In Ovo Supplementation of Primalac and the Effects on Performance and Immune Response of Broilers

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ABSTRACT

Probiotics are live nonpathogenic microorganisms capable of positively impacting the host by improving the natural gut microbial balance and promoting animal health. In ovo technology represents one means to administer probiotics and promote early colonization of beneficial bacteria for stimulating intestinal and immune system development, and warding off enteric threats. Three studies were conducted to evaluate the effects of in ovo administration of Primalac in broilers. The objective of the first study was to determine the effects of administering probiotics in ovo on hatchability, post-hatch performance and expression of immune-related genes in the ileum and cecal tonsils. On embryonic day 18, 360 eggs were injected with water, $1x10^5$, $1x10^6$, or $1x10^7$ probiotic bacteria. Another 90 eggs remained uninjected as a negative control group. Measurements and tissue samples were taken on day of hatch (DOH) and days (d) 4, 6, 8, 15, and 22. A subsequent study was conducted to evaluate the effects of administering Primalac in ovo and in the diet on broiler chick hatchability, post-hatch performance, immune organ weights and ileal immune-related gene expression. At embryonic day 18, 1977 eggs were either not injected (negative control), dry-punched, or injected with $1x10^6$ or $1x10^7$ probiotic bacteria. Performance parameters were measured on DOH and d4, 6, 8, 14, 19, and 42, while immune organ weights and tissue samples were taken on DOH and d4, 6, 8, 14, and 20. A third study investigated the effects of in ovo administration of Primalac on hatchability, performance, immune organ weights, and lesion scores in broiler chicks exposed to *Eimeria* sp. At embryonic day 18, 210 eggs were injected with either sterile water or $1x10^6$ probiotic bacteria. On d3 post-hatch, half of the chicks from each treatment group were

challenged with a mixed inoculum of *Eimeria acervulina*, *E. maxima*, and *E. tenella*. Measurements and tissue samples were taken on DOH and d3, 9 and 15. The results of these studies suggest that in ovo Primalac supplementation does not negatively impact hatchability, enhances performance, modulates intestinal gene expression, and provides protection against a mixed *Eimeria* infection.

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Dedication

To my family and friends. This would have been impossible without you!

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List of abbreviations

APCs: Antigen presenting cells

BW: Body weight

BWG: Body weight gain

CFU: Colony forming unit

Chall: Challenged with *Eimeria*

CMI: Cell-mediated immunity

d: Day

DOH: Day of hatch

FCR: Feed conversion ratio

FI: Feed intake

GALT: Gut-associated lymphoid tissues

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GM-CSF: Granulocyte macrophage colony stimulating factor

IEC: Intestinal epithelial cells

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

iNOS: Inducible nitric oxide synthase

LITAF: Lipopolysaccharide-induced tumor necrosis factor-α

LPS: Lipopolysaccharide

LS Means: Least squares means

M cells: Microfold cells

MALT: Mucosa-associated lymphoid tissues

MAMP: Microbe-associated molecular patterns

MHC: Major histocompatability complex

Muc: Mucin

NK: Natural killer

Not Chall: Not challenged

PP: Peyer's patches

PRRs: Pathogen recognition receptors

qRT-PCR: Quantitative real-time polymerase chain reaction

SE: Standard error

Tc: Cytotoxic T cell

TFF-2: Trefoil factor-2

Th: Helper T cell

Th1: Type 1 helper T cell

Th2: Type 2 helper t cell

TLR: Toll-like receptor

Chapter I

INTRODUCTION

Sub-therapeutic doses of antibiotics have been included in the diets of livestock animals for several decades to improve growth, feed efficiency, and general health. There has been increasing debate over their use in animal feed due to concerns that this practice may result in microbial resistance to human antibiotics employed in treating infections, thus causing a human health concern. The controversy and potential risk have resulted in the ban of many antibiotics as feed additives by the European Union as of January 1, 2006, which unfortunately has resulted in a decline of animal health (Castanon, 2007). The risk of the ban spreading overseas to the U.S. has created an impetus for finding alternatives capable of maintaining animal health without impeding performance.

Probiotics are one of the options that have been evaluated and shown to have potential in reducing the amount and severity of enteric infections in poultry and subsequent contamination of poultry products (Patterson and Burkholder, 2003). A 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). There are several microbial species that are utilized as probiotics including those of *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, and *Pediococcus* (Gaggia et al., 2010). Probiotics may be composed of one or a combination of many strains.

The primary function of the gastrointestinal tract is to digest and absorb nutrients in order to meet metabolic demands for maintenance, normal growth, and development, but it also acts as a vital barrier preventing the entry of several potentially harmful pathogens from the external environment (Beal et al., 2006). It is well known that a well-balanced gut microflora is essential

for animal health and performance. Probiotics are used to help maintain a healthy microbial balance within the intestine and promote gut integrity. This is accomplished through three main mechanisms: competitive exclusion, bacterial antagonism, and stimulation of the immune system (Ohimain and Ofongo, 2012). Introduction of probiotics is believed to prevent the colonization of pathogenic bacteria and attenuate enteric disease, thereby resulting in enhanced performance in poultry (Kabir et al., 2004).

Traditionally, probiotics have been administered in the feed or water supply to 1-day-old chicks. However, as soon as the chick hatches and is exposed to the external environment, it quickly begins to establish the microbial community in the intestine (Pedroso et al., 2005). This resident microflora may affect the establishment of the probiotic microorganisms, and in order to promote early establishment of probiotic strains, employing in ovo technology may be the answer.

Though there is a plethora of research in the areas of probiotic supplementation and in ovo feeding, very few researchers have entertained the idea of extending the concept of in ovo administration to probiotics. The first attempts of connecting these concepts demonstrated promising results where *Salmonella typhimurium* colonization was reduced in chicks administered an undefined cecal culture of bacteria in ovo (Cox et al., 1992). Unfortunately, negative results in terms of hatchability, performance, and mortality have been noted, but these consequences may be attributed to the probiotic strain used as well as the injection site (Cox et al., 1992; Meijerhof and Hulet, 1997). Edens et al. (1997) demonstrated positive results when using *Lactobacillus reuteri* for in ovo administration in hatching chickens and turkey poults. They found no differences among treatment groups when comparing the hatchability of embryos injected with *L. reuteri* either in the air cell or the amniotic fluid to uninocculated controls.

Similar results were also seen in turkey embryos. Additionally, they observed beneficial effects in terms of performance in broiler chicks and reduced cecal load of *Salmonella* in poults indicating that *L. reuteri* is a potential candidate for in ovo supplementation (Edens et al., 1997).

The objective of this dissertation project was to determine the effects of in ovo administration of the commercial product Primalac on broiler hatchability, post-hatch performance, and immune competency as evaluated by immune organ weights, intestinal immune-related gene expression, and lesion scores in the presence and absence of an *Eimeria* challenge.

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Chapter II

Literature Review

Avian Immune System

The structure of the avian immune system is comparable to its mammalian counterpart in terms of organization and mechanisms of action. 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 create physical barriers that are generally impermeable to most infectious agents. Most pathogens gain access through the respiratory and gastrointestinal tracts. If a pathogen is inhaled, ciliated epithelium and mucus found within the respiratory tract act as filters to assist in keeping the airways clear (Goldsby et al., 2000). 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). Because of 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 also maintains a dense microbial flora capable of preventing colonization by invading organisms through competitive exclusion and by producing soluble factors capable of inhibiting the growth and development of pathogens in the gut (Sharma, 2003). The lower pH found within the gastrointestinal tract also serves as a powerful chemical defense against ingested pathogens (Goldsby et al., 2000). In addition, high body temperature found in avian species is capable of prohibiting many infectious agents from causing disease (Butcher and Miles, 2001). Entry of the majority of pathogens is prohibited by these physical and chemical barriers, except in situations where these layers are compromised and the pathogens can gain 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 contains proteins such as perforin and proteases in order to kill virus-infected and tumor cells (Sharma, 2003).

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. Toll-like receptors are an invaluable class of PRRs that recognize microbial pathogens and induce an immediate response by innate immune cells. 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, and stimulate a response to the pathogen. Because of this presenting function, these innate immune cells are also called 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 and dictating the differentiation of naïve lymphocytes into appropriate effector cells in order to defeat specific types of pathogens (Lee and Iwasaki, 2007).

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. The antigen is first processed by the cells of the innate immune system and then is recognized by the key players of the adaptive immune system: B and T lymphocytes. 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), and, depending on the characteristics of the pathogen, the immune system will utilize the humoral branch, CMI, or a combination of the two in order to clear the offending pathogen. Lymphocytes are produced and develop in the primary lymphoid organs such as the thymus and bursa of Fabricius. Once developed, lymphocytes differentiate into effector cells in the secondary lymphoid organs such as the spleen and mucosal associated lymphoid tissues, where they come into contact with potential pathogens or other antigens (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 (Sharma, 2003).

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 (Sharma, 2003).

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 Th cells) and CD8+ (cytotoxic T cells) subpopulations (Viertlboeck and Gobel, 2008). Helper T cells are activated by recognition of a class II MHC coupled with processed antigen on an APC. Once activated, Th cells divide and produce a variety of cytokines in order to activate B and T lymphocytes as well as other immune cells. Depending on the cytokine profile present in the environment, proliferating Th cells will differentiate into one of two major subtypes known as Type 1 and Type 2 helper T cells (Th1 and Th2, respectively). The Th1 cells are primarily responsible for producing cytokines that encourage inflammation and activate B and T lymphocytes and macrophages, while inhibiting the function of Th2 cells, in order to generate immunity to intracellular pathogens. Cytokines secreted by Th2 cells stimulate B lymphocyte proliferation and antibody production while inhibiting Th1 cell function to enhance immunity to extracellular pathogens (Tizard, 2009). A third and more recently discovered Th cell lineage has been identified as Th17 due to the production of its signature pro-inflammatory cytokine IL-17. Cytotoxic T (Tc) 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-related 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. The Toll-like receptor (TLR) family is a highly conserved group of proteins that act as pathogen recognition receptors (PRRs)

by recognizing microbe-associated molecular patterns (MAMPs) expressed on infectious agents. They play a fundamental role in pathogen detection and are responsible for the initiation and regulation of the innate response. TLRs are expressed by a variety of cell types in order to recognize specific microbial components and trigger an appropriate immune response. TLR-2 and TLR-4 recognize various bacterial cell wall components such as peptidoglycan and lipoproteins from Gram positive bacteria and lipopolysaccharide from Gram negative bacteria, respectively (Kannaki et al., 2010).

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 (Tizard, 2009). The mucin (Muc)-2 gene encodes mucin production, which is mediated by T lymphocytes and Th2 cytokines (Beum et al., 2005). Mucin is made up of glycoproteins and serves a protective function by binding to pathogens, preventing their adhesion to the intestinal surface. 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 Th1 cell differentiation, suppressing Th2 cell activity, and enhancing innate immune cell activation and function (Kaiser and Staheli, 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 ligand superfamily (Hong et al., 2006b). Interleukin (IL)-4, a representative of Th2 cytokines, plays a fundamental role in the stimulation of B lymphocytes, T lymphocyte proliferation and the differentiation of CD4+ T cells into Th2 cells (Fietta and Delsante, 2009). The functions of IL-13, also characterized as a Th2 cytokine, overlap considerably with those of IL-4. Both IL-4 and IL-13 function by inhibiting the production of pro-inflammatory modulators.

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 (Beal et al., 2006). 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, contain 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 (Beal et al., 2006).

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 loss 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). 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. 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 even more. 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. 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. 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. There are two classes of anticoccidials; coccidiostats and coccidiocidals. Coccidiostats are dietary additives fed continuously at low levels, and they act by arresting the growth of intracellular coccidia. Coccidiocidals, on the other hand, act by actively destroying the parasites during their development (McDougald, 2003). However, growing consumer concerns about chemical residues in poultry products and microbial resistance have caused producers to shift to other methods of control, including vaccination. It has been established that natural immunity is acquired after an initial coccidial infection. This knowledge has resulted in the development of several anticoccidial vaccines in order to abate coccidiosis. Unfortunately, live, non-attenuated vaccines tend to reduce performance due to the initial infection and attenuated vaccines do not appear to be as effective as their more virulent counterparts (Williams, 2002). 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, 2006).

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). It has been shown that 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 boosted 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 accession 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 in concert 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 Th1 cytokines IFN-γ, IL-1β, IL-6, IL-12, IL-15, IL-17 and IL-18, are up-regulated as well as the Th2 cytokines IL-3, 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*, *IL-3*, 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 Th1 cytokines IL-2, IL-18 and IFN-γ. However, they observed an increase in gene expression of Th2 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 Th1 and Th2 subsets in controlling *Eimeria* infections.

Probiotics and Poultry

Prophylactic use of anti-coccidial 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 (Patterson and Burkholder, 2003). 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 gut microflora is an essential component involved in the overall health and well-being of the bird. Alterations in the microbial profile can influence the development, physiology and immunology of the gastrointestinal tract as well as resistance to enteric infections through their interactions 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. 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 maintain normal intestinal microflora and inhibit establishment of pathogenic bacteria through competition for space, attachment sites, and available nutrients. Probiotic bacteria may also produce antimicrobial substances such as volatile fatty acids, bacteroicins, 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 permeabilizing their cellular membranes or hindering essential enzymes. Furthermore, many probiotics, predominantly those containing *Lactobacillus* strains, 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) and turkeys (Torres-Rodriguez et al., 2007). 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; Seifert et al., 2011; Wolfenden et al., 2011). It has been suggested that these discrepancies could be due to a variety of factors including, but not limited to, strain(s) of bacteria utilized, composition and viability of the probiotic, preparation method, dosage, application method, frequency of application, overall diet, drug interactions, and condition of the animal (Huang et al., 2004; Mountzouris et al., 2007).

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 (Dunham et al., 1993; Samanya and Yamauchi, 2002; Markovic et al., 2009). The increase in villus length suggests a greater surface area with increased absorptive capacity. The intestinal crypt is where enterocytes undergo continuous proliferation in order to replace cells lost at the villus tip due to normal sloughing or inflammation from pathogens (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 (Markovic 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

The innate immune system is characterized by non-specific defense mechanisms and acts as the first line of defense providing immediate protection against an impending foreign challenge. Several studies have provided evidence that specific strains of probiotics are able to stimulate many aspects of innate immunity. Farnell and colleagues (2006) found that heterophils isolated from broiler chicks fed probiotic isolates of *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 Biomin PoultryStar had greater levels of heterophil oxidative burst (Stringfellow et al., 2010). The avian heterophil is considered to be equivalent to the mammalian neutrophil. These innate immune cells are highly phagocytic and make up the first line of cellular defense against a broad spectrum of microbial pathogens. Oxidative burst and degranulation are bactericidal mechanisms used by heterophils to kill phagocytized pathogens (Harmon, 1998).

Macrophages are phagocytic immune cells that play a key role in detecting and processing antigens and 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 1 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. Furthermore, abdominal exudate cells from *Salmonella*-challenged birds had greater phagocytic capacity when treated with probiotics (Higgins et al., 2007). The up-regulation of host innate immunity, including enhanced antimicrobial defenses and phagocytosis, may result in the reduction and/or 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 New Castle 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 IgG, against tetanus toxoid in the intestine. The administration of probiotics also augmented serum levels of IgG and IgM antibodies reactive against 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 and that those 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; Lee et al., 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 as shown 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-1β, IL-6, IL-17α, 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 have been shown to 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; Lee et al., 2007b; Santosh and Gupta, 2011). Moreover, *Bacillus*-based probiotics have been shown to reduce 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). Protection against a *Salmonella* challenge by probiotic supplementation has also been noted in turkey poults (Grimes et al., 2008; Wolfenden et al., 2011). 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; Knap et al., 2010). 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.

Probiotics and In Ovo Technology

For a commercial broiler, the incubation and neonatal periods represent approximately 50% of the productive lifespan of the bird. The time from embryonic day 18 to 4 days post-hatch is very critical for the survival and development of the chick and represents the metabolic and physiological transition from egg-supplied nutrition to feed. Around embryonic day 18, the chick will have its first meal when it consumes the amniotic fluid before internal pipping (Ferket, 2006). In ovo feeding is the administration of liquid feed components into the amnion of an egg several days before hatch. Intake of exogenous feed enhances intestinal and organ development, so providing a nutritional substance to the birds at an early age will stimulate development of the intestinal tract sooner and allow more efficient digestion upon hatch (Yegani and Korver, 2008). Some common nutrients used for in ovo feeding include easily digestible carbohydrates, protein,

individual amino acids, and β -hydroxy-β-methylbutyrate (Tako et al., 2004; Siwicki et al., 2006).

In 1992, Embrex, Inc. developed the first commercially available egg injection system capable of inoculating between 20,000 and 30,000 eggs per hour (Johnston et al., 1997; Jochemsen and Jeurissen, 2002). It was originally designed to administer vaccines but has since been explored for other technologies such as in ovo feeding. Using this system, nutrients are injected into the amnion around embryonic day 18 in chicken eggs. Rhythmic respiratory movements in chicken embryos begin between embryonic days 17 and 18 (Windle and Barcroft, 1938). This rhythmic movement causes the amnion and any injected nutrients to be taken into the mouth. After ingestion, injected substances move into the lungs via the trachea and into the intestine (Jochemsen and Jeurissen, 2002).

In ovo feeding has been shown to elicit beneficial effects on bird health and performance. In ovo feeding increases liver glycogen stores, increases activity of brush border enzymes, improves nutrient uptake, accelerates intestinal development, and increases body weight at hatch (Tako et al., 2004; Uni and Ferket, 2004; Foye et al., 2006). These improvements to chick quality and intestinal function may play a key role in bird performance by increasing livability and live market weight and meat yield, as well as reducing mortality and the time required to reach market weight (Uni and Ferket, 2004).

