreta samples were collected from either side of the feeder, either side of the water supply, and from the center of the pen. Samples were kept in separate airtight plastic bags. Starting excreta weights were recorded for each sample for later calculations of oocysts per gram of excreta as described by Dalloul and colleagues (2003). After homogenization, samples were stored at 4°C until assessed for oocyst counts, which were determined by dilution and counts via microscope using a McMaster counting chamber and expressed as oocysts per gram of excreta. Weekly litter samples were collected from each pen to assess moisture content. Five samples were taken from each pen once a week and stored in airtight plastic bags. The samples were transferred to paper bags and placed in a drying oven at 55°C for 24 hours, with both starting and final weights recorded. From d15-d24, excreta in each pen were evaluated and scored for bloody diarrhea as described by Youn and Noh (2001).

Performance

Pen and feed weights were taken on DOH, days 7, 15, 21, 28, 35 and 42. From these data, body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) were determined on a pen basis, and then averaged by treatment. Mortality was checked twice daily and feed conversion was corrected accordingly. One VNC pen was excluded from all calculations due to high mortality in the first weeks of the study.

Statistical Analysis

Data were analyzed using the Fit Model platform in JMP Pro 10.0 (SAS Institute Inc., Cary, NC). For performance measurements, oocyst shedding, and litter moisture analysis, the model included treatment with pen representing the experimental unit. Lesion score analysis was performed with treatment in the model with bird serving as the experimental unit. Differences among experimental treatments were tested using Tukey HSD following ANOVA. Values were considered statistically different at $P \leq 0.05$. Results are reported as Least Square Means (LS means) with standard errors of the mean (SEM).

Results

Eimeria Challenge

Lesion scores. On d21, a significant effect of treatment ($P < 0.0001$) was noted in lesion scores in the duodenum, the site of *E. acervulina* infection, as presented in Figure 4.1. The CON birds had significantly higher lesion scores than VNC and VPC birds ($P < 0.0001$). No lesions were observed in the duodenum of VPC birds, resulting in VPC being significantly different than all other treatments. The jejunum is one segment prone to damage from *E. maxima* and *E. necatrix*; the CON birds demonstrated significantly higher lesions in the jejunum than all other treatments ($P < 0.0001$). No lesions were observed in the ileum of VNC or NPC birds on d21. The average lesion scores in the ileum for CON and VPC birds were low, resulting in no significant differences among treatments. In addition, no lesions were observed in the ceca, the site of *E. tenella* infection, in any treatment (data not shown).

Oocyst shedding. Following the challenge administered on d15, a significant effect of treatment ($P = 0.0091$) on oocysts shed was noted seven days later (d22). Shown in Figure 4.2, VNC birds shed more oocysts per gram of excreta than CON birds only.

Litter moisture. Figure 4.3-A represents the significant effect of treatment ($P = 0.0119$) on litter moisture on d7. The litter present in the VNC pens had significantly less moisture when compared to CON and VPC pens. Litter moisture also demonstrated a significant effect of treatment on d14 (Figure 4.3-B) where VNC pens had significantly lower ($P < 0.0001$) litter moisture when compared to all other treatments. In addition, NPC pens had significantly higher percent moisture of the litter when compared to the CON pens ($P < 0.0001$). At the end of the trial, a significant effect of treatment ($P = 0.0245$) was noted on percent moisture of the litter. Shown in Figure 4.3-C, NPC pens had significantly higher percent moisture than VNC pens only.

Bloody diarrhea scores. No significant differences among treatments were noted regarding the presence of bloody diarrhea from days 15-24 (data not shown).

Performance

Results for performance parameters are summarized in Table 4.2.

Body weight (BW). A significant effect of treatment ($P = 0.00078$) was seen on d15, with NPC and VPC birds demonstrating significantly higher average BW than VNC birds. A significant effect of treatment was also seen on d21, with NPC birds having greater BW than the VNC birds ($P = 0.0434$). Average BW on d28 showed a significant effect of treatment, with VNC birds weighing significantly less than all other treatments ($P = 0.0008$).

Body weight gain (BWG). A significant effect of treatment was seen from DOH-d15 regarding BWG, with probiotic-treated birds (NPC and VPC) gaining significantly more weight than VNC

birds (P = 0.0079). Within the starter phase, from d7-d15, VPC birds gained significantly more weight than VNC birds only (P = 0.0289). The same trend was observed from d21-d28, where VPC birds had significantly higher BWG than VNC birds, with CON and NPC birds demonstrating comparable weight gains (P = 0.0032).

Feed intake (FI). A significant effect of treatment was seen from d7-d15 regarding FI, with VNC birds consuming more feed per day than CON birds (P = 0.0425).

Feed conversion ratio (FCR). For the period prior to challenge (DOH-d15), VNC birds had significantly higher FCR than CON birds (P = 0.0358). From d7-d15, VNC birds demonstrated significantly higher FCR than all other treatments (P = 0.0074).

Discussion

In this study, the combined protective effects of a probiotic and coccidia vaccine in the event of an *Eimeria* challenge were evaluated. Birds in the combined vaccine/probiotic treatment group had less severe duodenal lesion scores than all other treatments. Further, CON birds had lesions of greater severity than VNC birds in the duodenum, as well as lesion scores significantly greater than all other treatments in the jejunum. These findings suggest that probiotic supplementation, vaccine administration, and a combination of both help prevent damage to the intestine from coccidia. Similarly, Lee and colleagues (2010) reported that birds given a *Bacillus*-based direct-fed microbial had significantly lower lesion scores in the gut than birds given the non-supplemented diet following an *E. maxima* challenge. Studies investigating necrotic enteritis (NE) in broilers found birds given two different blends of direct-fed microbials had significantly reduced intestinal lesions due to NE than birds in the positive control (McReynolds et al., 2009). Li and colleagues (2005) found that birds receiving a coccidia

vaccine without therapeutic levels of an ionophore anticoccidial in the feed had less severe lesion scores in the upper and middle intestinal segments when compared to non-vaccinated, medicated birds following challenge with three *Eimeria* species. A second study found that birds given one of three different coccidiosis vaccine doses had less severe lesion scores than the positive non-vaccinated control (Li et al., 2005). Less severe lesion scores are indicative of less damage to the intestinal epithelium, leading to infected birds having a greater chance of recovery from disease. Numerous studies have found that probiotic supplementation leads to significant reductions in numbers of other intracellular pathogens present in the intestine, such as *Salmonella* Enteritidis and *Campylobacter jejuni* (Higgins et al., 2007; Ghareeb et al., 2012). Such effect of probiotic supplementation could prove to be exceptionally beneficial to the bird, as some microorganisms such as *Clostridium* and *Salmonella* may exacerbate *Eimeria* infections and vice versa (Chapman et al., 2002). Ultimately, the reduction in the presence of intracellular pathogens is indicative of a healthier intestine, with minimal damage done to the epithelium. An intact intestinal epithelium serves as the vital barrier preventing entry of potential pathogens and results in proper nutrient absorption and utilization, leading to optimal health and performance of the bird.

The presence of oocysts in the litter and feces after vaccination is crucial in vaccinated flocks, as it indicates proper vaccine uptake. Vaccine efficacy is dependent upon the infectivity and fecundity of oocysts, since protective immunity is induced after two to three consecutive infections (Vermeulen et al., 2001; Williams, 2002; Chapman et al., 2005). The re-infections are initiated by recycling of initially low doses of oocysts which result in gradual buildup and maintenance of immunity (Allen and Fetterer, 2002). While the only significant effect of treatment on oocyst shedding after challenge was seen on d7 post-challenge, where CON birds shed fewer oocysts than VNC birds, an interesting trend was observed d8 and d14 post-

challenge. Though not statistically significant, NPC and VPC birds shed fewer oocysts than CON and VNC birds, suggesting the supplementation of probiotic had an effect on susceptibility to infection. Corroborating our findings, Dalloul and colleagues (2003, 2005) found that broilers provided a *Lactobacillus*-based probiotic in the feed shed significantly fewer *E*. *acervulina* oocysts when compared to the challenged control. A reduction in oocysts shed in the feces indicates improved resistance of the bird to *Eimeria* species infection.

VNC birds shed more oocysts throughout the trial while percent moisture in VNC pens was significantly lower on d7, d14, and d42. Numerous studies have found that oocysts sporulate better in drier litter conditions, suggesting maximum sporulation rate and litter moisture are indirectly correlated (Waldenstedt et al., 2001; Chapman et al., 2002; Williams, 2002). As the infective state of the coccidia life cycle is the sporulated oocyst, birds on litter with lower percent moisture could be introduced to a greater number of infective oocysts, leading to a heavier infection. Bloody diarrhea is commonly associated with *E*. *tenella*, which was present in the challenge inoculum. However, as no significant differences were noted, the dose of *E*. *tenella* may not have been sufficient to cause extensive damage to the site of infection, as confirmed by the absence of lesions in the ceca.