Though research in the areas of probiotic supplementation and in ovo feeding is quickly growing in popularity, little has been done to extend the concept of in ovo administration to probiotics. Work done by Cox et al. (1992) is considered to be the first attempt at connecting the concepts of competitive exclusion and in ovo administration. The researchers administered an undefined cecal culture of bacteria in ovo to embryonated chicken eggs. At hatch, the chicks

were challenged with *Salmonella typhimurium* in doses ranging from 1,000 to 10 million cfu. Administration of a 1,000-fold dilution of the bacterial culture reduced *Salmonella* colonization in chicks given the 1,000 or 100,000 cfu challenge dose. Chicks given the 1,000,000-fold dilution of the cecal culture showed a subtle reduction of *Salmonella* colonization when challenged with 1,000 cfu, but not at higher challenge doses. Cox et al. (1992) also evaluated the effects of dose and delivery site (air cell and amnionic fluid) of in ovo administered cecal culture on hatchability. Dose of the culture and site of administration both significantly reduced hatchability. Air cell administration reduced hatching rate but allowed *Salmonella*-resistant chicks to hatch, but when culture was delivered into the amnionic fluid, the undiluted and the 1,000-fold dilution prevented hatching and at 1 million-fold dilution less than half of the chicks were able to hatch.

Meijerhof and Hulet (1997) conducted three experiments to assess the influence of in ovo injection of the competitive exclusion product Broilact on hatchability and protection against a *S. panama* challenge in broiler chicks. Injection of Broilact into the air cell significantly reduced hatchability while injection directly into the embryo caused almost complete loss of hatchability. Those chicks that were capable of hatching following injection into the air cells were subject to increased mortality mostly due to a reduction in yolk sac uptake. Following a *S. panama* challenge, chicks having received the Broilact injection into the air cell showed no significant improvement in protection, though numerically there were fewer birds that tested positive for *Salmonella* than in the challenged control group (Meijerhof and Hulet, 1997).

Edens et al. (1997) demonstrated the potential for the beneficial use of *Lactobacillus reuteri* for in ovo administration in hatching chickens and turkey poults. First they compared the hatchability of embryos injected with *L. reuteri* either in the air cell or the amniotic fluid to

uninocculated controls and found no differences among the treatment groups. Similar results were also seen in turkey embryos. They further looked at the effects of in ovo administered *L. reuteri* during a *S. typhimurium* challenge and performance post-hatch in chicks and poults. Though no significant differences were seen in chicks, turkey poults exposed to *L. reuteri* in ovo had a reduced cecal load of *Salmonella* than did the challenged control birds. In terms of performance, broiler chicks inoculated with *L. reuteri* and subsequently challenged with *Salmonella* displayed reduced mortality and increased bodyweight when compared to the challenge only birds. These findings indicate that *L. reuteri* is a safe organism that could be administered in ovo without having a negative impact on hatchability, and can provide protective benefits against enteric pathogens such as *S. typhimurium*, as well as help reduce production losses due to disease (Edens et al., 1997).

Probiotics supplementation has been shown to have beneficial effects in many animal species. In poultry, probiotics enhance performance, promote a healthy balance of microflora in the gut, and counteract the negative consequences of several enteric diseases. However, as soon as the chick hatches and is exposed to the external environment, it quickly begins to establish the microbial community in the intestine and this resident microflora, whether friendly or potentially harmful, may affect the colonization of the probiotic microorganisms. In ovo technology represents a means to take advantage of this crucial time and promote early colonization of beneficial bacteria in order to stimulate intestinal and immune system development. Unfortunately, little is known concerning the mechanistic actions of probiotics and how they alter the immune system. More research is required to further define the dynamic function of probiotics in terms of their immunomodulating capabilities. Understanding the embryo's response to probiotics and changes in the local immune responses is essential to enable the

manipulation of the microbiota for improved performance and intestinal health. To this end, the studies described herein were conducted to shed some light on the immunomodulating properties of probiotics and the effects of supplementing probiotics prior to hatch on post-hatch performance and health status.

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CHAPTER III

In Ovo Supplementation of Primalac and the Effects on Performance and Immune-Related Gene Expression in Broiler Chicks

ABSTRACT: Probiotics are live, nonpathogenic microorganisms known to have a positive effect on the host by improving the natural balance of gut microflora. The objective of this study was to determine the effects of administering probiotics (Primalac W/S) in ovo on hatchability, post-hatch performance and intestinal immune-related gene expression of broiler chicks. At embryonic day 18, 360 Cobb 500 eggs were injected with sterile water (sham), $1x10^5$, $1x10^6$, or $1x10^7$ (P1, P2, and P3 respectively) probiotic bacteria. Another 90 eggs remained uninjected to serve as a negative control. Measurements and tissue samples were taken on day of hatch (DOH) and days (d) 4, 6, 8, 15, and 22. No significant differences were seen among groups for hatchability, feed intake, feed conversion ratios, or mortality. Body weight (BW) of P2 was significantly greater than that of the negative control, sham and P1 on d4, and that of the negative control and P1 on d6. A similar pattern was observed for BW gain (BWG) from DOH to d4. Real-time PCR was used to investigate the expression of immune-related genes in the ileum and cecal tonsils. Other than an initial upregulation of inducible nitric oxide synthase on DOH, in ovo probiotic supplementation was associated with downregulated expression of Toll-like receptors-2 and -4, inducible nitric oxide synthase, trefoil factor-2, mucin-2, interferon-γ, and interleukins-4 and -13 in both the ileum and cecal tonsils, though expression patterns differed based on treatment, tissue, and time point evaluated. Taken together, these results indicate that in ovo supplementation of the probiotic product Primalac does not impact hatchability, can improve performance during the first week post-hatch, and is capable of modulating gene expression in the ileum and cecal tonsils.

INTRODUCTION

During the period immediately following hatch, the immune system of the chick is immature and inefficient, making the animal extremely vulnerable to infectious threats found in the environment. Due to the inadequacies in the neonatal chick immune system, immunomodulators are being sought after and studied in order to protect the chick during this immunologically sensitive time. Furthermore, with increasing concerns about antibiotic resistance, the European Union has placed a ban on the sub-therapeutic use of antibiotics as feed additives. Unfortunately, this ban has resulted in a decline of animal health (Castanon, 2007). The risk of a ban being enacted in the U.S. has created an impetus for finding alternatives capable of maintaining animal health without impeding performance.

Probiotics are one of the options under evaluation as antibiotic alternatives with their potential to modulate the immune system and reduce the rate and severity of enteric infections in poultry (Patterson and Burkholder, 2003). A 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).

A well-balanced gut microflora is essential for animal health and performance, and probiotics maintain this normal flora through a number of mechanisms including competitive exclusion. For poultry, supplementation of probiotics enhances growth and improves feed conversion in chickens and turkeys (Kabir et al., 2004). Manipulation of intestinal microflora through the use of probiotics may also influence the immune response. Dietary administration of probiotics may help maintain a healthy balance of microflora in the gut and provide protection against several enteric pathogens through stimulation of the mucosal immune system (Nava et al., 2005). In this respect, probiotics have been shown to enhance the innate immune system by

increasing heterophil oxidative burst and degranulation, as well as augmenting the phagocytic capacity of macrophages (Farnell et al., 2006; Higgins et al., 2007; Stringfellow et al., 2010). Not only do probiotics strengthen innate immunity, they also improve the adaptive immune system. Probiotics increase antibody production to many economically important disease agents such as *Eimeria*, New Castle disease virus, and infectious bursal disease virus (Khaksefidi and Ghoorchi, 2006; Lee et al., 2007a; Lee et al., 2007b; Nayebpor et al., 2007). Furthermore, probiotics have demonstrated their ability to increase T lymphocyte numbers and modulate production of several pro-inflammatory, T helper Type-1 (Th1) and T helper Type-2 (Th2) cytokines when provided in the diet, though discrepancies in cytokine production have been noted due to differences in the probiotic strains used (Dalloul et al., 2005; Brisbin et al., 2010; Lee et al., 2010). The immune enhancing capabilities of probiotics have resulted in the clearance of several economically important pathogens such as *Eimeria* spp., *Salmonella* spp., *Escherichia coli* and *Clostridium perfringens*, further asserting their potential use as an antibiotic alternative (Dalloul and Lillehoj, 2005; Knap et al., 2010) .

For a commercial broiler, the incubation and neonatal periods represent approximately 50% of the productive lifespan of the bird. The time from embryonic day 18 to 4 days post hatch is critical for the survival and development of the chick (Ferket, 2006), and in ovo technology represents one means to administer probiotics and promote early colonization of beneficial bacteria in order to stimulate intestinal and immune system development. The objective of the current study was to determine the effects of administering a *Lactobacillus*-based probiotic (Primalac) in ovo on hatchability, post-hatch performance, and intestinal immune-related gene expression of broiler chicks.

MATERIALS AND METHODS

Birds and in ovo treatments

This project was approved and conducted under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee. At embryonic day (d) 18, 450 fertile Cobb 500 eggs were obtained from a commercial hatchery (Pilgrim's Pride, Broadway, VA) and transported to the Virginia Tech Turkey Research Center. Prior to injection, all eggs were candled to determine position of the air cell. Eggs were sanitized by swabbing the large end (outside of the air cell) with 0.5% sodium hypochlorite and once dried, they were sprayed with 70% isopropyl alcohol. To create a guide and avoid cracking, a hole was made in the center of the air cell of those eggs receiving injections using an 18-gauge needle fitted with a rubber stopper to prevent the needle from piercing the air cell membrane. Needles were disinfected in between each injection by dipping in 0.5% sodium hypochlorite. Next, 360 eggs were injected in the amnion with 100 μL of either sterile water (sham), $1x10^5$, $1x10^6$, or $1x10^7$ (P1, P2, and P3 respectively) probiotic bacteria dissolved in sterile water (Primalac W/S, Star-Labs Inc.). Injections were performed using a 1 mL syringe equipped with a 22-gauge, 1-inch short bevel needle. A new syringe and needle were used for each injection. The remaining 90 eggs were not injected and served as a negative control. Eggs were placed into one of 3 replicate hatching trays (30 eggs/tray). On day of hatch (DOH), birds were individually tagged, placed into floor pens with clean shavings (3 replicate pens/treatment), and provided water and standard unmedicated starter feed in mash form ad libitum.

Hatchability and post-hatch performance parameters

On DOH, percent hatchability was recorded, and birds were weighed prior to placement and on days (d) 4, 6, 8, 15 and 22. Mean body weight (BW) for each treatment group was

calculated from the individual bird replicates for each weigh day. Average body weight gains (BWG) were calculated for each period and cumulatively based on individual bird weights. Feed intake (FI) of each group was measured at the same time periods as body weights (d4, 6, 8, 15, 22) with cumulative averages calculated. Feed conversion ratio (FCR) was calculated using BW and FI and adjusted for mortality. Mortality was recorded on a daily basis throughout the trial.

Tissue sampling for gene expression analysis

On DOH, d4, 6, 8, 15, and 22, 6 birds per treatment were euthanized by cervical dislocation. A section of the ileum, defined as the area posterior to the Meckel's diverticulum to the ileo-cecal junction, and cecal tonsils were sampled, rinsed in cold PBS, and placed in RNA*later* (Qiagen, Germantown, MD) for subsequent gene expression analysis. The samples were stored at -80°C until analysis.

Intestinal gene expression

Intestinal samples were removed from -80°C and a 20-30 mg aliquot of each sample was weighed, placed into a 2 mL microcentrifuge tube along with a 5 mm stainless steel bead and 600 µL of RLT buffer, and homogenized using the TissueLyser II system (Qiagen) according to manufacturer's recommendation. Total RNA was extracted from individual intestinal tissues using the RNeasy mini kit following the animal tissue protocol (Qiagen). Following extraction, RNA was eluted by rinsing the column membrane twice with 25 µL of RNase-free water. Total RNA concentration was determined at OD 260 nm (NanoDrop-1000, Thermo Fisher Scientific, Waltham, MA) and RNA purity was verified by evaluating the ratio of OD 260 nm to OD 280 nm. Total RNA was diluted to 0.2 µg/µL in nuclease-free water. Reverse transcription was accomplished using the high capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's protocol, and the cDNA was stored at -20°C.

Quantitative real-time PCR (qRT-PCR) was performed using an ABI 7500 FAST Real-Time PCR System (Applied Biosystems). The cDNA was diluted 1:30 in nuclease-free water then 1 µL of the diluted cDNA was added to each well of a 96-well plate. Next, 9 µL of real-time PCR master mix containing 5 µL FAST SYBR Green Master Mix (Applied Biosystems), 0.5 µL each of 2 µM forward and reverse primers and 3 µL of sterile nuclease-free water per reaction were added to each well for a final volume of 10 µL. During the PCR reaction, samples were subjected to an initial denaturation phase at 95°C for 20 sec followed by 40 cycles of denaturation at 95°C for 3 sec and annealing and extension at 60°C for 30 sec. Gene expression for interleukin (IL)-4, IL-13, interferon (IFN)-γ, inducible nitric oxide synthase (iNOS), lipopolysaccharide-induced tumor necrosis factor-α (LITAF), mucin(Muc)-2 , trefoil factor(TFF)-2, Toll-like receptor (TLR)-2 and TLR-4 was analyzed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. Each reaction was run in duplicate. Primers were designed (Table 3.1) using the Primer Express 3.0 software (Applied Biosystems) and synthesized by MWG Operon (Huntsville, AL). Results from qRT-PCR were analyzed using the 7500 Real-Time PCR software (Applied Biosystems). Average gene expression relative to the *GAPDH* endogenous control for each sample was calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001). The calibrator for each gene was the average ΔCt value from the negative control group for each sampling day for each respective tissue.

Statistical analysis

Results were analyzed using the Fit Model platform in JMP 9.0 (SAS Institute Inc., Cary, NC). For BW and BWG data, the model included in ovo treatment as a fixed effect and pen as a random effect. For hatchability, mortality, FI, and FCR, the model included in ovo treatment with pen representing the experimental unit. Percent hatchability and mortality data were arc

sine transformed prior to analysis. Gene expression analysis was performed with in ovo treatment in the model with bird serving as the experimental unit. Differences among experimental treatments were tested using Student's *t*-test following ANOVA. Values were considered statistically different at $P \leq 0.05$. Results are reported as least squares means with standard errors.

RESULTS

Hatchability and post-hatch performance

In ovo treatment had no significant effect on hatchability during this study (Figure 3.1). Body weight on DOH was not affected by in ovo treatment, but on d4 the P2 group had increased BW when compared to the negative control, sham and P1 groups (P = 0.01) (Table 3.2). A similar pattern was observed on d6 where the P2 birds had higher BW than negative control and P1 birds, but not the sham group. The P3 group also had a greater BW than the P1 group on d6 (P = 0.03). Body weight gain of P2 was higher than that of the negative control and P1 from DOH to d4 (P = 0.03) (Table 3.3). Differences were not noted at any other time point for BW or BWG. In ovo supplementation of Primalac did not significantly alter FI, FCR, or mortality in this study (Table 3.3 and Figure 3.2).

Intestinal gene expression

Ileal *TLR-2* expression was downregulated on d4 in the P2 and P3 groups (P = 0.045) and on d6 in the sham, P1 and P3 groups when compared to the negative control (P = 0.02) (Figure 3.3a). There was a significant effect of in ovo treatment in the cecal tonsils on DOH where the sham injected birds had decreased levels of *TLR-2* expression (P = 0.001) (Figure 3.3b). On d4, all injected groups had lower *TLR-2* expression than the negative control (P = 0.02). Levels of *TLR-2* were decreased in P2 chicks on d6 (P = 0.001) as well as in P3 chicks on d8 (P = 0.01).

On DOH, *TLR-4* expression was decreased in the ileum of sham injected and P2 birds when compared to the negative controls (P = 0.01) (Figure 3.4a). Expression of *TLR-4* was also downregulated on d6 in the ileum of P3 birds when compared to the negative control and sham birds (P = 0.03). In the cecal tonsil, *TLR-4* expression on DOH was upregulated in P3 chicks when compared to sham, P1 and P2, but not the negative control (P = 0.02) (Figure 3.4b). Expression of *TLR-4* was decreased in P3 on d4 (P = 0.02) and on d6 in P1 and P2 when compared to the controls (P = 0.002). Levels of *TLR-4* mRNA were also decreased in P3 birds on d6, but only in comparison to the negative control. On d22, *TLR-4* expression was downregulated in P3 when compared to the negative control, sham, and P1groups (P = 0.03).

Expression of *iNOS* was augmented in the ileum of P3 birds on DOH when compared to the negative control and sham groups, and in P1 and P2 when compared to the negative control only (P = 0.01) (Figure 3.5a). On d6, expression levels of *iNOS* were decreased in P1 when compared to the negative control, sham, and P2 groups, and in P2 and P3 when compared to the negative control only (P = 0.0004). Expression of *iNOS* was also reduced on d8 in P3 birds when compared to the negative control and sham injected groups, while all probiotic supplemented groups had lower expression than the sham inoculated group (P = 0.01). When looking at *iNOS* expression in the cecal tonsils, levels were decreased in all injected groups on d8 (P = 0.006) and in P3 on d22 (P = 0.0001) (Figure 3.5b).