VPC birds demonstrated significantly greater BWG from DOH-d15 when compared to VNC birds, suggesting the addition of probiotics helped the birds counter the reduction in growth associated with administration of a coccidiosis live vaccine. However, the lack of a significant difference among treatments regarding BW at the end of the trial indicates VNC birds experienced compensatory growth following the initial setback from vaccination. These results coincide with the findings of Li and colleagues (2005), in which a "reaction" caused by some doses of vaccine resulted in delayed growth and coccidial lesions during the two weeks

following vaccination. However, the vaccinated birds exhibited a compensatory weight gain that brought them to weights almost equal to the unchallenged control by 5-6 weeks of age (Li et al., 2005). The effect of *Eimeria* challenge on BW and BWG is not surprising, as coccidial infections are known to cause damage to the intestinal mucosa and enterocytes during the progression of their lifecycle. Significant damage causes nutrient malabsorption and subsequent reduced performance. Furthermore, parasitic infections result in nutrient resource allocation shifting from growth to immune response, which can also lead to noticeable differences in growth (Allen and Fetterer, 2002; Dalloul and Lillehoj, 2005). Numerous studies investigating probiotics as dietary additives in poultry have resulted in varying effects of those probiotics on performance. Some reported that probiotic supplementation in the diet can improve BWG and FCR in chickens (Kabir et al., 2004; Nayebpor et al., 2007; Apata, 2008; Talebi et al., 2008; Ignatova et al., 2009; Sen et al., 2012), while others found no significant benefit to probiotic addition (Rahimi et al., 2011; Wolfenden et al., 2011). These differences could be due to a variety of factors that can alter the efficacy of a probiotic such as strain(s) of bacteria utilized, composition and viability of the probiotic bacteria, and the preparation methods. Further, other factors may include probiotic dosage, method and/or frequency of application, overall diet, condition and age of the birds, presence or absence of disease challenges, potential drug interactions, as well as environmental stress factors such as temperature and stocking density (Patterson and Burkeholder, 2003; Mountzouris et al., 2007).

Live vaccines offer a route of protection that circumvents the issue of developing drug-resistant coccidia. In fact, evidence shows that vaccines with drug-sensitive parasites can ameliorate drug resistance of wild-type coccidial infections on farms where drug-resistant strains dominate (Chapman et al., 2002; Williams, 2002). As vaccination induces protective immunity

due to exposure of the immune system to *Eimeria* antigens, the birds may respond with a strong immune response more quickly to a field strain *Eimeria* infection (Chapman et al., 2005; Stringfellow et al., 2011). In conclusion, the administration of probiotics (PoultryStar) and coccidiosis vaccine (Immucox) resulted in an enhanced protective effect against *E. acervulina* and *E. maxima* challenge. The results of this study suggest that the combination of probiotics and coccidiosis vaccine can, when compared to untreated controls, result in better performance and intestinal response in early oocyst cycling. Administration of probiotics early in life may stabilize the intestinal tract at the time of vaccination, reducing the potential negative effect, and/or have an adjuvant effect by modulating the host immune system (Stringfellow et al., 2011). Poultry researchers are investigating the latest alternatives that will protect flocks from disease while not hindering performance or negatively impacting profit margins. Early establishment of beneficial microbiota by probiotics in poultry can lead to increased overall health and wellbeing while decreasing the need for prophylactic and preventative use of antibiotics. Beneficial intestinal microbiota can inhibit pathogens, and probiotics can potentiate this protective effect and also increase host resistance to infection (Rolfe, 2000; Patterson and Burkeholder, 2003; Ritzi et al., 2014). As PoultryStar is a product that contains multiple probiotic species of bacteria, there is a greater potential that such probiotics can be active in a wider range of conditions, similar to other multi-strain probiotics, resulting in greater efficacy (Fuller, 1989; Timmerman et al., 2006). Probiotics may enhance host defenses and improve vaccine response as a result of the influence of beneficial bacteria on host immunity and intestinal integrity against enteric pathogens (Dalloul et al., 2003; Stringfellow et al., 2011). Together, probiotics and coccidiosis vaccines can benefit performance and provide an augmented protective effect in the event of an *Eimeria* challenge.

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Table 4.1. Composition of broiler diets during 3 growing phases.

Item	Starter (DOH to d15)	Grower (d15 to d35)	Finisher (d35 to d42)
Ingredient, %			
Corn	60.55	65.63	69.90
Soybean meal	22.42	16.43	10.76
Distiller's grain	7.00	8.00	9.00
Poultry by-product meal	5.00	5.00	4.00
Grease (yellow)	1.91	2.12	2.79
Dicalcium phosphate	1.15	0.90	0.78
L-Lysine	0.63	0.60	0.80
Limestone	0.58	0.54	0.70
DL-Methionine	0.18	0.30	0.80
Salt	0.27	0.17	0.16
L-Threonine	0.10	0.10	0.10
Southern States vitamin premix	0.10	0.10	0.10
Southern States trace mineral premix	0.10	0.10	0.10
Optiphos	0.01	0.01	0.01
Total	100.00	100.00	100.00
Calculated nutrient level			
ME, kcal/kg	3,036.00	3,102.00	3,157.00
CP, %	21.00	19.00	17.00
Ca, %	0.90	0.80	0.76
Available P, %	0.45	0.40	0.35
Total P, %	0.71	0.64	0.57
Digestible Lys, %	1.50	1.33	1.32
Digestible Meth, %	0.50	0.60	1.06
Digestible Thr, %	0.89	0.81	0.71
Digestible Trp, %	0.22	0.19	0.16

Table 4.2. Effect of administration of PoultryStar probiotic and Immucox coccidiosis vaccine on performance.

	Treatment[1]					
Variable	CON	VNC	NPC	VPC	SEM	*P*-value
DOH-d7						
DOH BW, g	37.41	37.37	38.23	37.39	0.25	0.6090
d7 BW, g	150.85	142.37	154.17	150.50	3.31	0.1039
BWG, g	113.44	105.00	116.33	113.11	3.28	0.1202
FI, g/bird/day	30.01	28.14	37.62	37.61	3.31	0.1111
FCR	1.86	1.85	2.27	2.33	0.21	0.2525
d7-d15						
d15 BW, g	362.93^{AB}	341.23^{B}	379.42^{A}	379.22^{A}	7.88	0.0078
BWG, g	212.08^{AB}	198.87^{B}	225.25^{AB}	228.72^{A}	7.08	0.0289
FI, g/bird/day	46.82 ^{B}	59.89 ^{A}	48.38 ^{AB}	49.03 ^{AB}	3.31	0.0425
FCR	1.55 ^{A}	2.14 ^{B}	1.50 ^{A}	1.51 ^{A}	0.13	0.0074
d15-d21						
d21 BW, g	621.35^{AB}	583.33^{B}	709.36^{A}	610.90^{AB}	30.23	0.0434
BWG, g	258.41	242.10	329.94	231.68	28.10	0.0898
FI, g/bird/day	78.70	88.16	86.77	82.44	6.08	0.6864
FCR	1.90	2.02	1.52	3.34	0.84	0.4629
d21-d28						
d28 BW, g	1292.32^{A}	1121.92^{B}	1352.72^{A}	1394.23^{A}	38.37	0.0008
BWG, g	670.97^{AB}	531.92^{B}	643.36^{AB}	783.33^{A}	38.68	0.0032
FI, g/bird/day	296.79	292.01	357.28	351.49	28.96	0.2824
FCR	3.07	4.14	4.10	3.13	0.52	0.3192
d28-d35						
d35 BW, g	1935.92	1847.17	2025.32	2018.63	47.01	0.0648
BWG, g	643.61	725.25	672.61	624.40	43.82	0.4593
FI, g/bird/day	150.40	201.94	155.85	187.38	27.80	0.5428
FCR	1.74	2.01	1.67	2.06	0.32	0.7782
d35-d42						
d42 BW, g	2799.75	2749.44	2874.86	2835.99	56.11	0.4954
BWG, g	863.83	902.28	849.54	817.36	47.24	0.6835
FI, g/bird/day	183.59	176.74	188.32	179.27	6.29	0.6151
FCR	1.50	1.39	1.56	1.54	0.05	0.1237
DOH-d15						
BWG, g	325.52 ^{AB}	303.86 ^{B}	341.58^{A}	341.82^{A}	7.84	0.0079
FI, g/bird/day	38.62	41.17	43.10	43.50	2.68	0.5680
FCR	1.84 ^{B}	2.44^{A}	1.93 ^{AB}	1.93 ^{AB}	0.15	0.0358
d15-d35						
BWG, g	1572.99	1497.69	1645.91	1639.41	47.24	0.1589
FI, g/bird/day	164.29	183.29	189.41	192.51	11.64	0.3432
FCR	2.11	2.08	2.32	2.76	0.35	0.5153
DOH-d42						
BWG, g	2762.33	2712.07	2837.03	2798.59	56.22	0.4999
FI, g/bird/day	107.01	113.48	122.96	120.47	4.40	0.0775
FCR	1.63	1.76	1.82	1.81	0.06	0.1383

[1]CON : control, VNC : vaccine administration only, NPC : probiotic administration only, VPC : vaccine and probiotic administration

[A-B]Means within rows that do not have a common superscript differ significantly ($P \leq 0.05$).