Relative to the negative control, *TFF-2* expression in the ileum was decreased on d6 in all injected groups (P = 0.004) (Figure 3.6a). Expression of *TFF-2* in the cecal tonsils was downregulated in P2 and P3 birds on d6 (P = 0.001) (Figure 3.6b). Additionally, *TFF-2* was downregulated on d22 in P2 and P3 when compared to the negative control with P3 also exhibiting lower expression than P1 (P = 0.01).

Expression of *Muc-2* in the ileum was not affected by in ovo treatment at any time point during this study (Figure 3.7a). On DOH, there was a significant effect of in ovo treatment in the cecal tonsils where *Muc-2* expression was decreased in P2 and P3 chicks when compared to both control groups ($P = 0.001$) (Figure 3.7b). Expression of *Muc-2* on d6 was downregulated in the sham, P1 and P2 groups when compared to the negative controls, but upregulated in P3 when compared to the sham and P1 birds ($P = 0.01$). Birds in P2 had increased expression of *Muc-2* on d22 in relation to the negative controls, while P3 birds had decreased expression in comparison to the sham, P1 and P2 groups ($P = 0.02$).

On d6, *IFN-γ* expression in the ileum was lower in P3 birds than both the negative control and sham injected groups (Figure 3.8a). All injected groups displayed reduced *IFN-γ* levels when compared to the negative controls ($P < 0.0001$). Expression of *IFN- γ* was decreased on d8 in the cecal tonsils of P3 birds when compared to the negative control and sham birds ($P = 0.03$) (Figure 3.8b).

Ileal expression of *LITAF* on d6 was downregulated in P1 and P2 birds when compared to the sham injected birds, but not the negative control birds ($P = 0.04$) (Figure 3.9a). Significant differences were not observed for *LITAF* expression in the cecal tonsils (Figure 3.9b).

Interleukin-4 expression in the ileum was decreased on d4 in all groups when compared to the negative control ($P = 0.002$) (Figure 3.10a). On d6, the P1 group had lower *IL-4* expression than both the negative control and sham groups, while expression in P2 and P3 were only found to be less than the negative control ($P = 0.0003$). Furthermore, expression of *IL-4* on d15 was lower in P3 than in the negative control, sham and P1 groups. Expression of *IL-4* was also reduced in P2 birds on d15, but only when compared to the sham and P1 groups ($P = 0.01$). In the cecal tonsils, *IL-4* expression was decreased on d22 in P2 and P3 chicks when compared to

both control groups and in P1 chicks when compared to the sham group only (P = 0.002) (Figure 3.10b).

On d6, *IL-13* expression was downregulated in the ileum of P1 and P3 birds when compared to the negative control and sham injected birds (P = 0.04) (Figure 3.11a). Expression levels of *IL-13* were decreased in the cecal tonsils on DOH in P3 chicks relative to the negative control, sham and P1 groups and in P2 chicks relative to the negative controls and P1 (P = 0.0002) (Figure 3.11b). Following a similar pattern, *IL-13* levels on d4 were lower in P3 birds than either of the control groups while P2 was lower than the negative controls only (P = 0.001). All probiotic supplemented groups showed downregulation of *IL-13* on d8 in comparison to the sham group , but only P2 and P3 were found to be different from the negative controls (P = 0.003).

Discussion

The aim of this study was to explore the effects of in ovo administration of the probiotic product Primalac W/S (Water Soluble) in broiler chicks. In this experiment, in ovo supplementation of probiotics had no effect on hatchability. Corroborating our findings, Edens et al. (1997) compared the hatchability of broiler embryos injected with *Lactobacillus reuteri* either in the air cell or the amniotic fluid to uninocculated controls and found no differences among the treatment groups. Similar results were also seen in turkey embryos (Edens et al., 1997). Alternatively, other researchers have found that in ovo injection of some probiotic bacteria can lead to significantly reduced hatchability (Cox et al., 1992; Meijerhof and Hulet, 1997). These findings suggest that the probiotic bacteria in Primalac can be safely administered in ovo without negatively impacting hatchability.

Probiotics may be utilized to alter the gut microbial profile in order to create conditions favorable to enhancing performance. During this study, augmented BW and BWG were noted in P2 birds on d4 and d6. Supporting our finding, Edens and colleagues (1997) found that broiler chicks inoculated with *L. reuteri* and subsequently challenged with *Salmonella typhimurium* or *Escherichia coli* displayed increased BW when compared to the challenge only birds. Dietary supplementation of probiotics has also been shown to result in improved BWG and FCR 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) and turkeys (Torres-Rodriguez et al., 2007). Despite the plethora of data demonstrating the positive effects of probiotics on performance, several researchers reported no significant enhancements due to probiotic supplementation (Rahimi et al., 2011; Seifert et al., 2011; Wolfenden et al., 2011). These discrepancies could be due to a variety of factors including, but not limited to, strain(s) of bacteria utilized, composition and viability of the probiotic, preparation method, dosage, application method, frequency of application, overall diet, drug interactions, and condition of the animal (Huang et al., 2004; Mountzouris et al., 2007).

Modulation of the immune system by in ovo supplementation of probiotics in poultry and early establishment of beneficial microflora may lead to increased overall health and well-being while decreasing the need for prophylactic antibiotic use due to reduced infection rates. In spite of considerable amount of published data regarding the efficacy of probiotics in poultry, the exact mechanism of how probiotics modulate the immune system is still not fully understood. Additional research is required to further define the dynamic function of probiotics in terms of their immunomodulating capabilities against pathogens.

Our goal was to identify patterns of gene expression underlying the effects of in ovo probiotic supplementation on the immune system. We assayed both ileum and cecal tonsil tissues for differences in gene expression using real-time PCR. To observe the effects of probiotics on innate immunity, we evaluated the expression of *TLR-2*, *TLR-4*, *iNOS*, *Muc-2* and *TFF-2*. The TLR family is a highly conserved group of proteins that act as pathogen recognition receptors (PRR) recognizing microbe-associated molecular patterns (MAMPs) that are expressed on infectious agents. They play a fundamental role in pathogen detection and are responsible for the initiation and regulation of the innate response. When exposed to antigens or chemotactic agents, macrophages will begin to produce 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 (Tizard, 2009). The *Muc-2* gene, which is mediated by T lymphocytes and Th2 cytokines, is responsible for encoding mucin production (Beum et al., 2005). Mucin is made up of glycoproteins and serves a protective function by binding to pathogens, thus preventing their adhesion to the intestinal surface. Trefoil factor-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).

To evaluate effects on the adaptive immune response, we analyzed gene expression of *IFN-γ*, *LITAF*, *IL-4* and *IL-13*. Interferon-γ is a vital cytokine that plays a central role in regulating the innate and adaptive immune responses, and is responsible for promoting Th1 cell differentiation, suppressing Th2 cell activity, and enhancing innate immune cell activation and function (Kaiser and Staheli, 2008). Expression of *LITAF* is principally in the spleen of chickens as well as in intestinal intraepithelial lymphocytes. The LITAF protein is a transcription factor

that mediates the expression of members of the tumor necrosis factor ligand superfamily (Hong et al., 2006). Interleukin-4, a representative of Th2 cytokines, plays a fundamental role in the stimulation of B lymphocytes, T lymphocyte proliferation and the differentiation of CD4+ T cells into Th2 cells (Fietta and Delsante, 2009). The functions of IL-13, also characterized as a Th2 cytokine, overlap considerably with those of IL-4. Both IL-4 and IL-13 function by inhibiting the production of pro-inflammatory modulators.

On DOH, all probiotic treatment levels resulted in increased expression of *iNOS* in the ileum when compared to the negative control, while the highest probiotic dosage resulted in levels higher than that of both the negative control and sham injected groups. These data suggest that probiotics may promote antimicrobial activity in the host by increasing intestinal *iNOS* gene expression and thus provide a means of protection against pathogens soon after hatch when the chick is immunologically most vulnerable.

Other than *iNOS* on DOH, our results suggest the primary effect of probiotics is to downregulate immune-related gene expression within the intestine. The downregulation of these genes in the probiotic-treated groups may be the result of inhibitory effects of probiotic bacteria on pathogen colonization. A reduction in intestinal colonization by pathogenic bacteria may have eliminated the need for the induction of these genes. Probiotics have demonstrated their ability to modulate the levels of several cytokines; however, discrepancies have been noted due to differences in the probiotic strains, combinations of probiotic strains and presence or absence of a challenge (Dalloul et al., 2005; Brisbin et al., 2010; Lee et al., 2010). Correlating with our findings, several studies have noted decreases in immune-related factors. Akbari et al. (2008) found the expression of antimicrobial peptides in the cecal tonsils to be downregulated due to probiotic supplementation during a *Salmonella* infection. The author speculated that the

observed decrease in gene expression could be a result of a reduced S*almonella* load in the intestine (Akbari et al., 2008). 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 and that those levels were not different from those of the negative controls. Haghighi et al. (2008) evaluated gene expression in the cecal tonsils of chicks treated with probiotics and challenged with *Salmonella* and found that *IL-12* and *IFN-γ* levels were repressed by probiotic treatment and these results were correlated with reduced intestinal *Salmonella* colonization. While evaluating the effects of feeding a *Lactobacillus*-based probiotic during an *Eimeria acervulin*a infection, Dalloul and colleagues (2003) observed that probiotic supplemented groups displayed reduced oocyst production and lower antibody levels against a recombinant coccidial antigen in intestinal secretions than the non-supplemented group. The immunosuppressive effects seen in these studies could be a result of lowered invasive potential of the pathogens, enhanced clearance and accelerated recovery caused by the probiotic treatments. Furthermore, the bacterial strains used as probiotics are normal inhabitants of the chicken intestine and it has been speculated that commensal bacteria have the ability to produce proteins with immunosuppressive effects (Brisbin et al., 2010). It is of note that no challenge was utilized during this study, but these results provide rationale for further analysis to elucidate the impact of this application on development of the immune system during infection, thus providing better evidence of potentially higher degree of protection against enteric challenges.

In conclusion, our data demonstrate that in ovo supplementation of Primalac does not impact hatchability and can enhance performance of broilers during the first week post-hatch.

Furthermore, in ovo inoculation of Primalac acts as an immunomodulator by altering the expression of several immune-related genes within the ileum and cecal tonsils. The underlying mechanisms of probiotic activity are multi-faceted with effects on both the innate and adaptive immune systems. To our knowledge, this is the first study to evaluate the effects of in ovo administration of the probiotic product Primalac W/S on performance and immune profile in broiler chicks. This study reveals the immunoregulatory effect of probiotics on intestinal immunity in poultry and provides justification for further study to investigate the various effects of *Lactobacillus*-based probiotics in poultry and in ovo supplementation as a means of promoting early establishment of beneficial bacteria and immune system development.

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Table 3.1. Primers used for relative real-time PCR[1]

Target	Accession No.	Nucleotide sequence (5' → 3')
GAPDH_F	NM_204305	CCTAGGATACACAGAGGACCAGGTT
GAPDH_R		GGTGGAGGAATGGCTGTCA
IL-4_F	NM_001007079	GCTCTCAGTGCCGCTGATG
IL-4_R		GAAACCTCTCCCTGGATGTCAT
IL-13_F	NM_001007085	CATGACCGACTGCAAGAAGGA
IL-13_R		CCGTGCAGGCTCTTCAGACT
IFN-γ_F	NM_205149	GCTCCCGATGAACGACTTGA
IFN-γ_R		TGTAAGATGCTGAAGAGTTCATTCG
iNOS_F	D85422	CCTGTACTGAAGGTGGCTATTGG
iNOS_R		AGGCCTGTGAGAGTGTGCAA
LITAF_F	AY765397	TGTTCTATGACCGCCCAGTTC
LITAF_R		AGACGTGTCACGATCATCTGGTTA
Muc-2_F	XM_421035	TTCATGATGCCTGCTCTTGTG
Muc-2_R		CCTGAGCCTTGGTACATTCTTGT
TFF-2_F	XM_416743.2	TGGTCCCCCAGGAATCTCA
TFF-2_R		CACCGACGCATTGAAGCA
TLR-2_F	NM_204278	GCGAGCCCCCACGAA
TLR-2_R		GGAGTCGTTCTCACTGTAGGAGACA
TLR-4_F	NM_001030693	CCACACACCTGCCTACATGAA
TLR-4_R		GGATGGCAAGAGGACATATCAAA

[1]Primers designed by Primer Express software (Applied Biosystems, Foster City, CA).

Table 3.2. Effect of in ovo probiotic supplementation on body weight (g).

	DOH	d4	d6	d8	d15	d22
Neg[1]	44.2 ± 0.24	78.4 ± 0.91^{bc}	116.9 ± 1.76^{bc}	171.9 ± 2.57	472.4 ± 7.10	940.7 ± 18.26
Sham[2]	44.2 ± 0.22	78.9 ± 0.89^{bc}	119.0 ± 1.73^{abc}	174.8 ± 2.50	477.1 ± 6.84	955.8 ± 18.37
P1[3]	43.9 ± 0.23	76.5 ± 0.91^{c}	113.9 ± 1.76^{c}	170.3 ± 2.55	470.9 ± 7.03	954.1 ± 18.24
P2[4]	44.2 ± 0.23	81.5 ± 0.90^{a}	123.7 ± 1.76^{a}	178.1 ± 2.56	480.7 ± 7.04	961.5 ± 18.46
P3[5]	44.5 ± 0.24	80.2 ± 0.91^{ab}	120.5 ± 1.75^{ab}	174.1 ± 2.54	477.1 ± 7.03	932.6 ± 18.29
P -Value	0.58	0.01	0.03	0.31	0.86	0.95

Different letters within a column indicate significant difference among treatment groups

[1]Neg = negative control

[2]Sham = sham water injection

[3] P1 = $1x10^5$ probiotic bacteria

[4]P2 = $1x10^6$ probiotic bacteria

[5]P3 = $1x10^7$ probiotic bacteria

Table 3.3. Effect of in ovo probiotic supplementation on body weight gain, feed intake and feed conversion ratios.

	DOH-d4	d4 - d6	d6 - d8	d8 - d15	d15 - d22	DOH - d22
Body Weight Gain						
Neg	34.2 ± 0.87[bc]	38.7 ± 1.18	54.9 ± 1.05	300.5 ± 5.13	462.2 ± 13.30	890.3 ± 17.06
Sham	34.7 ± 0.85[abc]	39.9 ± 1.17	56.1 ± 1.01	302.1 ± 5.13	465.3 ± 13.30	899.4 ± 17.09
P1	32.5 ± 0.86[c]	37.5 ± 1.18	56.8 ± 1.04	300.7 ± 5.13	468.1 ± 13.30	894.5 ± 16.91
P2	37.3 ± 0.86[a]	41.6 ± 1.18	54.6 ± 1.04	302.1 ± 5.13	464.6 ± 13.30	899.6 ± 17.06
P3	35.7 ± 0.86[ab]	40.2 ± 1.18	53.3 ± 1.04	302.7 ± 5.13	454.4 ± 13.30	889.1 ± 17.22
P -Value	0.03	0.18	0.6	0.99	0.96	0.99
Feed Intake						
Neg	57.1 ± 6.88	66.1 ± 4.91	70.1 ± 2.88	396.6 ± 10.61	654.3 ± 19.78	1244.1 ± 31.82
Sham	63.1 ± 6.88	66.2 ± 4.91	67.6 ± 2.88	401.4 ± 10.61	640.1 ± 19.78	1238.5 ± 31.82
P1	45.3 ± 6.88	71.4 ± 4.91	64.2 ± 2.88	406.8 ± 10.61	666.0 ± 19.78	1253.7 ± 31.82
P2	48.2 ± 6.88	65.0 ± 4.91	67.4 ± 2.88	400.7 ± 10.61	654.4 ± 19.78	1235.8 ± 31.82
P3	56.8 ± 6.88	65.0 ± 4.91	70.7 ± 2.88	411.4 ± 10.61	652.7 ± 19.78	1256.6 ± 31.82
P -Value	0.40	0.87	0.56	0.87	0.92	0.99
Feed Conversion Ratio						
Neg	1.66 ± 0.19	1.75 ± 0.15	1.25 ± 0.07	1.33 ± 0.04	1.39 ± 0.07	1.39 ± 0.05
Sham	1.82 ± 0.19	1.53 ± 0.15	1.25 ± 0.07	1.32 ± 0.04	1.36 ± 0.07	1.35 ± 0.05
P1	1.39 ± 0.19	1.76 ± 0.15	1.21 ± 0.07	1.37 ± 0.04	1.43 ± 0.07	1.42 ± 0.05
P2	1.30 ± 0.19	1.70 ± 0.15	1.23 ± 0.07	1.35 ± 0.04	1.41 ± 0.07	1.39 ± 0.05
P3	1.59 ± 0.19	1.73 ± 0.15	1.25 ± 0.07	1.34 ± 0.04	1.48 ± 0.07	1.42 ± 0.05
P -Value	0.38	0.82	0.99	0.92	0.83	0.89

Different letters within a column indicate significant difference among treatment groups

[1]Neg = negative control

[2]Sham = sham water injection

[3] P1 = $1x10^5$ probiotic bacteria

[4]P2 = $1x10^6$ probiotic bacteria

[5]P3 = $1x10^7$ probiotic bacteria

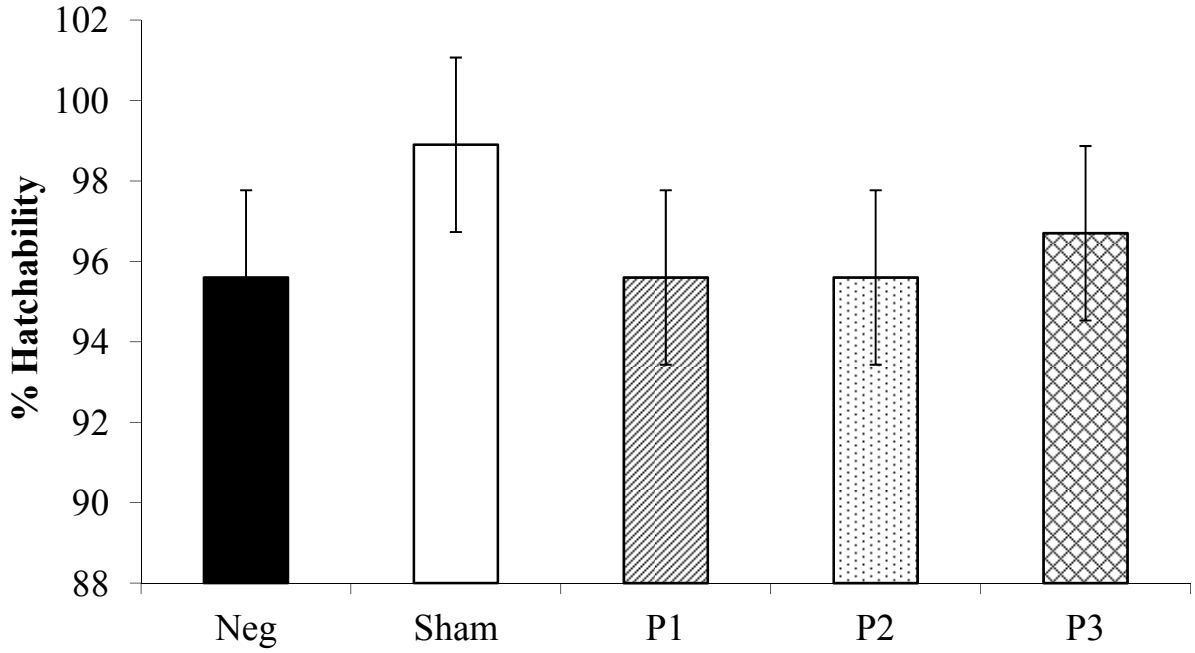

Figure 3.1. Effect of in ovo probiotic supplementation on hatchability of Cobb 500 chicks. Data are represented as least squares means ± SEM. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. (P = 0.77).