Figure Captions

Figure 4.1. Effect of administration of probiotics (PoultryStar) and coccidiosis vaccine (Immucox) on d21 lesion scores of Cobb 500 male broilers challenged with *Eimeria* species on d15. Data are presented as Least Square Means ± SEM; bars lacking a common letter differ significantly. There was a significant effect of treatment on lesion scores in the duodenum ($P < 0.0001$), the site of *E. acervulina* infection, and in the jejunum ($P < 0.0001$), one site of *E. maxima* and *E. necatrix* infection. There was not a significant effect of treatment observed in the ileum ($P = 0.1785$), a second potential site of infection for both *E. maxima* and *E. necatrix*. No lesions were observed in the ceca among any of the treatments. CON = control; VNC = vaccine administration only; NPC = probiotic administration only, VPC = both vaccine and probiotic administration.

Figure 4.1

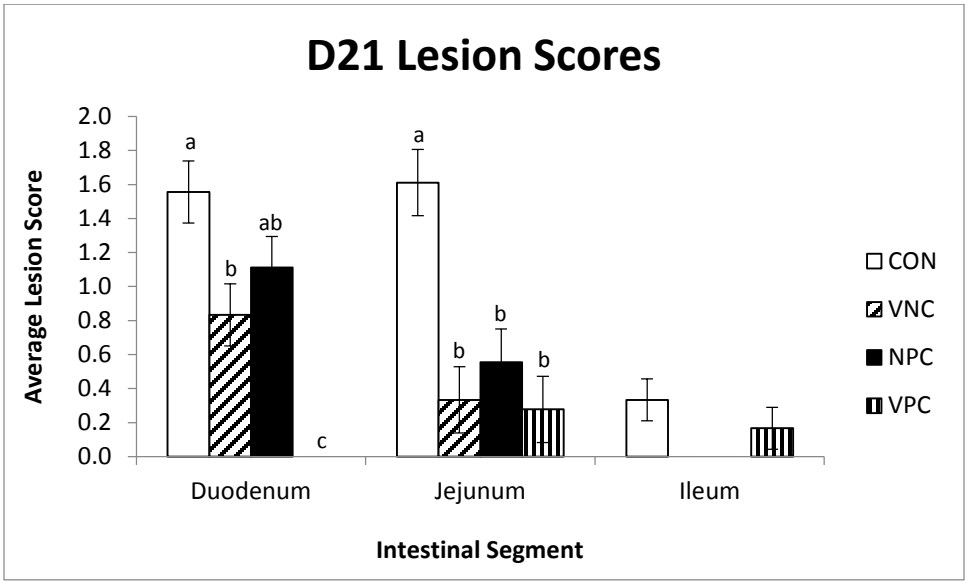

D21 Lesion Scores
Average Lesion Score
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
ab
c
a
b
b
b
Duodenum
Jejunum
Ileum
Intestinal Segment
CON
VNC
NPC
VPC

Figure 4.2. Effect of administration of probiotics (PoultryStar) and coccidiosis vaccine (Immucox) on post-challenge oocyst shedding in feces of Cobb-500 male broiler chickens. Data are presented as Least Square Means ± SEM; bars lacking a common letter differ significantly. There was a significant effect of treatment on oocysts shedding d7 post-challenge (P = 0.0091). CON = control; VNC = vaccine administration only; NPC = probiotic administration only, VPC = both vaccine and probiotic administration.

Figure 2

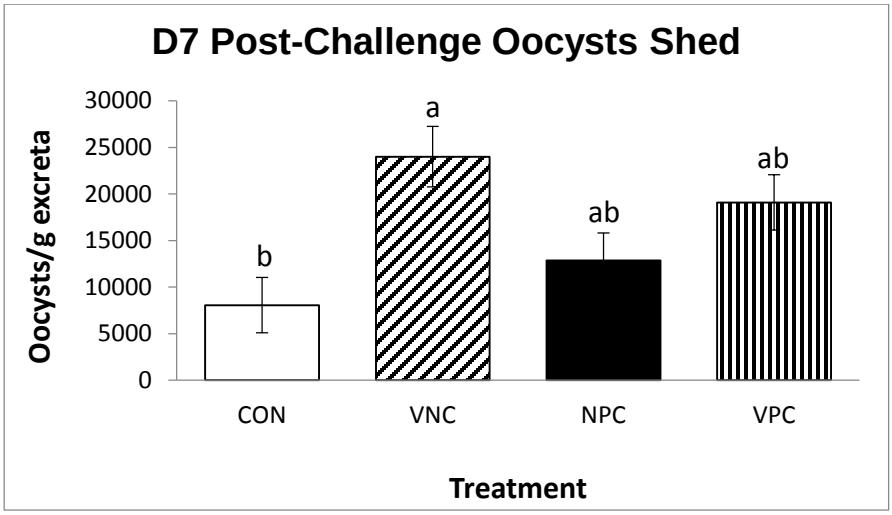

D7 Post-Challenge Oocysts Shed
Oocysts/g excreta
30000
25000
20000
15000
10000
5000
0
a
ab
ab
b
CON
VNC
NPC
VPC
Treatment

Figure 4.3. Effect of administration of probiotics (PoultryStar) and coccidiosis vaccine (Immucox) on litter moisture in pens of Cobb-500 male broiler chickens. Data are presented as Least Square Means ± SEM; bars lacking a common letter differ significantly. There was a significant effect of treatment on litter moisture on d7 (Fig. 4.3-A, $P = 0.0119$), on d14 (Fig. 4.3-B, $P < 0.0001$), and on d42 (Fig. 4.3-C, $P = 0.0245$). CON = control; VNC = vaccine administration only; NPC = probiotic administration only, VPC = both vaccine and probiotic administration.

Figure 4.3-A

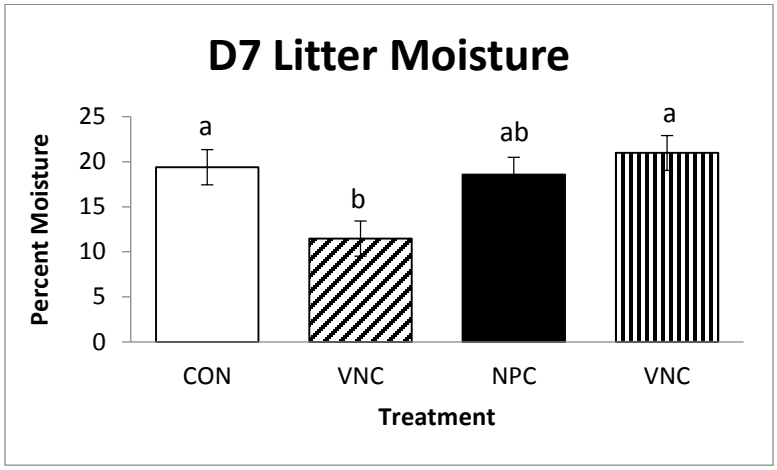


Figure 4.3-B

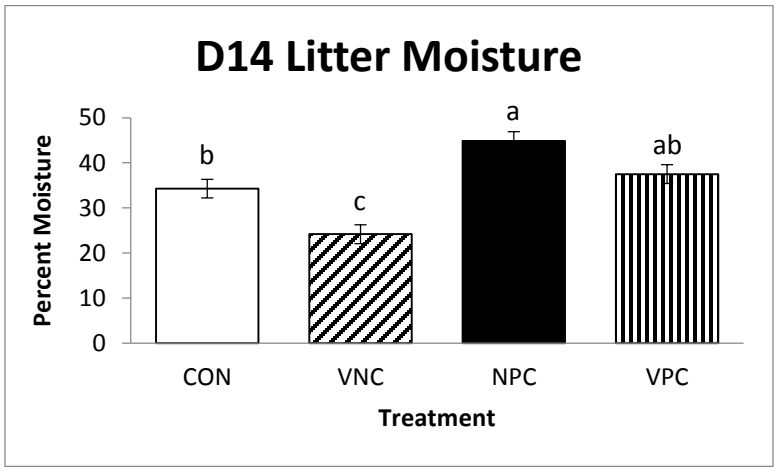
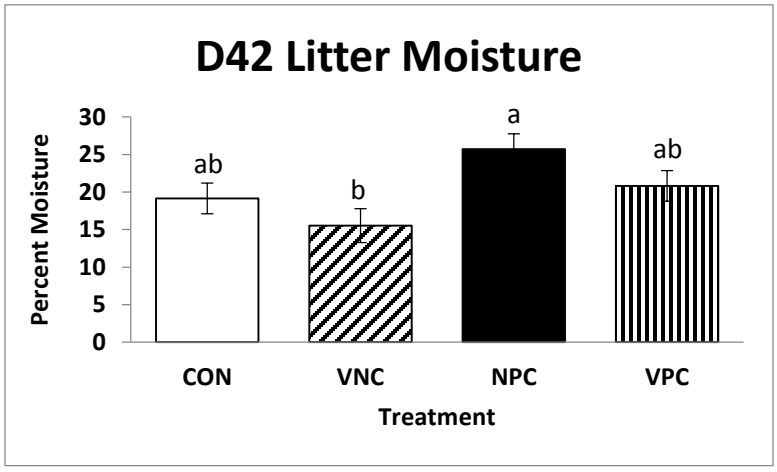


Figure 4.3-C



CHAPTER V

Epilogue

The small intestine is the largest lymphoid organ as well as a vital component of the digestive system. The integrity of the digestive tract is essential for protecting the host against enteric diseases such as coccidiosis. With the ban of antibiotics as feed additives by the European Union and threat of the ban being carried to the U.S., more consumer friendly and 'natural' alternatives need to be procured in the near future. The results of the studies presented in this thesis support the protective effects provided by probiotic administration, both in feed and water applications.

Probiotics are live microbial supplements that are administered to animals and humans to improve intestinal function by modifying the resident microflora. Supplementation of probiotics has been shown to enhance performance in poultry and provide protection against several economically important enteric pathogens. Additionally, manipulation of intestinal microflora through the use of probiotics has been noted to promote the development of the intestinal tract and local immune system. Over the course of these experiments, we demonstrated the safety and efficacy of probiotic administration in a challenge setting. The effects of probiotics in these studies included enhanced feed conversion and less intestinal epithelium damage during a cocci challenge. Further, certain aspects associated with live coccidiosis vaccination, such as delayed growth and early intestinal epithelial damage, were reduced with probiotic supplementation, resulting in healthier broiler chickens as exhibited in the second study. The early addition of probiotics in the chick's life cycle aided in rapid colonization of beneficial bacteria within the intestinal tract, which likely led to the reduction in vaccination effects.

The interaction between the probiotics and coccidiosis vaccine was very encouraging, as the data support the theory that the two together provide better protection against infection by *Eimeria* species than the administration of either on its own. As such, I would like to see a study designed on a larger scale that would further investigate the relationship between the two, including different administration methods of the probiotic formulation. It is important to note that at the start of the second study, the birds encountered an unintentional *E. coli* infection. The infection resulted in slight increases in mortality among the treatments, most notably in the control and vaccinated groups. The treatments that were provided with probiotic had fewer losses among treated birds, as well as recovered from the infection more rapidly. These aspects suggest the beneficial bacteria in the probiotic product helped the chicks combat the *E. coli* infection, possibly due to the early colonization of beneficial bacteria in the intestinal tract. An experiment that induced a coccidiosis challenge as well as a controlled *E. coli* challenge would likely yield remarkable results that could shed more light on the protective and beneficial mechanisms of probiotic action, as a combined challenge is a possibility in the poultry industry.

At the time this thesis was defended, intestinal samples from the first trial (Chapter III) were undergoing analyses for microbial profiling, gene expression, and histological changes. As probiotics have been reported to alter gene expression and intestinal histology in the literature, it would be interesting to see if there exist any consistent trends regarding this probiotic formulation, especially in the disease situation. As the probiotic product used in these experiments consisted of multiple probiotic species, the microbial profile analysis will potentially provide insight as to which species better colonize the intestinal tract, thus providing a more robust intestinal microbiota. Once completed, we should be able to better correlate

immune responses to the corresponding gut microbial profiles, which will help us paint a clearer overall picture of how probiotics exert their influence on the host immune system.

Appendix 1

Non-significant Data Associated with Chapter III

Table S3.1. NEG = negative control; POS = positive control; SAL = Salinomycin continuous in feed; WPC = probiotic at low dose continuous water administration; WPI = probiotic at high dose intermittent water administration; FSP = probiotic continuous in feed.

	Treatment							
Variable	**NEG**	**POS**	**SAL**	**WPC**	**WPI**	**FSP**	**St. Error**	***P*-value**
d15 BW (kg)	0.49	0.49	0.49	0.48	0.49	0.48	0.005	0.3674
DOH-d15 BWG (kg/bird)	0.45	0.45	0.45	0.44	0.44	0.44	0.004	0.3685
d35-d42 BWG (kg/bird)	0.72	0.84	0.81	0.83	0.81	0.79	0.030	0.1539
d21-d35 FCR	1.60	1.80	1.80	1.80	1.90	1.70	0.060	0.0507
d35-d42 FCR	2.00	2.00	1.90	1.90	2.10	2.10	0.120	0.6892

Appendix 2

Non-significant Data Associated with Chapter IV

Figure S4.1. Effect of administration of probiotics (PoultryStar) and coccidiosis vaccine (Immucox) on d6 post-challenge oocyst shedding in feces of Cobb-500 male broiler chickens. Data are presented as Least Square Means ± SEM. CON = control; VNC = vaccine administration only; NPC = probiotic administration only, VPC = both vaccine and probiotic administration. *P*= 0.0869

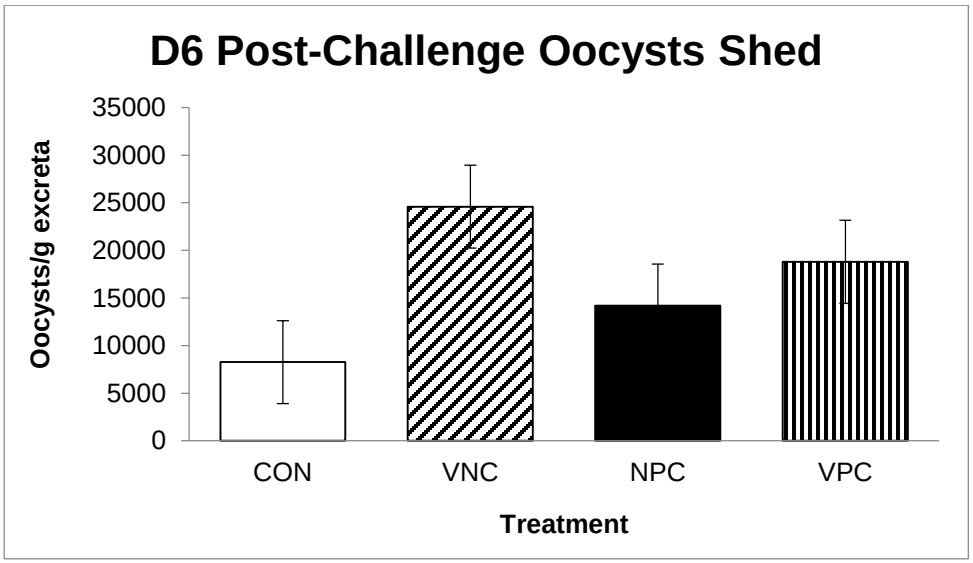


Figure S4.2. Effect of administration of probiotics (PoultryStar) and coccidiosis vaccine (Immucox) on d8 post-challenge oocyst shedding in feces of Cobb-500 male broiler chickens. Data are presented as Least Square Means ± SEM. CON = control; VNC = vaccine administration only; NPC = probiotic administration only, VPC = both vaccine and probiotic administration. *P*= 0.0733

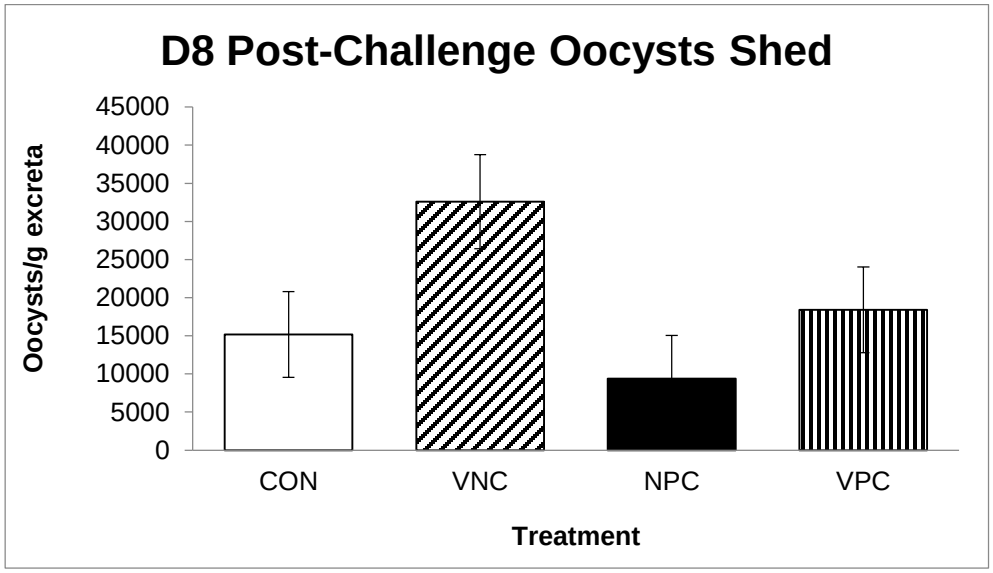
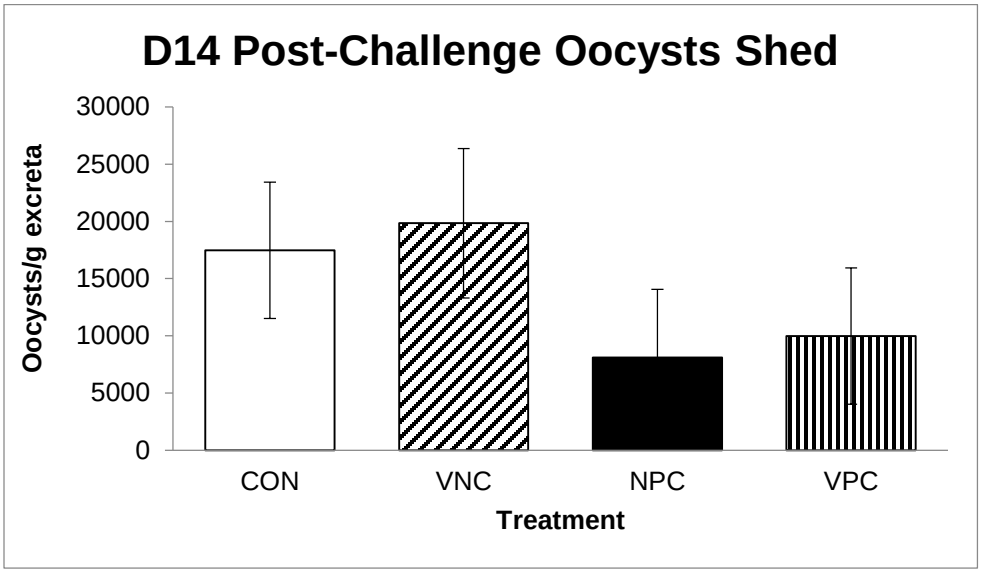