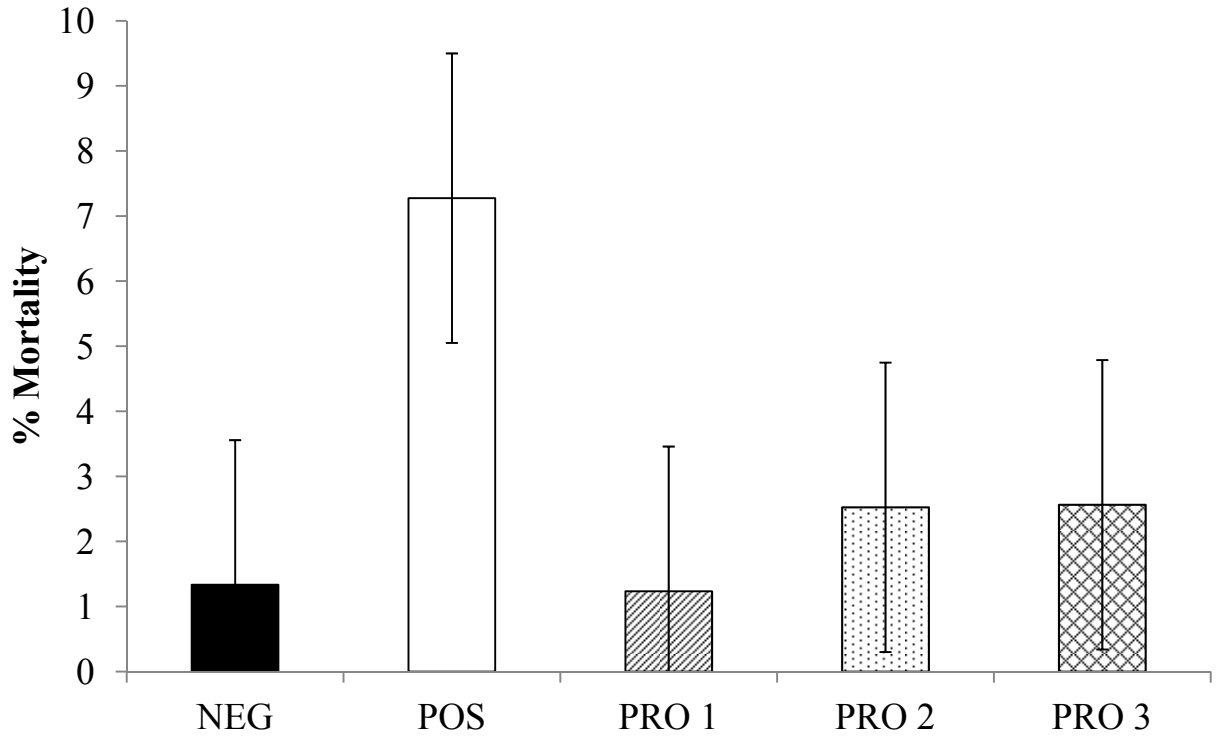

Figure 3.2. Effect of in ovo probiotic supplementation on mortality of Cobb 500 chicks. Data are represented as least squares means ± SEM. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. (P = 0.65).

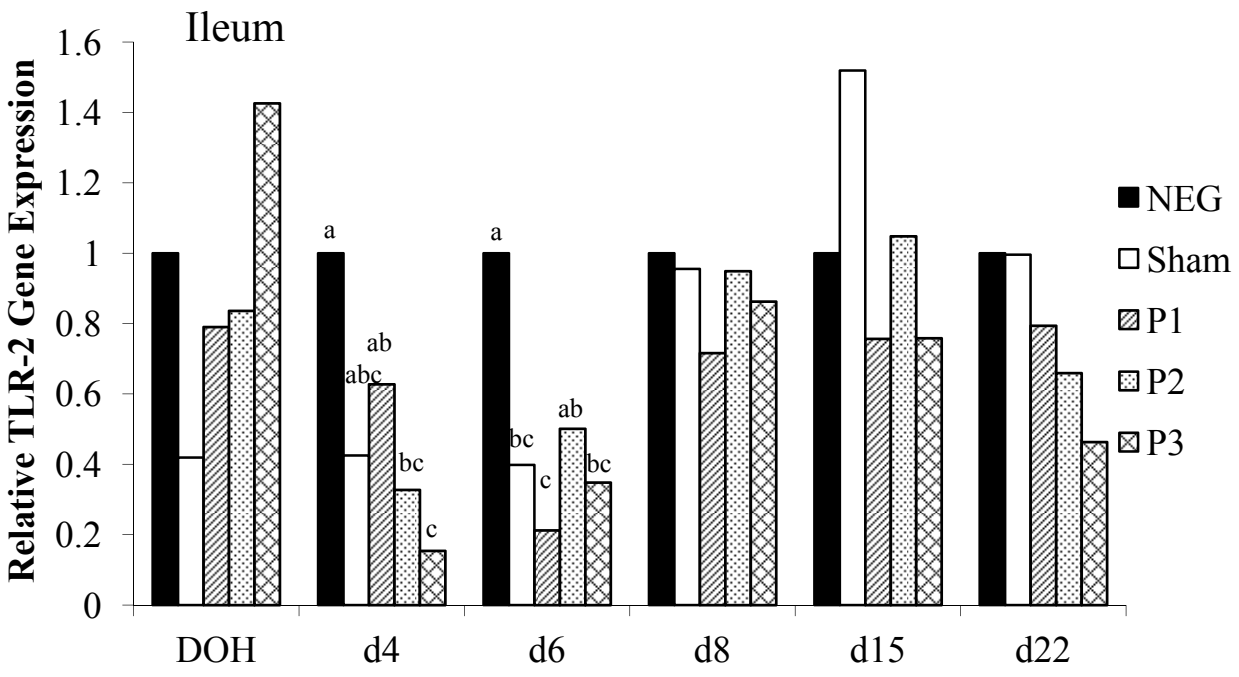

Figure 3.3a. Effect of in ovo probiotic supplementation on relative Toll-like receptor (TLR)-2 expression in the ileum of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on d4 (P = 0.045) and d6 (P = 0.02).

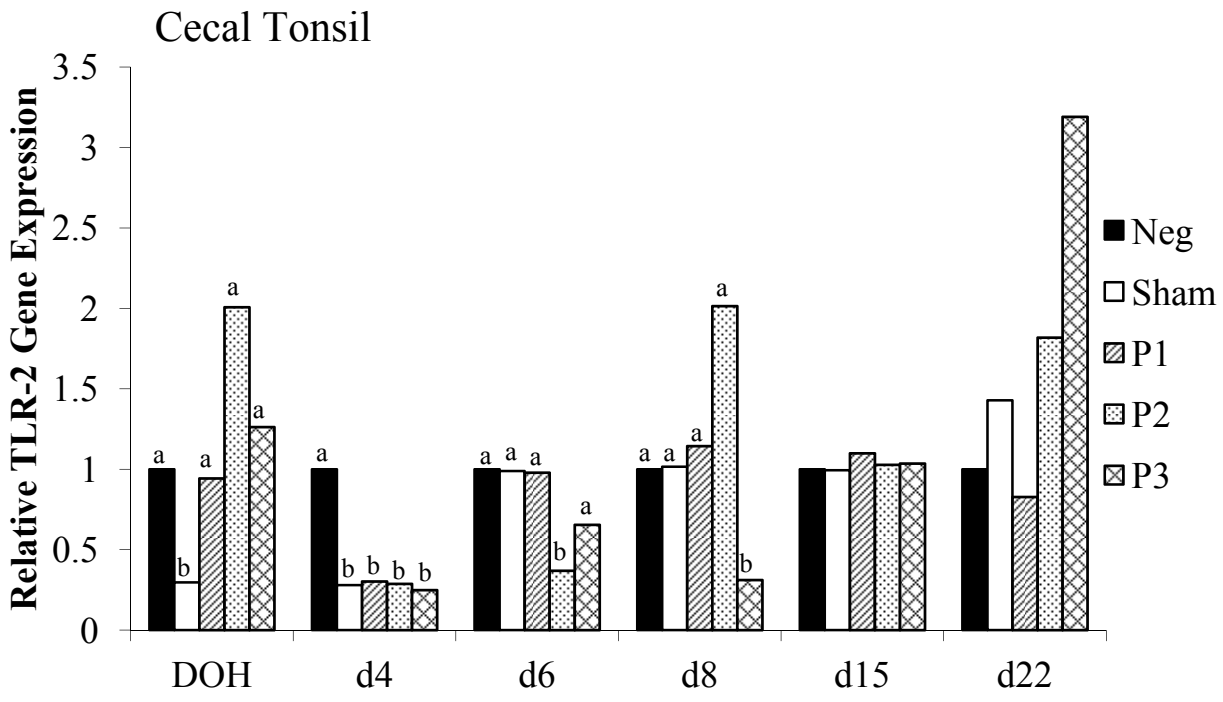

Figure 3.3b. Effect of in ovo probiotic supplementation on relative Toll-like receptor (TLR)-2 expression in the cecal tonsils of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on DOH (P = 0.001), d4 (P = 0.02), d6 (P = 0.001) and d8 (P = 0.01).

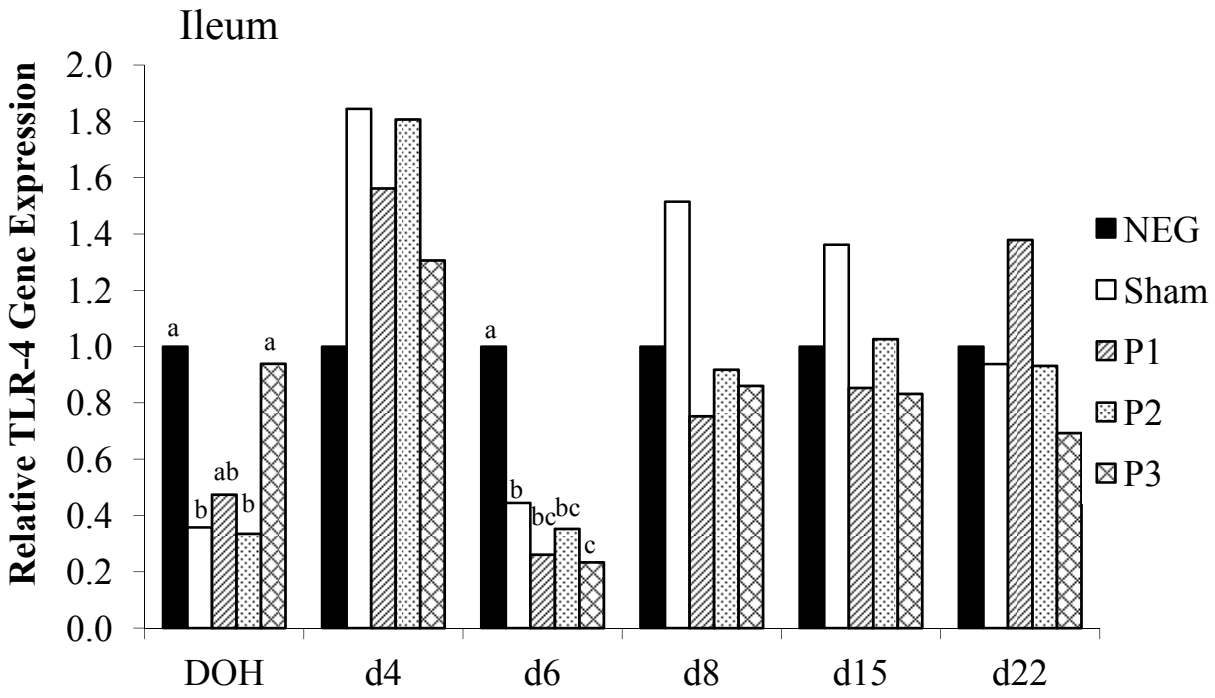

Figure 3.4a. Effect of in ovo probiotic supplementation on relative Toll-like receptor (TLR)-4 expression in the ileum of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on DOH (P = 0.01) and d6 (P = 0.004).

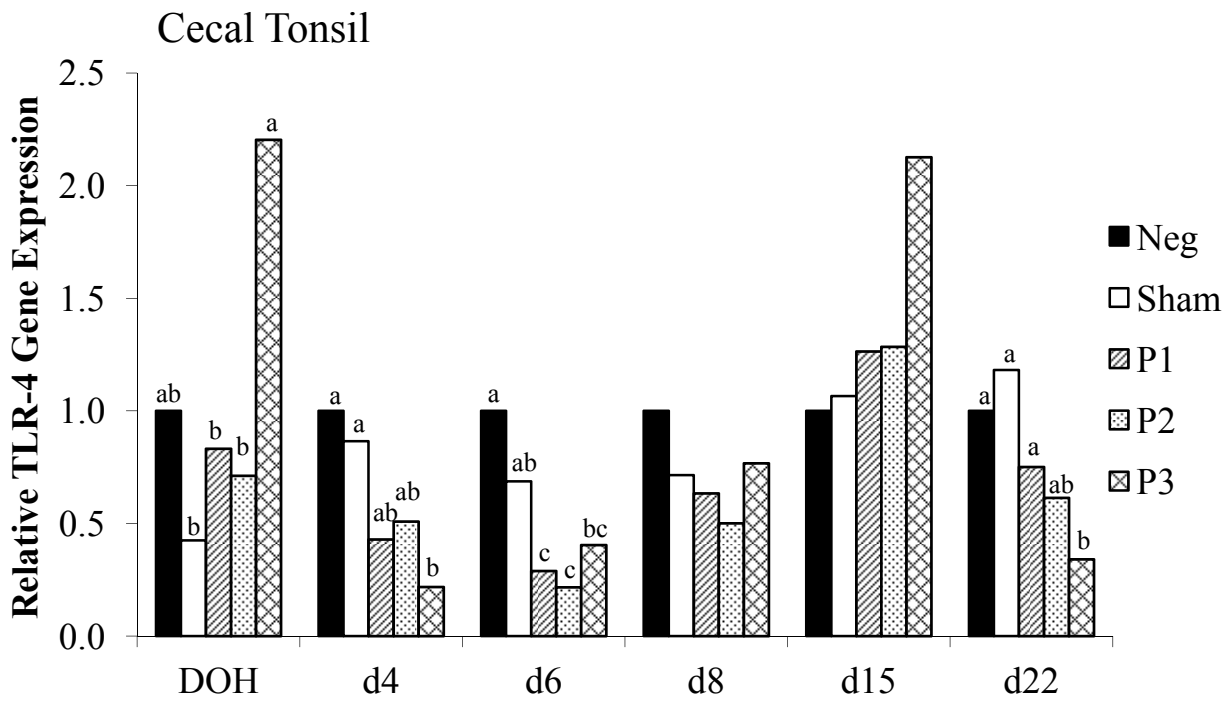

Figure 3.4b. Effect of in ovo probiotic supplementation on relative Toll-like receptor (TLR)-4 expression in the cecal tonsils of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on DOH (P = 0.02), d4 (P = 0.02), d6 (P = 0.002), and d22 (P = 0.03).