Figure S4.3. Effect of administration of probiotics (PoultryStar) and coccidiosis vaccine (Immucox) on d8 post-challenge oocyst shedding in feces of Cobb-500 male broiler chickens. Data are presented as Least Square Means ± SEM. CON = control; VNC = vaccine administration only; NPC = probiotic administration only, VPC = both vaccine and probiotic administration. *P*= 0.4812



Effects of probiotics and application methods on performance and response of broiler chickens to an *Eimeria* challenge

Miranda M. Ritzi,* Wael Abdelrahman,†‡ Michaela Mohnl,† and Rami A. Dalloul*[1]

*Avian Immunobiology Laboratory, Department of Animal and Poultry Sciences, Virginia Tech, Blacksburg 24061; †BIOMIN Holding GmbH, 3130 Herzogenburg 3130, Austria; and ‡Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt 41522

ABSTRACT Coccidiosis is an inherent risk in the commercial broiler industry and inflicts devastating economic losses to poultry operations. Probiotics may provide a potential alternative to the prophylactic use of anticoccidials in commercial production. This study evaluated the effects of probiotic applications (feed and water) on bird performance and resistance to a mixed *Eimeria* infection in commercial broilers. On day of hatch, 1,008 commercial male broilers (Cobb 500) were assigned to 1 of 6 treatments (8 replicate floor pens; 21 birds/pen), including noninfected negative control (NEG), *Eimeria*-infected positive control (POS), anticoccidial control (0.01% salinomycin, SAL), intermittent high-dose water-applied probiotic (WPI), continuous low-dose water-applied probiotic (WPC), and feed-supplemented probiotic (FSP). On d 15, all birds except those in NEG were challenged with a mixed inoculum of *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella*. Measurements were taken on d 7, 15, 21, 28, 35, and 42. Fecal samples were collected from d 20 to 24 for oocyst counts, and lesion scores were evaluated on d 21. Data were analyzed using the Fit Model platform in JMP Pro 10.0 (SAS Institute Inc.). Differences in experimental treatments were tested using Tukey's honestly significant difference following ANOVA with significance reported at $P \leq 0.05$. Overall, NEG birds outperformed all other groups. For performance, the probiotic groups were comparable with the SAL-treated birds, except during the 6 d immediately following the *Eimeria* species challenge, where the SAL birds exhibited better performance. The WPC birds had lower duodenal and jejunal lesion scores, indicating a healthier intestine and enhanced resistance to *Eimeria* species compared with POS. Birds in the WPI treatment shed fewer oocysts in the feces, although this was not a trend for all of the probiotic treatment groups. The results of this study suggest probiotic supplementation without anticoccidials can enhance performance and help alleviate the negative effects of a mixed *Eimeria* infection.

Key words: probiotic, coccidiosis, broiler, performance, *Eimeria*

2014 Poultry Science 93:2772–2778
http://dx.doi.org/10.3382/ps.2014-04207

INTRODUCTION

The decades-old practice of supplying food animals with subtherapeutic doses of antibiotics to protect against infections and improve general health has recently been under scrutiny. These practices are perceived to lead to microbial resistance to the drugs in use, resulting in consumer concerns regarding residues in food products. The relatively recent ban of subtherapeutic doses of certain antibiotics as feed additives in the European Union led to a general decline in animal health (Castanon, 2007). This outcome, as well as the threat of a domestic ban, has led researchers to explore the next promising alternatives.

Probiotics present a potential alternative to the prophylactic use of antibiotics in feed animals. Also known as direct-fed microbials, probiotics are classified as live nonpathogenic microorganisms that are capable of maintaining a normal gastrointestinal microbiota (Patterson and Burkholder, 2003; Ohimain and Ofongo, 2012). Probiotic, meaning "for life" in Greek, has been defined as "a live microbial feed supplement, which beneficially affects the host animal by improving intestinal balance" (Fuller, 1989). Probiotics can be composed of one or many strains of microbial species, with the more common ones belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, and *Pediococcus* (Gaggia et al., 2010).

Although the primary function of the gastrointestinal tract is to digest and absorb nutrients, a well-balanced

Received May 23, 2014.
Accepted July 23, 2014.
[1]Corresponding author: RDalloul@vt.edu

gastrointestinal microbiota is crucial for optimal animal health and performance. The gastrointestinal tract also serves as a vital barrier preventing the entry of potentially harmful pathogens and other environmental antigens (Kogut and Swaggerty, 2012). Because the gastrointestinal tract begins to be colonized within hours after the chick hatches, the earlier the introduction of nonpathogenic microorganisms, the more effective their establishment in the digestive tract (Timmerman et al., 2006; Torok et al., 2007). Probiotics help maintain a healthy balance of microorganisms within the intestine, which is accomplished through multiple modes of action. Those mechanisms include competitive exclusion, pathogen antagonism, and stimulation of the immune system (Ohimain and Ofongo, 2012). The presence of probiotics reduces colonization of the gastrointestinal tract by pathogenic bacteria and attenuates enteric diseases, which ultimately result in enhanced performance of poultry (Kabir et al., 2004).

Coccidiosis is an inherent risk in the commercial broiler industry and inflicts devastating economic losses to poultry operations. Caused by development and reproduction of multiple species of the *Eimeria* protozoa, coccidiosis is estimated to result in a loss of US $3 billion annually to the industry worldwide (Dalloul and Lillehoj, 2006). *Eimeria* species are unlike other protozoan parasites in that the primary target tissue is the intestinal epithelium, which results in considerable impairment of growth and feed utilization in poultry. Probiotics may provide a potential alternative to the prophylactic use of drugs in food animals due to their studied abilities to reduce enteric diseases in poultry (Patterson and Burkholder, 2003; Eckert et al., 2010). This study aimed to evaluate the effects of a multi-species, host-specific probiotic product containing *Enterococcus*, *Bifidobacterium*, and *Lactobacillus* species in the feed and water on performance parameters and resistance to coccidiosis (*Eimeria* infection) in commercial broiler chickens.

MATERIALS AND METHODS

Birds and Experimental Treatments

The study performed was a 42-d trial with broilers housed on floor pens, with 8 replicate pens per treatment and 21 birds per pen. On day of hatch (**DOH**), 1,008 commercial male broilers (Cobb 500) were assigned to 1 of 6 treatments, including noninfected negative control (**NEG**), *Eimeria*-infected positive control (**POS**), anticoccidial control [salinomycin as Sacox-60 (Huvepharma, Peachtree City, GA) at the rate of 0.01%, **SAL**], continuous low-dose water-applied probiotic (**WPC**), intermittent high-dose water-applied probiotic (**WPI**), and feed-supplemented probiotic (**FSP**). Birds in the low-dose WPC group were given probiotics at the rate of 2 mg/bird per day. Birds in the high-dose WPI treatment were given probiotics at the rate of 20 mg/bird per day intermittently. The WPI birds received probiotics on the first 3 d of life, once a week, during the week of *Eimeria* species challenge starting 1 d before inoculation, and 1 d before, the day of, and 1 d after feed changes. The diet for birds receiving probiotics in the feed was mixed and pelleted weekly. Probiotics for the feed application were microencapsulated to protect the probiotic bacteria from high pelleting temperature. All animal protocols were approved and conducted under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee.

Probiotic Mixture Preparation

The probiotic product used in the experiment was a multi-species, host-specific probiotic (PoultryStar, BIOMIN GmbH, Herzogenburg, Austria) containing *Bifidobacterium animalis* subspecies *animalis* DSM 16284, *Lactobacillus salivarius* subspecies *salivarius* DSM 16351, and *Enterococcus faecium* DSM 21913. For the WPC and WPI groups, the cfu content of the product was 5×10^{12} per kg. For the FSP group, the cfu content of the product was 10^{11} cfu per kg and was mixed into the finished feed at an inclusion rate of 1 g per kg of feed, giving a final concentration of 10^8 cfu per kg of feed. This dosage was determined based on recovery rate of probiotic bacterial counts in pelleted feed samples from previous studies.

Eimeria Challenge

On d 15 of age, all birds except those in the NEG group were challenged via oral gavage with a mixed inoculum of *Eimeria acervulina* (USDA isolate #12), *Eimeria maxima* (Tysons isolate), and *Eimeria tenella* (Wampler isolate). The dose was 1 mL per bird containing 50,000 *E. acervulina* oocysts, 10,000 *E. maxima* oocysts, and 2,500 *E. tenella* oocysts. The inoculation rates of salinomycin-sensitive *Eimeria* species were based on previous studies in our laboratories. On d 21 (6 d postinfection), 24 birds per treatment (3 birds from each replicate pen) were randomly selected and euthanized for scoring of intestinal lesions caused by *Eimeria* infection. Lesions in the duodenum, jejunum, and ceca were scored according to the method of Johnson and Reid (1970) by personnel blinded to treatment based on scores ranging from 0 (no gross lesion) to 4 (most severe lesion). Fecal samples were collected from each pen on d 20 to 24 (d 5–9 postinfection) and kept in separate airtight plastic bags. After homogenization, samples were stored at 4°C until assessed for oocyst counts, which were determined by dilution and counts via microscope using a McMaster counting chamber (JA Whitlock & Co., Eastwood, NSW, Australia) and expressed as oocysts per gram of excreta.

Performance Measurements

Pen and feed weights were taken on DOH, d 15, d 21, d 35, and d 42. From these data, BW, BW gain

(**BWG**), feed intake (**FI**), and feed conversion ratio (**FCR**) were determined on a pen basis, and then averaged by treatment.

Statistical Analysis

Data were analyzed using the Fit Model platform in JMP Pro 10.0 (SAS Institute Inc., Cary, NC). Values were considered statistically different at $P \leq 0.05$. Results are reported as least squares means (least squares means) with SEM.

RESULTS

Eimeria Challenge

Lesion Scores. On d 21, lesion scores in the duodenum caused by *E. acervulina* infection showed a significant effect of treatment ($P < 0.0001$), as presented in Figure 1A. The NEG treatment group had significantly lower lesion scores than all challenged treatment groups. The POS treatment group had higher lesion scores than the SAL group and WPC group. The WPC had lower lesion scores than the FSP treatment. Similar to the duodenum, lesion scores caused by *E. maxima* in the jejunum on d 21 (Figure 1B) showed a significant effect of treatment ($P < 0.0001$). The NEG treatment group had lower lesion scores compared with all challenged treatments. The POS treatment group had higher lesion scores than WPC and WPI, the 2 water-administered probiotics. No significant lesions were observed in the ceca (Figure 1C), which is the site of *E. tenella* infection ($P = 0.0473$).

Oocyst Shedding. Fecal samples were collected on d 20 to 24 from each pen for evaluating oocyst shedding in the feces as presented in Figure 2. There was a significant effect of treatment on number of oocysts shed ($P = 0.0014$). The NEG control birds shed significantly fewer oocysts than those in the POS and SAL treatments. The POS group shed significantly more oocysts than the NEG, WPI, and FSP treatments.

Performance

BW. Presented in Figure 3A, a significant effect of treatment on BW was noted on d 21 ($P < 0.0001$; d 6 after *Eimeria* infection). Birds in the NEG treatment had significantly higher BW than all other treatments, whereas the SAL birds exhibited higher BW than all 3 probiotic treatments and the POS group. A significant effect of treatment on BW was also noted on d 35 ($P < 0.0001$), shown in Figure 3B, and d 42 ($P < 0.0001$), presented in Figure 3C, with the NEG group showing heavier BW.

BWG. A significant effect of treatment was evident from d 15 to 21, with the NEG group showing a greater BWG compared with all other treatments ($P < 0.0001$), as presented in Figure 4A. The SAL group showed greater BWG compared with the POS and all probiotic product treatments, though still less than the NEG birds. From d 21 to 35, a significant effect of treatment was observed ($P < 0.0001$) where the NEG treatment had greater BWG than all other treatment groups (Figure 4B). When comparing total BWG through the course of the study (DOH to d 42), there was also a significant effect of treatment (Figure 4C). The NEG birds gained significantly more weight compared with all of the challenged treatment groups,

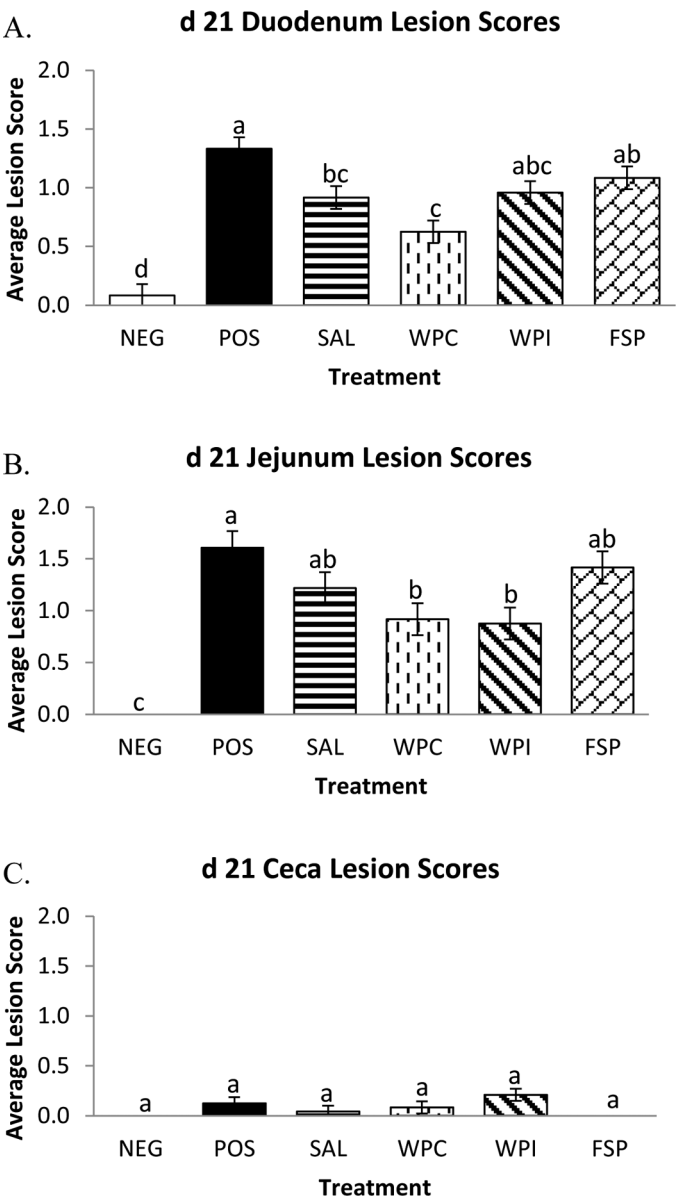


Figure 1. Effect of administration of probiotics (PoultryStar, BIOMIN GmbH, Herzogenburg, Austria) on d 21 lesion scores of Cobb 500 male broiler challenged with *Eimeria* species on d 15. Data are presented as least squares means ± SEM; bars lacking a common letter (a–d) differ significantly. There was a significant effect of treatment on lesion scores in the duodenum (A), the site of *Eimeria acervulina* infection ($P < 0.0001$), in the jejunum (B), the site of *Eimeria maxima* infection ($P < 0.0001$), but no significant lesions were observed in the ceca (C), the site of *Eimeria tenella* infection ($P = 0.0473$). NEG = negative control; POS = positive control; SAL = salinomycin continuous in feed; WPC = probiotic at low-dose continuous water administration; WPI = probiotic at high-dose intermittent water administration; FSP = probiotic continuous in feed.

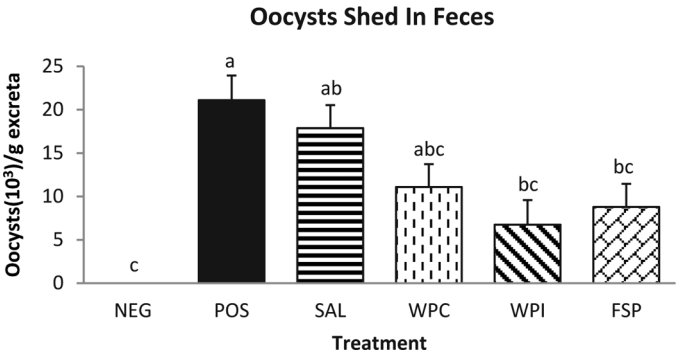


Figure 2. Effect of administration of probiotics (PoultryStar, BIOMIN GmbH, Herzogenburg, Austria) on d 20 to 24 oocyst shedding in feces of Cobb 500 male broiler chicks challenged with *Eimeria* species on d 15. Data are presented as means ± SEM; bars lacking a common letter (a–c) differ significantly. There was a significant effect of treatment ($P = 0.0014$). NEG = negative control; POS = positive control; SAL = salinomycin continuous in feed; WPC = probiotic at low-dose continuous water administration; WPI = probiotic at high-dose intermittent water administration; FSP = probiotic continuous in feed.

whereas the probiotic treatments gained a comparable weight as the SAL birds ($P < 0.0001$).

FI. As shown in Figure 5A, there was a significant treatment effect on FI from DOH to d 15 ($P = 0.0050$), where SAL birds consumed more feed per bird per day than POS, WPC, and WPI birds. From d 15 to 21, the NEG group consumed significantly more feed compared with all other treatments (Figure 5B). Also, the SAL birds consumed more feed than the WPC birds ($P < 0.0001$).