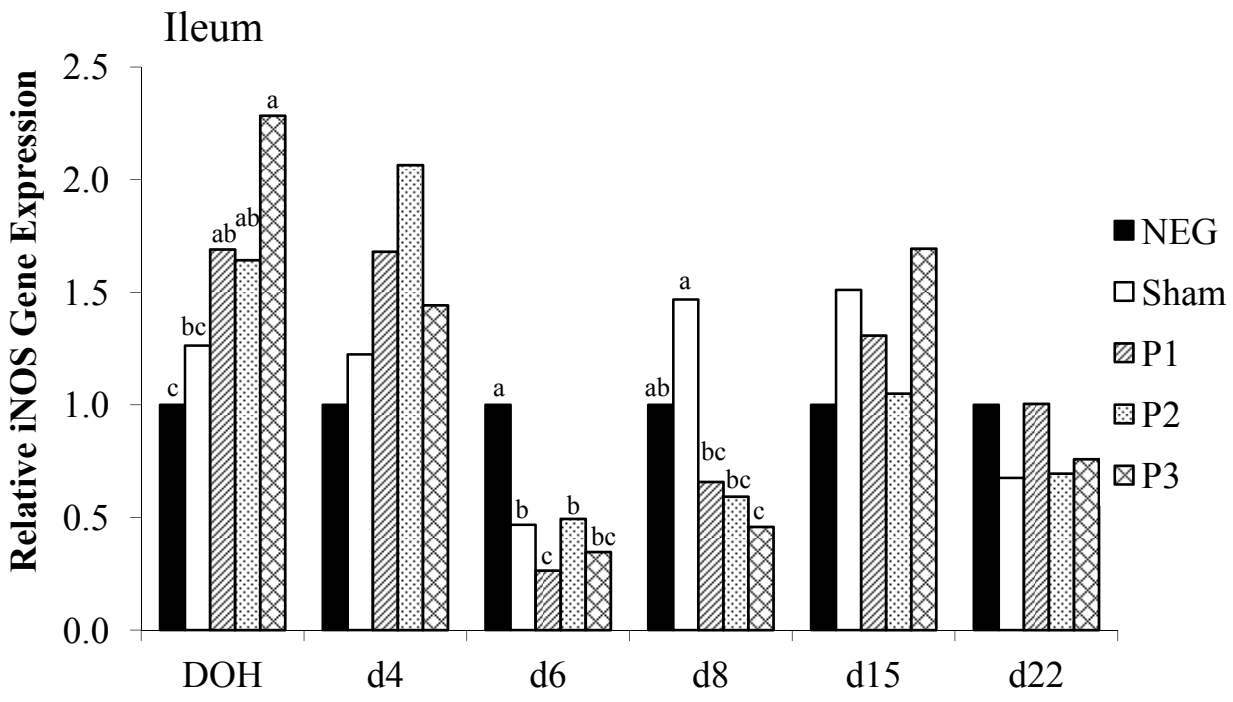

Figure 3.5a. Effect of in ovo probiotic supplementation on relative inducible nitric oxide synthase (iNOS) expression in the ileum of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on DOH (P = 0.01), d6 (P = 0.0004) and d8 (P = 0.01).

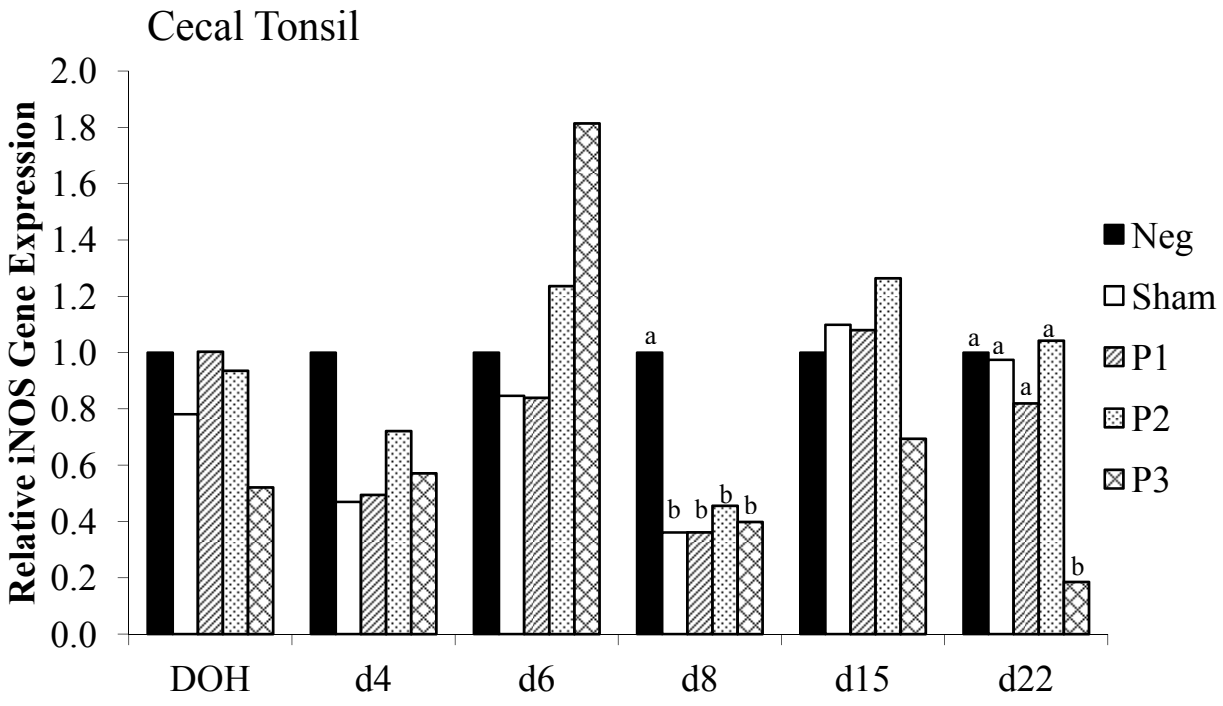

Figure 3.5b. Effect of in ovo probiotic supplementation on relative inducible nitric oxide synthase (iNOS) expression in the cecal tonsils of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on d8 (P = 0.01), and d22 (P = 0.0001).

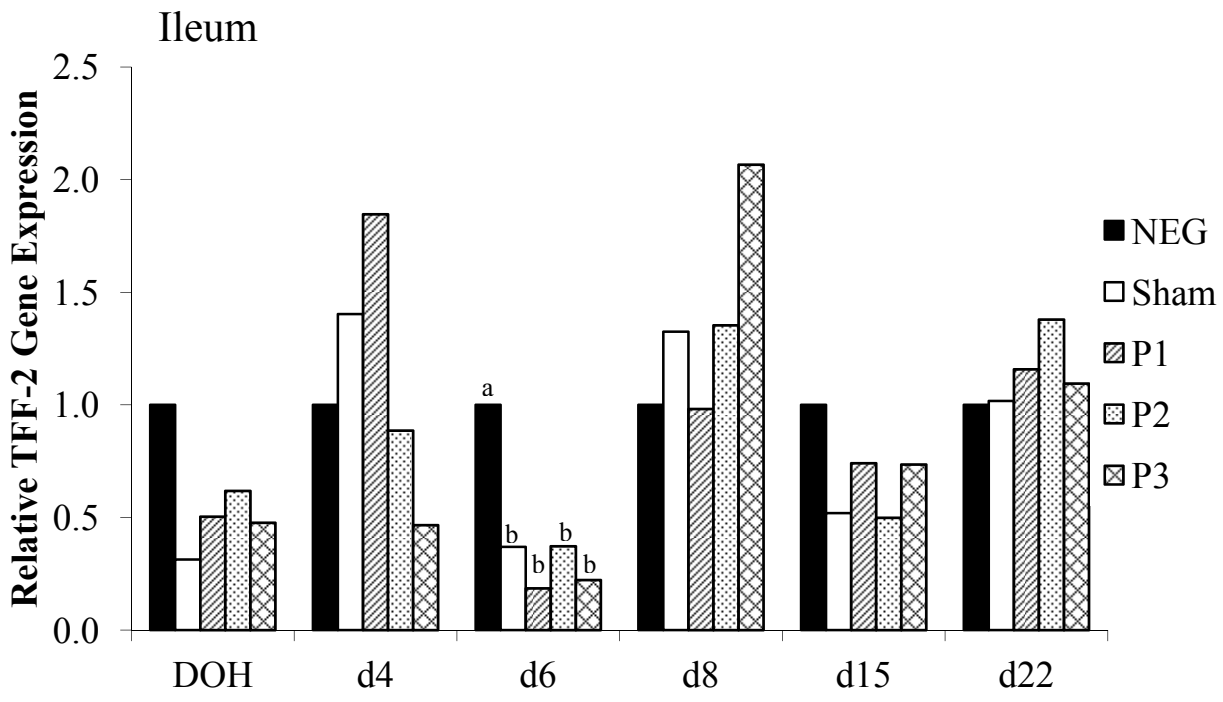

Figure 3.6a. Effect of in ovo probiotic supplementation on relative Trefoil factor (TFF)-2 expression in the ileum of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on d6 (P = 0.004).

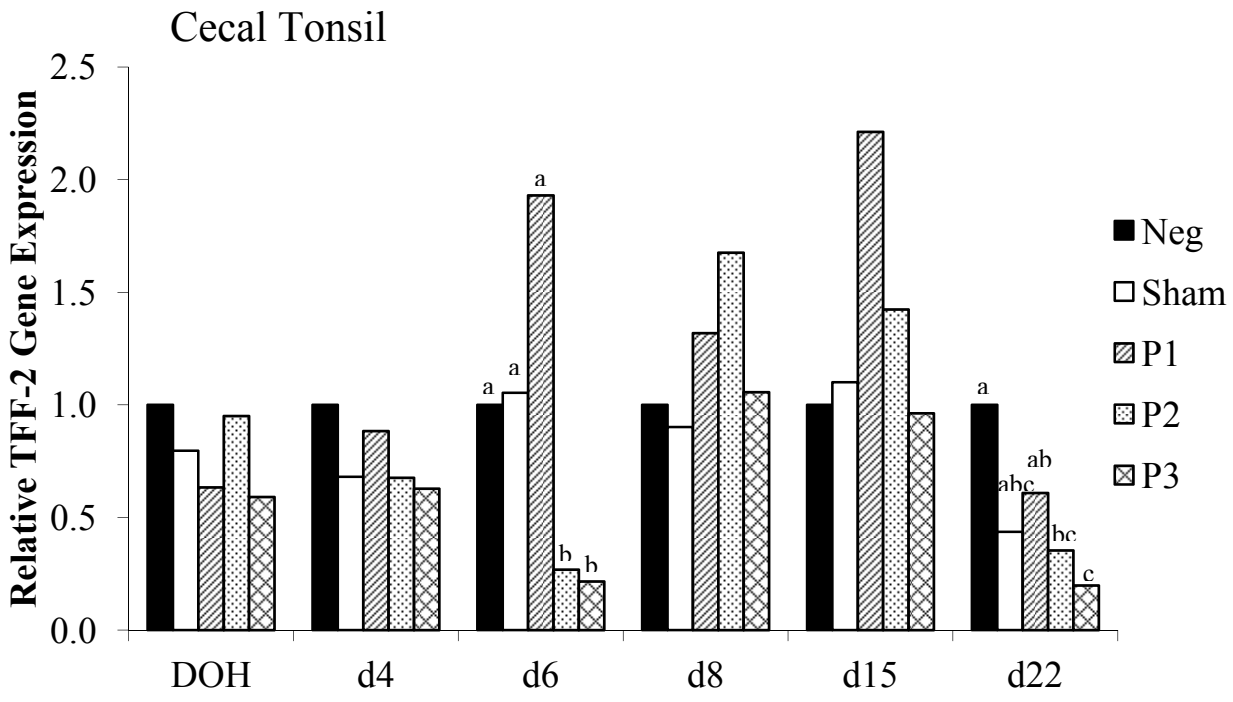

Figure 3.6b. Effect of in ovo probiotic supplementation on relative Trefoil factor (TFF)-2 expression in the cecal tonsils of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on d6 (P = 0.001) and d22 (P = 0.01).

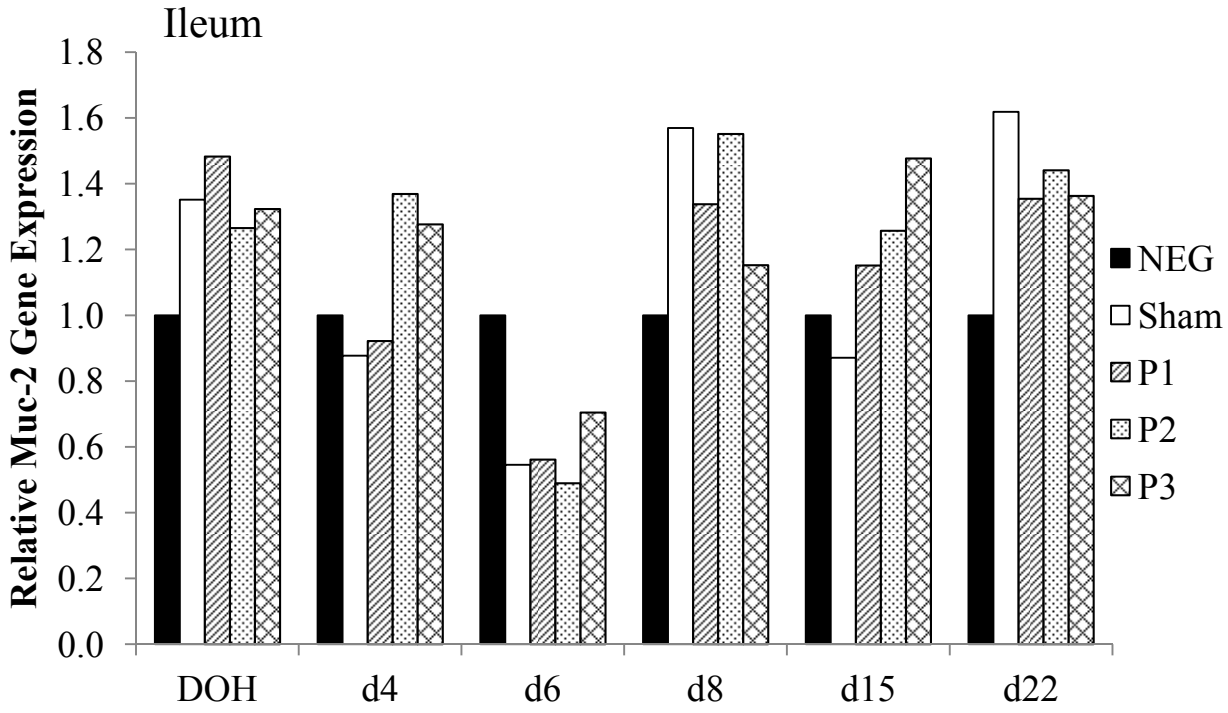

Figure 3.7a. Effect of in ovo probiotic supplementation on relative mucin (Muc)-2 expression in the ileum of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria.

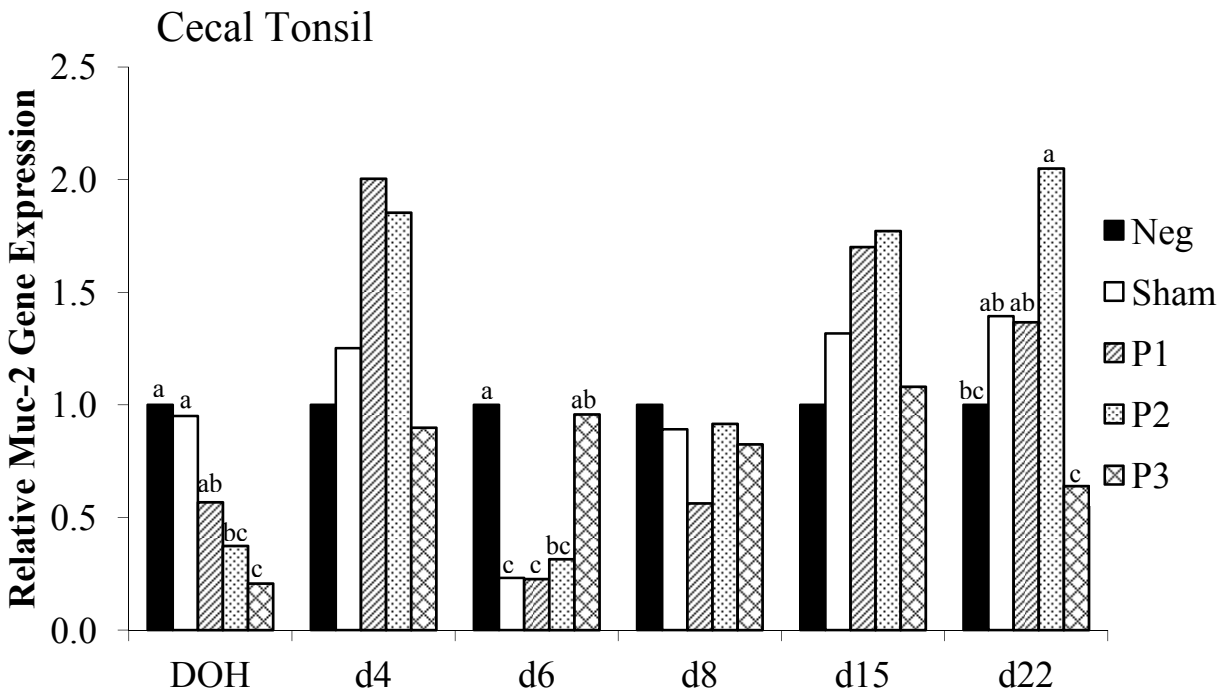

Figure 3.7b. Effect of in ovo probiotic supplementation on relative mucin (Muc)-2 expression in the cecal tonsils of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on DOH (P = 0.001), d6 (P = 0.01), and d22 (P = 0.02).