FCR. There was a significant effect of treatment on FCR during DOH to d 15 (Figure 6A). Treatment groups that received probiotics in drinking water had significantly lower FCR than the SAL group ($P = 0.0017$). Figure 6B presents the significant effect of treatment noted from d 15 to 21, with the NEG group exhibiting a significantly lower FCR compared with all other treatment groups ($P < 0.0001$). The POS group had a higher FCR compared with the SAL and NEG groups ($P < 0.0001$). The WPI treatment had a significantly higher FCR than the SAL group and the negative control ($P < 0.0001$), whereas treatment groups that received a low dose of probiotics continuously in drinking water and in feed did not differ significantly from SAL-treated group.

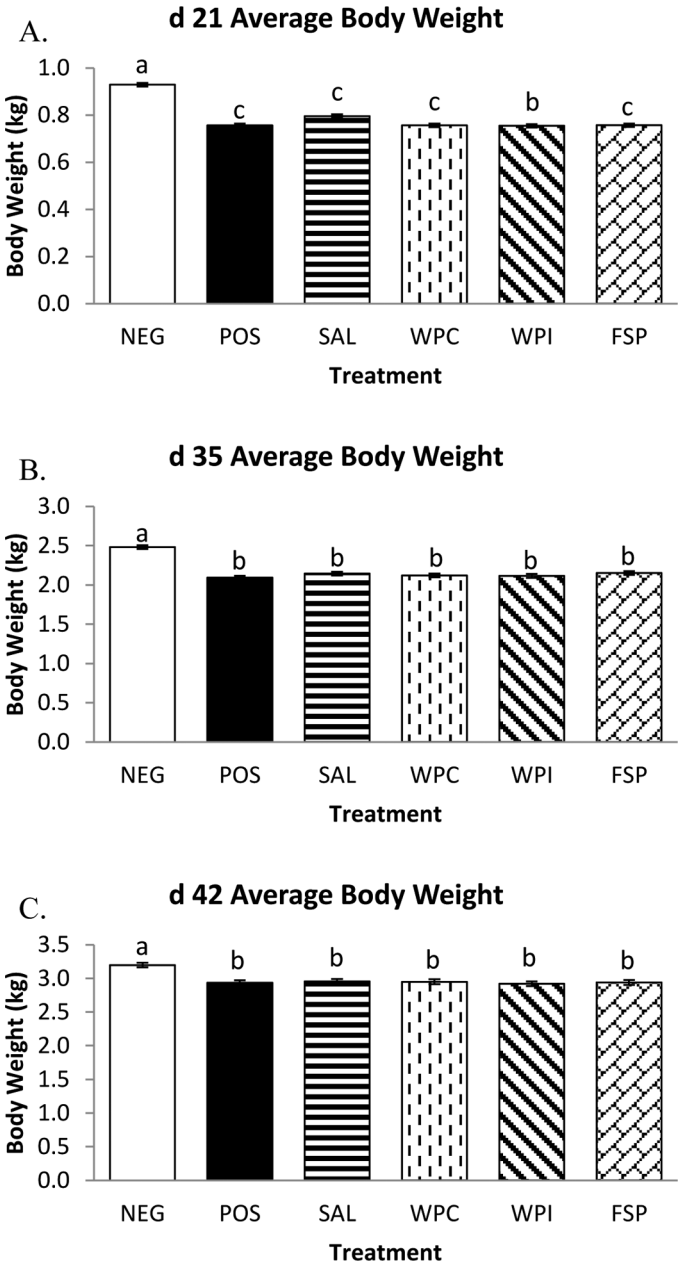


Figure 3. Effect of administration of probiotics (PoultryStar, BIOMIN GmbH, Herzogenburg, Austria) on BW of Cobb 500 male broiler chicks. Data are presented as least squares means ± SEM; bars lacking a common letter (a–c) differ significantly. There was a significant effect of treatment ($P < 0.0001$) on d 21 (A), on d 35 (B), and on d 42 (C). NEG = negative control; POS = positive control; SAL = salinomycin continuous in feed; WPC = probiotic at low-dose continuous water administration; WPI = probiotic at high-dose intermittent water administration; FSP = probiotic continuous in feed.

DISCUSSION

In this study, the effects of probiotic products in the feed and water of broilers and their resistance to an *Eimeria* infection were investigated. Birds in the WPC treatment group had less severe duodenal and jejunal lesion scores, indicating a healthier intestine. While present in the challenge inoculum, the dose of *E. tenella* may not have been sufficient to cause extensive damage to the site of infection, resulting in the absence of lesions in the ceca. Similar to present results, Lee et al. (2010) reported that birds given a strain of a *Bacillus*-based direct-fed microbial had significantly lower lesions scores in the gastrointestinal tract than birds given the nonsupplemented diet following an *E. maxima* challenge. Studies investigating necrotic enteritis in broilers found birds given 2 different blends of direct-fed microbials had significantly reduced intestinal lesions due to necrotic enteritis than birds in the positive control (McReynolds et al., 2009). Fewer and less severe lesion scores are indicative of less damage to the epithelium of the intestine, leading to infected

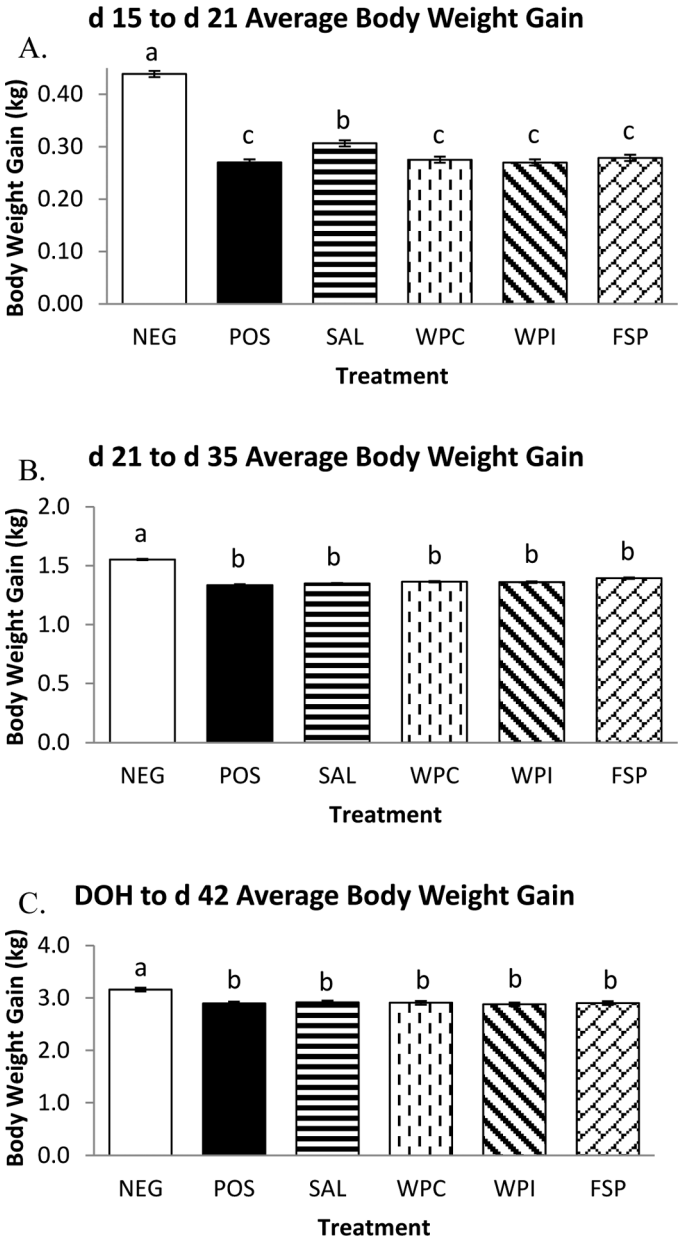


Figure 4. Effect of administration of probiotics (PoultryStar, BIOMIN GmbH, Herzogenburg, Austria) on BW gain of Cobb 500 male broiler chicks. Data are presented as least squares means ± SEM, bars lacking a common letter (a–c) differ significantly. There was a significant effect of treatment ($P < 0.0001$) on BW gain from d 15 to 21, the 6 d immediately following challenge (A), from d 21 to 35 (B), and through the course of the trial from day of hatch (DOH) to d 42 (C). NEG = negative control; POS = positive control; SAL = salinomycin continuous in feed; WPC = probiotic at low-dose continuous water administration; WPI = probiotic at high-dose intermittent water administration; FSP = probiotic continuous in feed.