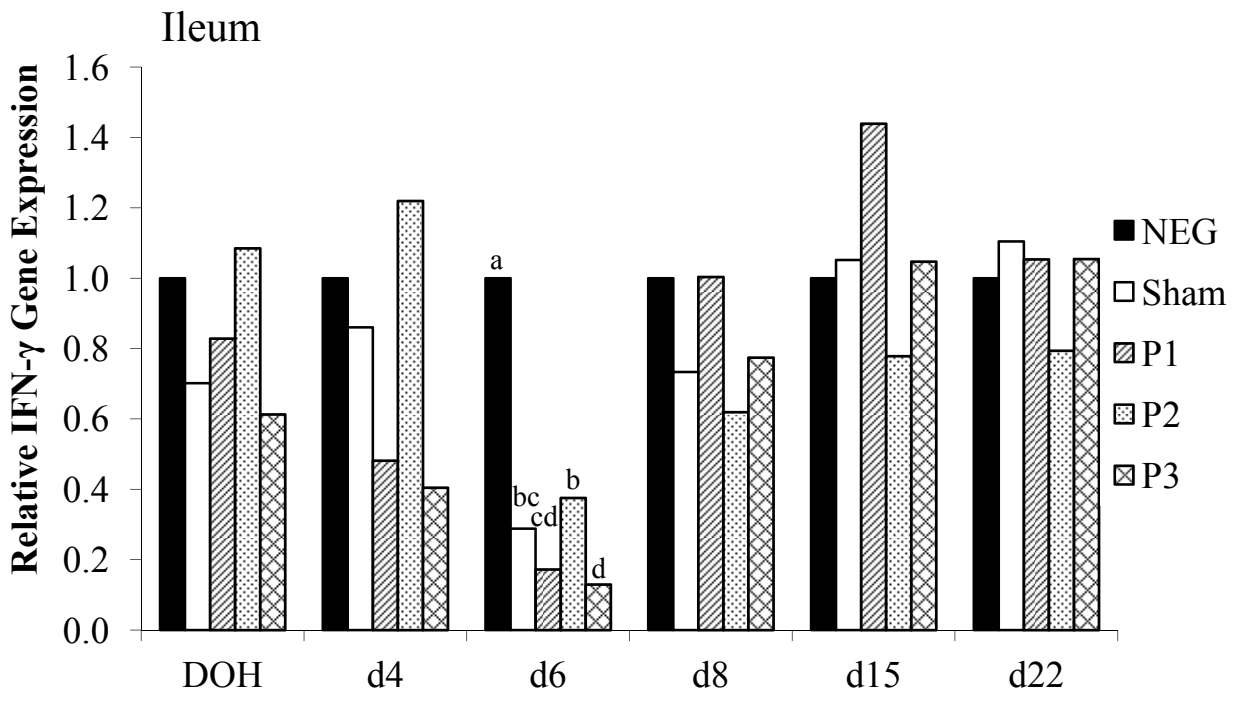

Figure 3.8a. Effect of in ovo probiotic supplementation on relative interferon (IFN)-γ expression in the ileum of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on d6 ($P < 0.0001$).

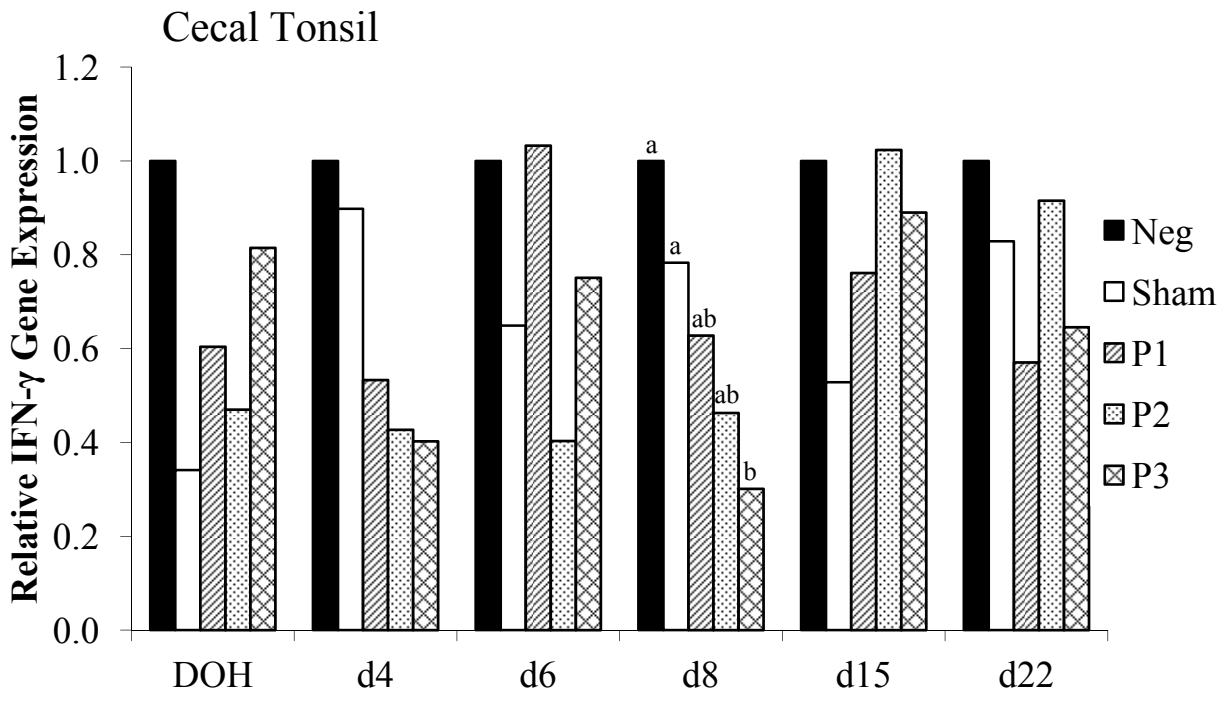

Figure 3.8b. Effect of in ovo probiotic supplementation on relative interferon (IFN)-γ expression in the cecal tonsils of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on d8 (P = 0.03).

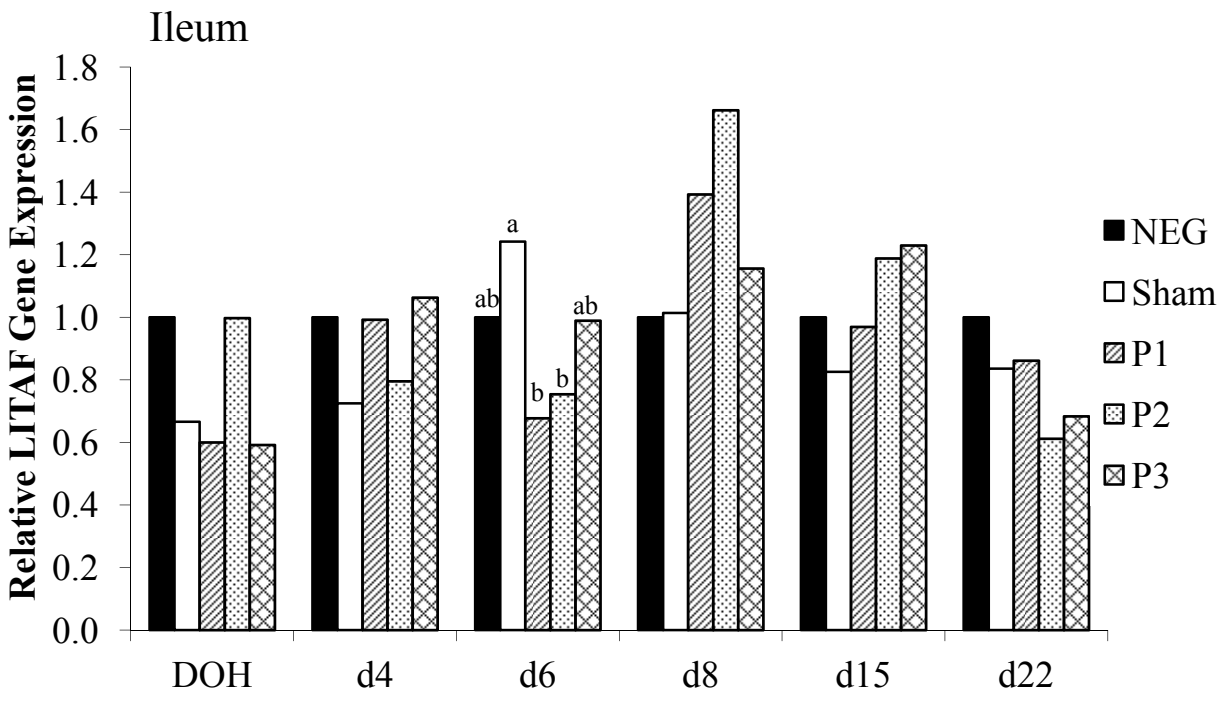

Figure 3.9a. Effect of in ovo probiotic supplementation on relative lipopolysaccharide-induced tumor necrosis factor-α factor (LITAF) expression in the ileum of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on d6 (P = 0.04).

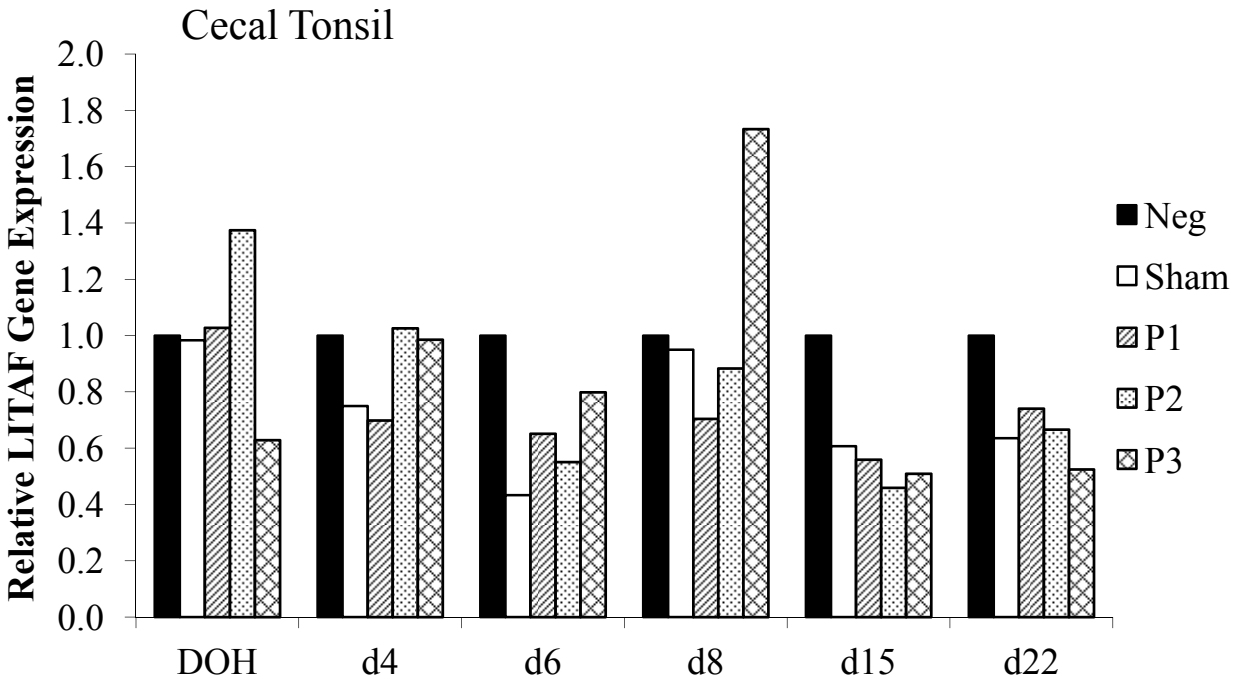

Figure 3.9b. Effect of in ovo probiotic supplementation on relative lipopolysaccharide-induced tumor necrosis factor-α factor (LITAF) expression in the cecal tonsils of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria.

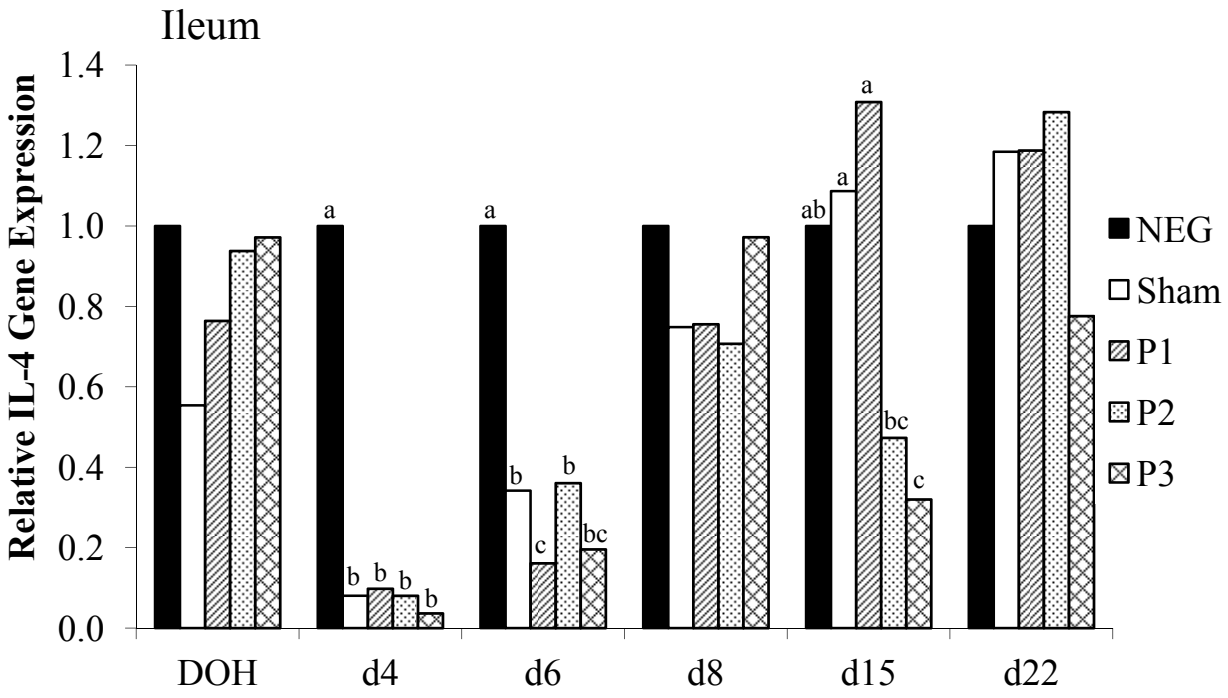

Figure 3.10a. Effect of in ovo probiotic supplementation on relative interleukin (IL)-4 expression in the ileum of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on d4 (P = 0.002), d6 (P = 0.0003) and d15 (P = 0.01).

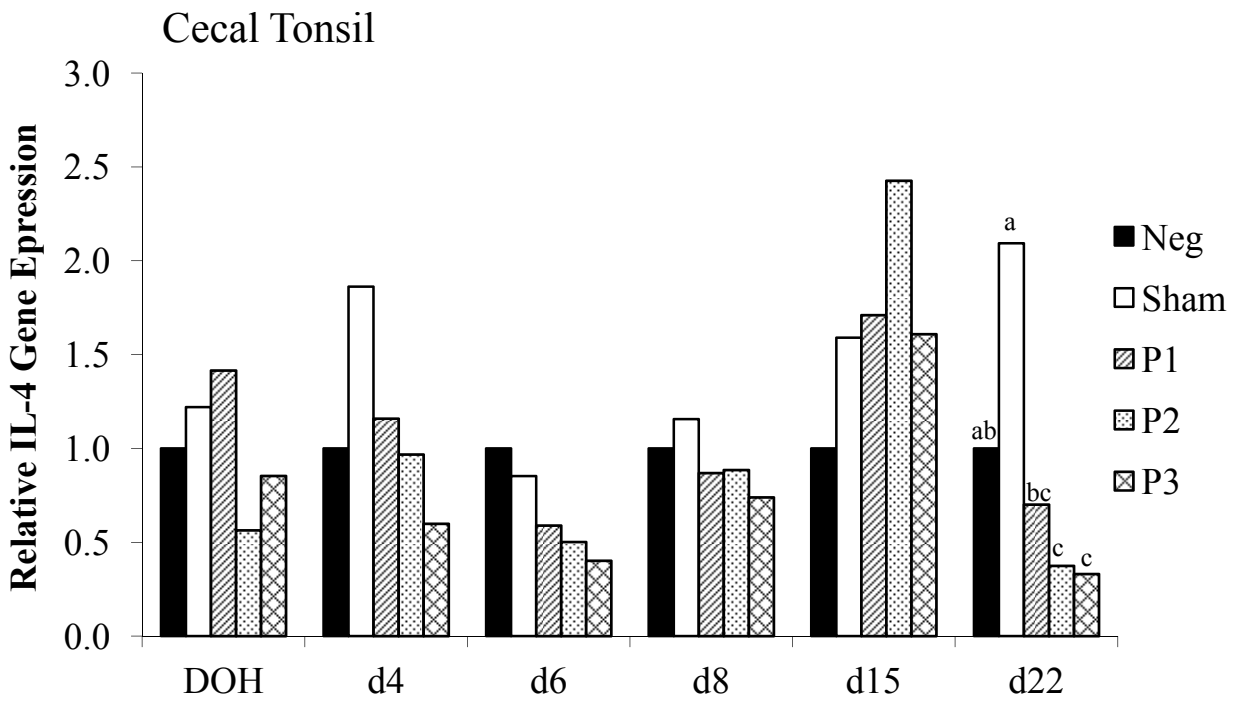

Figure 3.10b. Effect of in ovo probiotic supplementation on relative interleukin (IL)-4 expression in the cecal tonsils of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on d22 (P = 0.002).

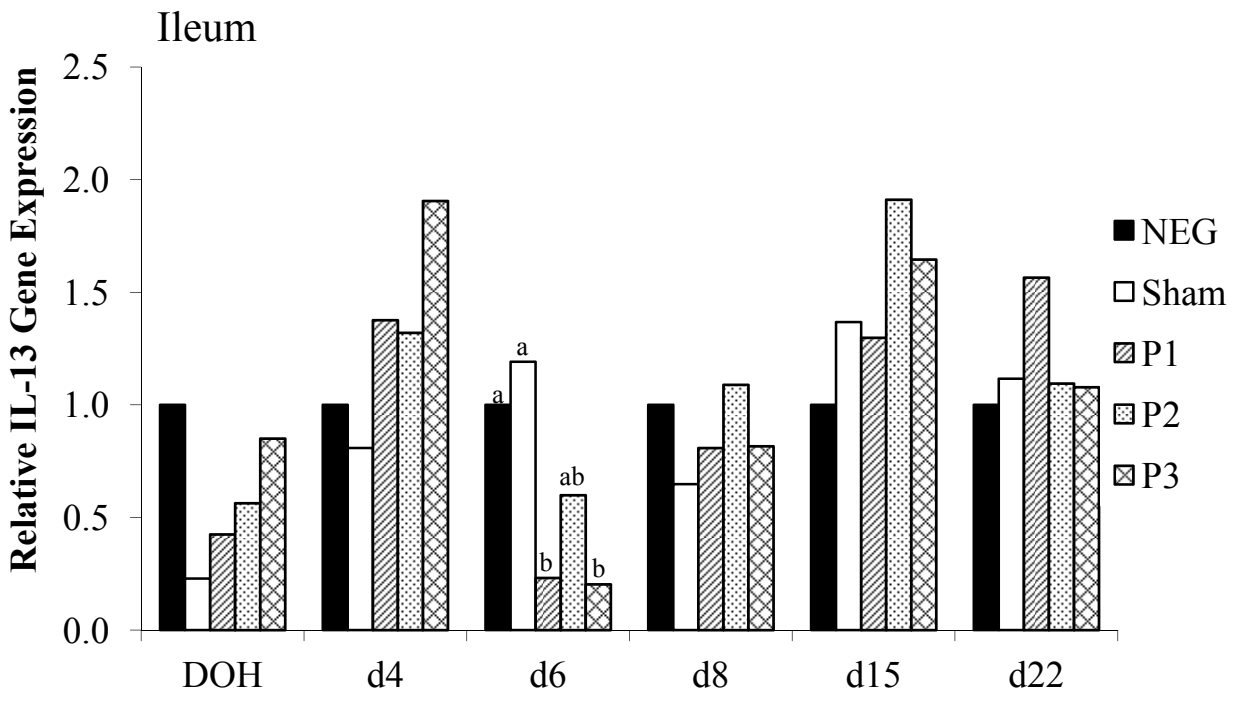

Figure 3.11a. Effect of in ovo probiotic supplementation on relative interleukin (IL)-13 expression in the ileum of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on d6 (P = 0.04).

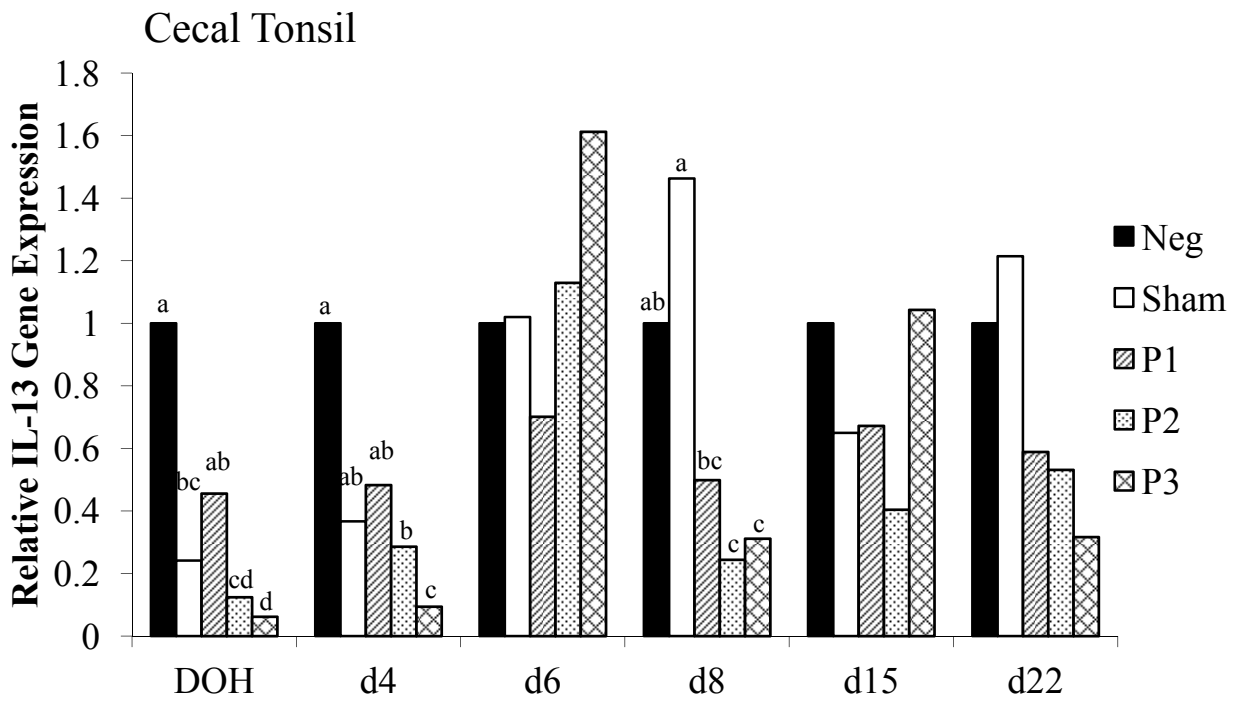

Figure 3.11b. Effect of in ovo probiotic supplementation on relative interleukin (IL)-13 expression in the cecal tonsils of Cobb 500 chicks. Relative gene expression (2-ΔΔCt) was calculated using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and the average ΔCt value for the negative control birds on each day as the calibrator. Data are represented as least squares means. Neg = negative control, Sham = sham water injection, P1 = $1x10^5$ probiotic bacteria, P2 = $1x10^6$ probiotic bacteria, P3 = $1x10^7$ probiotic bacteria. There was a significant effect of in ovo treatment on DOH (P = 0.0002), d4 (P = 0.001) and d8 (P = 0.003).

Chapter IV

In Ovo and Dietary Supplementation of Primalac and the Effects on Performance and Immune-Related Gene Expression in Broiler Chicks

ABSTRACT: During the first week post-hatch, the neonatal chick is immunologically vulnerable and subject to infectious threats found in the environment. Probiotics are live, nonpathogenic microorganisms known to have a positive effect on the host by improving the natural balance of gut microflora and promoting animal health. The objective of this study was to determine the effects of administering probiotics (Primalac) in ovo and in the diet on broiler chick hatchability, post-hatch performance, immune organ weights and ileal immune-related gene expression. At embryonic day 18, 1584 eggs were injected with either nothing (Dry), $1x10^6$, or $1x10^7$ (P1 and P2 respectively) probiotic bacteria. The remaining 393 eggs were left uninjected to serve as a negative control. Performance parameters were measured on day of hatch (DOH) and days (d) 4, 6, 8, 14, 19, and 42, while immune organ weights and tissue samples were taken on DOH and d4, 6, 8, 14, and 20. No differences were observed for hatchability, mortality, or body weight; however, from d6 to d8, both P1 and P2 gained more weight than the non-injected controls. Oral administration of probiotics reduced feed intake and improved feed conversion from DOH to d4 and from d8 to d14. Both groups receiving probiotics via in ovo injection from d6 to d8 and the P2 group from d19 to d42 had significantly improved feed conversion than the negative control and dry punch groups. Feed intake was also reduced in in ovo supplemented birds from d14 to d19, d19 to d42, and cumulatively from DOH to d42. Both in ovo and dietary administration of probiotics were able to modulate the expression of the immune-related genes in the ileum; however, expression patterns differed based on the gene, treatment, and time point evaluated. In conclusion, these results indicate that

in ovo supplementation of the probiotic product Primalac does not impact hatchability, can improve performance, and is capable of modulating gene expression in the ileum. Furthermore, in ovo administration of probiotics has an effect similar to that of dietary supplementation endorsing its usage to promote early colonization of beneficial bacteria to stimulate intestinal and immune system development.

INTRODUCTION

The immune system of the neonatal chick is immature and inefficient during the first week of life, making the bird extremely vulnerable to infectious threats found in the environment (Farnell et al., 2006). Immunomodulators are currently being studied and sought after to counteract the inadequacies of the chick immune system and promote host defense during this immunologically liable time. Furthermore, changes in the poultry industry, such as increasing concerns about antibiotic resistance, the banning of sub-therapeutic antibiotic usage by the European Union, and the potential banning of these drugs in the United States, have created an impetus for finding alternatives capable of maintaining animal health without having negative consequences on performance and profit margins.

Probiotics have received increasing attention as an antibiotic alternative with their potential to stimulate the immune system and reduce the rate and severity of enteric infections in poultry (Patterson and Burkholder, 2003). A 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). There are several microbial species that are commonly utilized as probiotics including those of *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Bacillus*, and *Pediococcus* (Gaggia et al., 2010). Probiotic products may contain only one or several different bacterial species.

The primary function of the gastrointestinal tract is to digest and absorb nutrients in order to meet metabolic demands for maintenance, normal growth, and development, but it also acts as a vital barrier preventing the entry of several potentially harmful pathogens from the external environment (Beal et al., 2006). The introduction of commensal microflora to the gastrointestinal tract is critical to the development of the gut-associated lymphoid tissues (GALT). Interactions between the microflora, the host intestinal tract, and the associated immune tissues are necessary for the complete and robust development of the gastrointestinal system (Dibner et al., 2008). Furthermore, a well-balanced gut microflora is essential for animal well-being and optimal performance.

The gut microbial profile can be manipulated through the use of probiotics in order to create conditions favorable to enhancing performance. In poultry, supplementation of probiotics has been shown to enhance growth and improve feed conversion (Kabir et al., 2004). Probiotics promote a healthy balance of microflora in the gut and enhance host defense against several enteric pathogens through stimulation of the mucosal immune system (Nava et al., 2005). Probiotics have been shown to enhance both innate and adaptive aspects of the immune systems. Oral administration of probiotics results in enhanced heterophil oxidative burst and degranulation as well as augmented phagocytic capacity of macrophages (Farnell et al., 2006; Higgins et al., 2007; Stringfellow et al., 2010). Additionally, probiotics augment the humoral immune response by increasing antibody production to many disease agents such as *Eimeria*, New Castle disease virus, and infectious bursal disease virus (Khaksefidi and Ghoorchi, 2006; Lee et al., 2007a; Lee et al., 2007b; Nayebpor et al., 2007). Probiotics have also demonstrated their ability to influence cell-mediated immune response by increasing T lymphocyte numbers and modulating the production of several pro-inflammatory, T helper Type-1 (Th1) and T helper Type-2 (Th2) cytokines, though discrepancies in cytokine production have been noted due to differences in the probiotic strains used (Dalloul et al., 2005; Brisbin et al., 2010; Lee et al.,

2010). Probiotics have proven their ability to enhance the immune response by promoting the clearance of several economically important pathogens such as *Eimeria* spp., *Salmonella* spp., *Escherichia coli* and *Clostridium perfringens*, further asserting their potential use as an antibiotic alternative (Dalloul and Lillehoj, 2005; Knap et al., 2010).

Traditionally, probiotics are administered in the feed or water supply to 1-day-old chicks. However, as soon as the chick hatches and is exposed to the external environment, it quickly begins to establish the microbial community in the intestine (Pedroso et al., 2005). This resident microflora may affect the establishment of the probiotic microorganisms. In order to promote early establishment of beneficial probiotic strains, employing in ovo technology may be the solution. In ovo technology represents a means to take advantage of this crucial time and promote early colonization of beneficial bacteria in order to stimulate intestinal and immune system development. Few researchers have entertained the idea of administering probiotics in ovo. The earliest attempts of connecting these concepts demonstrated promising results where *Salmonella typhimurium* colonization was reduced in chicks administered an undefined cecal culture of bacteria in ovo (Cox et al., 1992). Unfortunately, negative results in terms of hatchability, performance, and mortality have been noted, though these consequences may be attributed to the probiotic strain used and injection site (Cox et al., 1992; Meijerhof and Hulet, 1997). Edens et al. (1997) demonstrated positive results administering *Lactobacillus reuteri* in ovo in hatching chicks and turkey poults. They found no differences among treatment groups when comparing the hatchability of embryos injected with *L. reuteri* either in the air cell or the amniotic fluid to non-inocculated controls. Similar results were also seen in turkey embryos. Additionally, beneficial effects were observed in terms of performance in broiler chicks as well as reduced cecal load of *Salmonella* in poults (Edens et al., 1997).

The objective of this study was to determine the effects of administering a *Lactobacillus*-based probiotic (Primalac) in ovo and in the diet on broiler chick hatchability, post-hatch performance, immune organ weights, and intestinal immune-related gene expression.

MATERIALS AND METHODS

Birds and treatments

This study was approved and conducted under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee. At embryonic day (d) 18, 1980 fertile Cobb 500 eggs were obtained from a commercial hatchery (Pilgrim's Pride, Broadway, VA) and transported to the Virginia Tech Turkey Research Center. Prior to injection, all eggs were candled to determine position of the air cell. Eggs were sanitized by swabbing the large end (outside of the air cell) with 0.5% sodium hypochlorite and once dried, they were sprayed with 70% isopropyl alcohol. To create a guide and avoid cracking, a hole was made in the center of the air cell of those eggs receiving injections using an 18-gauge needle fitted with a rubber stopper to prevent the needle from piercing the air cell membrane. Needles were disinfected in between each injection by dipping in 0.5% sodium hypochlorite. Next, 1584 eggs were evenly divided and injected with either nothing (dry), or $1x10^6$ or $1x10^7$ (P1 and P2 respectively) probiotic bacteria dissolved in sterile water (Primalac W/S, Star-Labs Inc.). Injections were performed using a 1 mL syringe equipped with a 22-gauge, 1-inch short bevel needle. A new syringe and needle were used for each injection. The remaining 396 eggs were not injected and served as a negative control. Eggs were placed into one of 6 replicate hatching trays (66 eggs/tray). On day of hatch (DOH), birds were individually tagged, divided in half and placed into floor pens relative to treatment group. Half of the pens (6 pens/in ovo treatment) received supplemental probiotic (Primalac 454 FG, Star-Labs Inc.) in the feed at 2 lbs/ton fed

continuously throughout the trial. The basal diet consisted of a standard unmedicated broiler starter feed in crumble form provided from DOH to d19 and a standard unmedicated broiler grower feed in pellet form provided from d19 to d42. Feed and water were offered ad libitum throughout the study.

Hatchability and post-hatch performance parameters

On DOH, percent hatchability was recorded, and birds were weighed prior to placement and on days (d) 4, 6, 8, 14, 19 and 42. Mean body weight (BW) for each treatment group was calculated from the individual bird replicates for each weigh day. Average body weight gains (BWG) were calculated for each period and cumulatively based on individual bird weights. Feed intake (FI) of each group was measured at the same time periods as BW (d4, 6, 8, 14, 19, 42) with cumulative averages calculated. Feed conversion ratio (FCR) was calculated using BWG and FI and adjusted for mortality. Mortality was recorded on a daily basis throughout the trial.

Immune organ weights

On DOH, d4, 6, 8, 14, 20 and 42, 6 birds per treatment were randomly selected and euthanized by cervical dislocation. The bursa of Fabricius and spleen were excised and weighed, and relative organ weights were expressed as a percentage of live BW.

Tissue sampling for gene expression analysis

On DOH, d4, 6, 8, 14, and 20, the same 6 birds per treatment were sampled for gene expression analysis. The ileum, defined as the area posterior to the Meckel's diverticulum to the ileo-cecal junction, was sampled, rinsed in cold PBS, and placed in RNA*later* (Qiagen, Germantown, MD) for subsequent gene expression analysis. The samples were stored at -80°C until analysis.

Ileal gene expression

Intestinal samples were removed from -80°C and a 20-30 mg aliquot of each sample was weighed, placed into a 2 mL microcentrifuge tube along with a 5 mm stainless steel bead and 600 μL RLT Buffer, and homogenized using the TissueLyser II system (Qiagen) according to manufacturer's recommendation. Total RNA was extracted from individual intestinal tissues using the RNeasy mini kit following the animal tissue protocol (Qiagen). Following extraction, RNA was eluted by rinsing the column membrane twice with 25 μL of RNase-free water. Total RNA concentration was determined at OD 260 (NanoDrop-1000, Thermo Fisher Scientific, Waltham, MA) and RNA purity was verified by evaluating the ratio of OD 260 to OD 280. Total RNA was diluted to 0.2 μg/μL in nuclease-free water. Reverse transcription was accomplished using the high capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) following the manufacturer's protocol, and the cDNA was stored at -20°C.