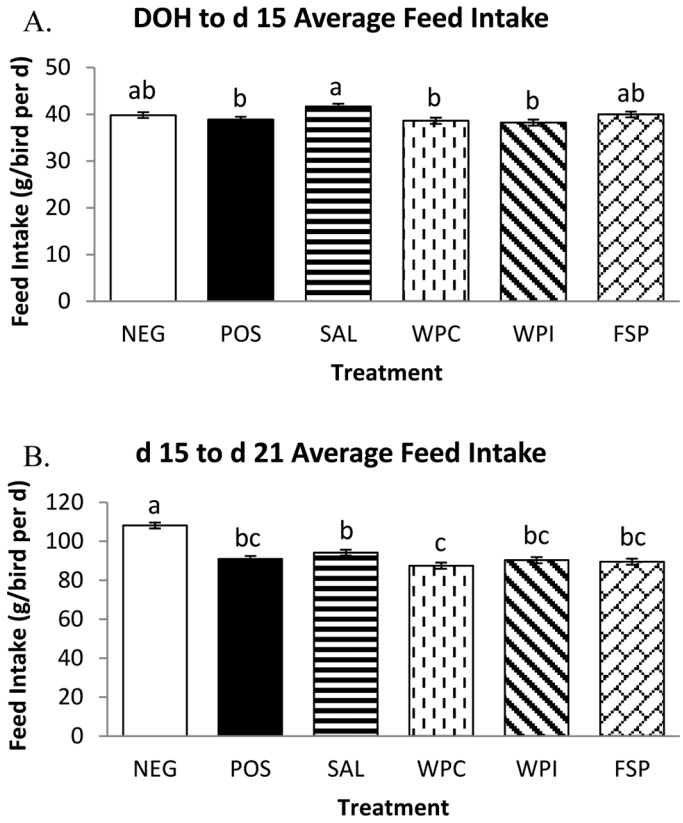


Figure 5. Effect of administration of probiotics (PoultryStar, BIOMIN GmbH, Herzogenburg, Austria) on feed intake per bird per day of Cobb 500 male broiler chicks. Data are presented as least squares means ± SEM. Bars lacking a common letter (a–c) differ significantly. A: There was a significant effect of treatment on feed intake from day of hatch (DOH) to d 15 (A, $P = 0.0050$) and from d 15 to 21 (B, $P < 0.0001$). NEG = negative control; POS = positive control; SAL = salinomycin continuous in feed; WPC = probiotic at low-dose continuous water administration; WPI = probiotic at high-dose intermittent water administration; FSP = probiotic continuous in feed.

birds having a greater chance of recovery from disease. Numerous studies have found probiotic supplementation leads to significant reductions in numbers of other intracellular pathogens present in the intestine, such as *Salmonella* Enteritidis and *Campylobacter jejuni* (Higgins et al., 2007; Ghareeb et al., 2012). The pathogen load reductions could be due to multiple mechanisms of action employed by direct-fed microbials, depending on strains present in various products employed in those studies. Ultimately, the reduction in the presence of intracellular pathogens is indicative of a healthier intestine, with minimal damage done to the epithelium. An intact intestinal epithelium serves as the vital barrier preventing entry of potential pathogens and results in proper nutrient absorption and utilization, leading to optimal health and performance of the bird.

Birds receiving a high probiotic dose in the water supply on intermittent days (WPI) and in the feed (FSP) shed fewer oocysts in the feces than the positive control. Corroborating our findings, Dalloul et al. (2003, 2005) found that broilers provided a *Lactobacillus*-based probiotic in the feed shed significantly fewer *E. acervulina* oocysts compared with the challenged control. A reduction in oocysts shed in the feces indicates improved resistance of the bird to *Eimeria* species infection. Not surprisingly, the birds receiving the anticoccidial in the feed handled the challenge during the peak infection period better than probiotic-treated birds with regard to performance. However, as the study progressed, the probiotics helped the birds to overall perform as well as the birds receiving anticoccidial, and better than birds in the positive control.

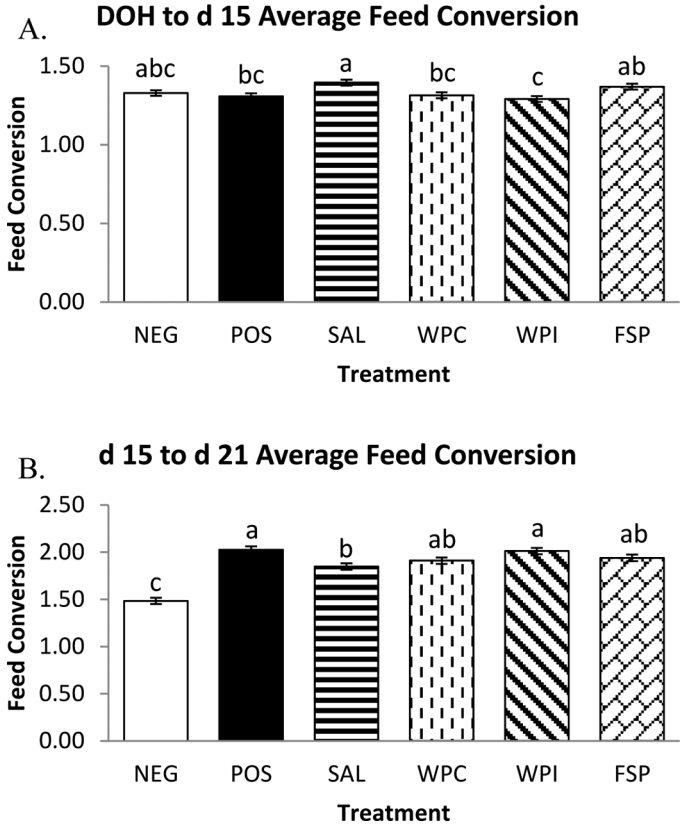


Figure 6. Effect of administration of probiotics (PoultryStar, BIOMIN GmbH, Herzogenburg, Austria) on feed conversion ratio of Cobb 500 male broiler chicks. Data are presented as least squares means ± SEM; bars lacking a common letter (a–c) differ significantly. There was a significant effect of treatment on feed conversion ratio from day of hatch (DOH) to d 15 (A, $P = 0.0017$) and from d 15 to 21 (B, $P < 0.0001$). NEG = negative control; POS = positive control; SAL = salinomycin continuous in feed; WPC = probiotic at low-dose continuous water administration; WPI = probiotic at high-dose intermittent water administration; FSP = probiotic continuous in feed.

The reductions in BW and BWG due to the *Eimeria* challenge were not surprising because coccidial infections are known to cause significant damage to the intestinal mucosa and enterocytes during the progression of their lifecycle. This extensive damage causes nutrient malabsorption and subsequent reduced performance. Furthermore, parasitic infections result in nutrient resource allocation shifting from growth to immune response, which can also lead to noticeable differences in growth (Allen and Fetterer, 2002; Dalloul and Lillehoj, 2005). Supporting the current findings, Mountzouris et al. (2007) found that broilers receiving probiotic in the feed performed as well as broilers receiving the coccidiostat in terms of BWG and FCR over the duration of the trial. Numerous studies investigating probiotics as dietary additives in poultry have resulted in varying effects of probiotics on performance. Some studies reported that probiotic supplementation in the diet can improve BWG and FCR in chickens (Kabir et al., 2004; Nayebpor et al., 2007; Apata, 2008; Talebi et al., 2008; Ignatova et al., 2009; Sen et al., 2012), whereas others found no significant benefit to probiotic addition (Rahimi et al., 2011; Wolfenden et al., 2011). These differences could be due to a variety of factors that can alter the efficacy of a probiotic such as strain(s) of bacteria used, composition and viability of the probiotic bacteria, and the preparation methods. Further, other factors may include probiotic dosage, method or frequency of application (or both), overall diet, condition and age of the birds, potential drug interactions, as well as environmental stress factors such as temperature and stocking density (Patterson and Burkholder, 2003; Mountzouris et al., 2007).

Concurrent with previous studies (Mountzouris et al., 2007; Karimi Torshizi et al., 2010), our data suggest that water administration of the probiotic product would be the method of choice, especially in a coccidiosis challenge situation. Although not examined in the current study, Karimi Torshizi et al. (2010) speculated that probiotics in water survive the demanding conditions in the upper gastrointestinal tract for a few reasons. The first possibility is a shorter transit time compared with solid feed. The second potential explanation is the water may limit the negative effects of gastric acid and digestive secretions on the microorganisms.

In conclusion, our data suggest that probiotic treatment (PoultryStar) helped alleviate the negative effects of the *Eimeria* species infection and may be used as a promising and beneficial anticoccidial. Poultry researchers are investigating the latest alternatives that will protect flocks from disease while not hindering performance or negatively affecting profit margins. Early establishment of beneficial microbiota by probiotics in poultry may lead to increased overall health and well-being while decreasing the need for prophylactic antibiotic use. Numerous studies have demonstrated that commensal intestinal microbiota inhibit pathogens and that probiotics can increase resistance to infection (Rolfe, 2000; Patterson and Burkeholder, 2003). Because PoultryStar is a product that contains multiple probiotic species of bacteria, there is a greater promise that such probiotics will be active in a wider range of conditions, similar to other multistrain probiotics, resulting in greater efficacy (Fuller, 1989; Dalloul et al., 2003, 2005; Timmerman et al., 2006). Future research evaluating pertinent gene expression within the intestinal and immune tissues, microbial profiles, histological changes, and other measurable parameters will provide further understanding of the probiotic effects and their mechanisms of action.

ACKNOWLEDGMENTS

The authors acknowledge Ray Fetterer (USDA-Agricultural Research Service, Beltsville, MD) for providing *Eimeria* oocysts and Diego Paiva (Virginia Tech, Blacksburg) for technical assistance.

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