Quantitative real-time PCR (qRT-PCR) was performed using an ABI 7500 FAST Real-Time PCR System (Applied Biosystems). The cDNA was diluted 1:30 in nuclease-free water then 1 μL of the diluted cDNA was added to each well of a 96-well plate. Next, 9 μL of real-time PCR master mix containing 5 μL FAST SYBR Green Master Mix (Applied Biosystems), 0.5 μL each of 2 μM forward and reverse primers and 3 μL of sterile nuclease-free water per reaction were added to each well for a final volume of 10 μL. During the PCR reaction, samples were subjected to an initial denaturation phase at 95°C for 20 sec followed by 40 cycles of denaturation at 95°C for 3 sec and annealing and extension at 60°C for 30 sec. Gene expression for interferon (IFN)-γ, interleukin (IL)-4, IL-13, inducible nitric oxide synthase (iNOS), lipopolysaccharide-induced tumor necrosis factor-α (LITAF), mucin (Muc)-2, trefoil family factor (TFF)-2, Toll-like receptor (TLR)-2 and TLR-4 was analyzed using glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) as an endogenous control. Each reaction was run in duplicate. Primers were designed (Table 4.1) using the Primer Express 3.0 software (Applied Biosystems) and synthesized by MWG Operon (Huntsville, AL). Results from qRT-PCR were analyzed using the 7500 Real-Time PCR software (Applied Biosystems). Average gene expression relative to the *GAPDH* endogenous control for each sample was calculated using the 2-ΔΔCt method (Livak and Schmittgen, 2001). The calibrator for each gene was the average ΔCt value from the negative control group for each sampling day for each respective tissue.

Statistical analysis

Results were analyzed as a 4x2 factorial using the Fit Model platform in JMP 9.0 (SAS Institute Inc., Cary, NC). Percent hatchability and mortality data were arc sine transformed prior to analysis. Differences in experimental treatments were tested using Student's *t*-test following ANOVA. Values were considered statistically different at $P \leq 0.05$. Results are reported as least squares means with standard errors.

RESULTS

Hatchability, post-hatch performance and immune organ weights

No significant differences were observed for hatchability, mortality, or BW during this study (Figure 4.1, Figure 4.2 and Table 4.2 respectively). From d6 to d8, however, there was a main effect of in ovo treatment where probiotic supplemented birds gained more weight than the negative control ($P = 0.01$) (Table 4.3). There was a main effect of dietary treatment from DOH to d4 for FI ($P = 0.0002$) and FCR ($P = 0.001$) (Tables 4.4 and 4.5 respectively). Birds receiving the probiotic supplemented diet had lower FI and thus more efficient FCR than the birds receiving the basal diet. A similar pattern was observed for FI ($P = 0.03$) and FCR ($P = 0.01$)

from d8 to d14. From d6 to d8, there was a main effect of in ovo treatment on FCR ($P < 0.0001$). Both groups receiving probiotics via in ovo injection had significantly improved FCR than the negative control and dry punch groups with P1 also having a lower FCR than P2. There was a main effect of in ovo treatment on FI from d14 to d19 ($P = 0.01$), d19 to d42 ($P = 0.03$), and cumulatively from DOH to d42 ($P = 0.02$). From d14 to d19, P1 birds ate significantly less than the negative control and P2 birds. From d19 to d42 and from DOH to d42, P2 birds consumed less than the negative control and the dry punch groups. In ovo treatment did significantly affect FCR from d19 to d42 with P2 having a more efficient FCR than all other groups ($P = 0.03$). Neither in ovo supplementation nor diet had an effect on bursa weight at any time point during the study (Table 4.6). On d6, in ovo treatment and diet resulted in a 2-way interaction on spleen weight with the P2 birds receiving the probiotic supplemented diet having a larger spleen than all other groups except P1 birds given the control diet ($P = 0.02$) (Table 4.7).

Ileal gene expression

On d14, in ovo treatment and diet resulted in a 2-way interaction on *TLR-2* expression ($P = 0.048$) (Table 4.8). Expression of *TLR-2* was downregulated in P1 birds given the unsupplemented diet when compared to the birds receiving the dry punch and the same diet. When given probiotic supplementation in the diet, however, P1 birds displayed increased *TLR-2* expression. There was a main effect of in ovo treatment ($P = 0.01$) and diet ($P < 0.001$) on *TLR-4* expression on d8 (Table 4.9). *TLR-4* levels were higher in P2 than the negative control and dry punch groups, while P1 had higher levels than the dry punch group. Additionally, chicks given the probiotic supplemented diet had augmented *TLR-4* expression. On d14, in ovo treatment and diet presented a 2-way interaction on *TLR-4* gene expression ($P = 0.01$). When looking at the groups given the basal diet, P1 birds displayed downregulated expression of *TLR-4* when

compared to the dry punch group. In those groups given the probiotic supplemented diet, however, P1 birds demonstrated an increase in *TLR-4* expression when compared to the dry punch and P2 groups. Dietary supplementation resulted in a decrease in *TLR-4* expression in the dry punch and P2 birds.

On d20, there was a main effect of dietary treatment on *iNOS* gene expression where levels were decreased due to probiotic supplementation ($P = 0.04$) (Table 4.10). Neither in ovo treatment nor diet altered *TFF-2* expression during this study (Table 4.11). On d4, there was a main effect of diet on *Muc-2* expression in the ileum where levels of *Muc-2* were elevated due to probiotic supplementation ($P < 0.0001$) (Table 4.12). A similar pattern was observed on d14 ($P = 0.003$) and d20 ($P < 0.0001$). There was a main effect of in ovo treatment on *Muc-2* expression on d6 where levels in P1 were significantly higher than the negative control and dry punch groups ($P = 0.01$).

Expression of *IFN-γ* was downregulated on d4 due to dietary probiotic supplementation ($P = 0.0002$) (Table 4.13). On d14, in ovo treatment and diet resulted in a 2-way interaction for *IFN-γ* expression ($P = 0.0002$). Within the groups receiving the basal diet, P1 and P2 exhibited a downregulation of *IFN-γ* expression. In the groups receiving the supplemented diet, however, P1 displayed an increase in *IFN-γ* expression when compared to all other groups. Dietary supplementation of probiotics resulted in decreased *IFN-γ* levels in the negative control and dry punch birds.

In ovo treatment and diet presented a 2-way interaction on *LITAF* expression on d20 ($P = 0.02$) (Table 4.14). Expression of *LITAF* was significantly downregulated in P2 birds given the unsupplemented diet when compared to negative control and P1 birds given the same diet. No

differences were noted among groups given the supplemented diets, but dietary supplementation did reduce *LITAF* levels in the negative control and P1 chicks.

There was a main effect of dietary treatment on *IL-4* gene expression on d4 (P = 0.001) and d20 (P = 0.02) where *IL-4* levels were decreased due to probiotic supplementation (Table 4.15). Neither in ovo treatment nor diet altered *IL-13* expression during this study (Table 4.16).

DISCUSSION

The aim of this study was to explore the effects of in ovo administration of the probiotic product Primalac W/S in broiler chicks and evaluate those effects with and without dietary probiotic supplementation. In this experiment, in ovo supplementation of probiotics had no effect on hatchability which corroborates our previous studies (Chapter III, unpublished data). Very few studies have been published regarding the concept of administrating probiotics via the in ovo route. Edens et al. (1997) compared the hatchability of broiler embryos injected with *Lactobacillus reuteri* either in the air cell or the amniotic fluid to uninocculated controls and found no differences among the treatment groups. Similar results were also seen in turkey embryos (Edens et al., 1997). Alternatively, other researchers have found that in ovo injection of some probiotic strains can negatively impact hatchability (Cox et al., 1992; Meijerhof and Hulet, 1997). Our findings suggest that the probiotic bacteria in Primalac can be safely administered in ovo without having a negative impact on hatchability.

During this study, improvements in performance due to probiotic supplementation were observed in both the in ovo and dietary treatment groups. Corresponding with our findings, Edens and colleagues (1997) found that broiler chicks inoculated in ovo with *L. reuteri* and subsequently challenged with *Salmonella typhimurium* or *Escherichia coli* displayed increased BW when compared to the challenge only birds. Additionally, dietary supplementation of

Primalac has also been reported to improve growth performance and feed efficiency of broiler chickens and turkeys (Nayebpor et al., 2007; Grimes et al., 2008; Talebi et al., 2008)

The relative size of the bursa was unaffected by in ovo or dietary supplementation during this study. In ovo treatment and diet did, however, present a 2-way interaction for relative spleen weight on d6 with P2 birds given the supplemented diet having larger spleens than all other groups, except P1 birds given the control diet. Larger spleens have also been observed in studies where probiotics were added to the diets of broilers, suggesting that probiotics have an effect on the systemic immune system (Kabir et al., 2004; Ahmadi, 2011). There are conflicting reports, however, suggesting that probiotics do not affect immune organ weights (Al-Barwary et al., 2012; Naseem et al., 2012).

In spite of the considerable amount of published data regarding the efficacy of probiotics in poultry, the exact mechanism of how probiotics alter the immune system is still not fully understood. Our goal was to identify patterns of gene expression underlying the effects of probiotic supplementation on the immune system, particularly at the gut level. To observe the effects of probiotics on the innate immunity, we evaluated the expression of *TLR-2*, *TLR-4*, *iNOS*, *Muc-2* and *TFF-2* in the ileum. The TLR family is a highly conserved group of proteins that act as pathogen recognition receptors (PRR), recognizing microbe-associated molecular patterns (MAMPs) that are expressed on infectious agents. They play a fundamental role in pathogen detection and are responsible for the initiation and regulation of the innate response. On d14, *TLR-2* expression was downregulated in P1 birds given the control diet when compared to the dry punch group, but expression was upregulated when those P1 birds were given the supplemented diet. On d8, *TLR-4* expression was increased in response to probiotic treatment regardless of administration method. On d14, however, *TLR-4* levels were reduced due to

dietary supplementation in the dry punch and P2 groups. Though it is evident that probiotic supplementation can alter expression patterns of these TLRs, and thus the innate immune system, it cannot be conclusively determined how probiotics influence localized innate responses under healthy conditions. As such, the presence of an enteric challenge might shed some light on the mechanistic functions of probiotic bacteria, and help us determine their impact on TLR pathways consistent with appropriate responses to those challenges.

When exposed to antigens or chemotactic agents, macrophages will begin to produce 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 (Tizard, 2009). Though no effects were observed in the in ovo treatment groups, dietary administration of probiotics resulted in a downregulation of *iNOS* gene expression on d20, suggesting Primalac may possess an anti-inflammatory function.

The *Muc-2* gene is responsible for encoding mucin production, which is mediated by T lymphocytes and Th2 cytokines (Beum et al., 2005). Mucin is made up of glycoproteins and serves a protective function by binding to pathogens, thus preventing their adhesion to the intestinal surface. Trefoil factor-2 is a stable secretory protein expressed in gastrointestinal mucosa responsible for protecting the mucosa from insults, stabilizing the mucus layer and promoting the healing of the epithelium (Jiang et al., 2011). While no differences were noted for *TFF-2* expression, *Muc-2* levels were increased due to in ovo treatment on d6 and dietary treatment on d4, 14, and 20. Previous studies have shown that Primalac is able to modulate the processes of mucin synthesis by altering the intestinal bacterial populations (Smirnov et al., 2005). Primalac has also been reported to increase the goblet cell number and mucus secretion in the small intestine of turkeys, which may protect intestinal epithelia from adverse factors

including pathogens (Rahimi et al., 2009). The upregulation of *Muc-2* also suggests the favoring of a Th2 mediated response.

To evaluate effects on the adaptive immune response, we analyzed gene expression of *IFN-γ*, *LITAF*, *IL-4* and *IL-13*. IFN-γ is a vital cytokine, secreted mostly by Th1 cells, that plays a central role in regulating the innate and adaptive immune responses, and is responsible for promoting Th1 cell differentiation, suppressing Th2 cell activity, and enhancing innate immune cell activation and function (Kaiser and Staheli, 2008). Expression of *IFN-γ* was downregulated due to dietary probiotic supplementation on d6 and by in ovo and dietary adminsitration on d14. Expression of *LITAF* is principally in the spleen of chickens, as well as in intestinal intraepithelial lymphocytes. The LITAF protein is a transcription factor that mediates the expression of members of the tumor necrosis factor ligand superfamily (Hong et al., 2006). On d20, expression of *LITAF* was reduced due to both probiotic administration methods. This, coupled with the decrease in *IFN-γ* levels, further supports the thought that probiotics may promote an anti-inflammatory environment, suppress Th1 activity, and promote a Th2 mediated response.

Interleukin-4, a representative of Th2 cytokines, plays a fundamental role in the stimulation of B lymphocytes, T lymphocyte proliferation, and the differentiation of CD4+ T cells into Th2 cells (Fietta and Delsante, 2009). The functions of IL-13, also characterized as a Th2 cytokine, overlap considerably with those of IL-4. Both IL-4 and IL-13 function by inhibiting the production of pro-inflammatory modulators. Dietary-administered probiotic, but not in ovo administration, resulted in decreased *IL-4* expression on d4 and d20, while no differences were observed in *IL-13* expression levels.

Other than *Muc-2*, the general trend appears to be a downregulation of host immune-

related genes. The decreased transcription of these genes in the probiotic-treated groups may be a response to the inhibitory effects of probiotic bacteria on pathogen colonization. A reduction in intestinal colonization by pathogenic bacteria may have eliminated the need for the induction of these genes. Several studies have demonstrated the ability of probiotics to modulate the levels of several cytokines; however, discrepancies have been noted due to differences in the bacterial strains, combinations of probiotic strains, and presence or absence of a challenge (Dalloul et al., 2005; Brisbin et al., 2010; Lee et al., 2010). Further supporting our findings, many reports have noted decreases in immune-related factors. Akbari et al. (2008) found the expression of antimicrobial peptides in the cecal tonsils to be downregulated due to probiotic supplementation during a *Salmonella* infection. The authors speculated that the observed decrease in gene expression could be a result of a reduced S*almonella* load in the intestine. Mountzouris et al. (2009) found that avilamycin and probiotic treatment result in reduced levels of plasma IgA and IgG and intestinal IgA against *Salmonella enteritidis* when compared to the challenged control and that those levels were similar to the non-challenged controls. Dalloul and colleagues (2003) observed similar results when they evaluated antibody secretion during a coccidiosis infection between control and probiotic fed birds. They found that probiotic supplemented birds had significantly lower antibody levels in intestinal secretions when compared to non-supplemented birds as well as reduced oocyst production. When evaluating the effects of probiotic treatment on gene expression in the cecal tonsils of chicks challenged *Salmonella*, Haghighi et al. (2008) found that *IL-12* and *IFN-γ* levels were suppressed by probiotic treatment, which correlated with reduced intestinal *Salmonella* colonization. The immunosuppressive effects seen in these studies could be a result of the reduced colonization capacity of pathogenic bacteria, enhanced clearance, and accelerated recovery caused by the probiotic treatments.

Based on the results presented in this study, in ovo administration of probiotics has an effect similar to that of dietary probiotic supplementation, reinforcing its potential usage to promote early colonization of beneficial bacteria to stimulate intestinal and immune system development. Immunomodulation by in ovo supplementation of probiotics in poultry, and early establishment of beneficial microflora, may lead to increased overall health and well-being, while decreasing the need for prophylactic antibiotic use due to reduced infection rates. It is important to note that no challenge was utilized during this study, but these results provide rationale for further analysis to elucidate the impact of this application on development of the immune system during infection, thus providing better evidence of potentially higher degree of protection against enteric challenges.

In conclusion, our data demonstrate that in ovo supplementation of the commercial probiotic product Primalac does not impact hatchability and can enhance performance of broilers much like that seen with dietary supplementation. In ovo inoculation of Primalac acts as an immunomodulator by altering the expression of several immune-related genes within the ileum. The mode by which the probiotic bacteria within Primalac alter the immune system is multi-faceted with effects on both innate and adaptive immunity. Excluding few exceptions, these results support our previous findings in terms of enhancing performance and general downregulation of immune-related genes. This study further elucidates the immunoregulatory effect of probiotics on intestinal immunity in poultry, which may be more pronounced under more challenging conditions such as in commercial settings. Moreover, it provides justification for further research to investigate the beneficial effects of *Lactobacillus*-based probiotics in poultry and the use of in ovo technology as a means of promoting early establishment of beneficial bacteria, immune system development, gut health, and animal well-being.

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Table 4.1. Primers used for relative real-time PCR[1]

Target	Accession No.	Nucleotide sequence (5' → 3')
GAPDH_F	NM_204305	CCTAGGATACACAGAGGACCAGGTT
GAPDH_R		GGTGGAGGAATGGCTGTCA
IL-4_F	NM_001007079	GCTCTCAGTGCCGCTGATG
IL-4_R		GAAACCTCTCCCTGGATGTCAT
IL-13_F	NM_001007085	CATGACCGACTGCAAGAAGGA
IL-13_R		CCGTGCAGGCTCTTCAGACT
IFN-γ_F	NM_205149	GCTCCCGATGAACGACTTGA
IFN-γ_R		TGTAAGATGCTGAAGAGTTCATTCG
iNOS_F	D85422	CCTGTACTGAAGGTGGCTATTGG
iNOS_R		AGGCCTGTGAGAGTGTGCAA
LITAF_F	AY765397	TGTTCTATGACCGCCCAGTTC
LITAF_R		AGACGTGTCACGATCATCTGGTTA
Muc-2_F	XM_421035	TTCATGATGCCTGCTCTTGTG
Muc-2_R		CCTGAGCCTTGGTACATTCTTGT
TFF-2_F	XM_416743.2	TGGTCCCCCAGGAATCTCA
TFF-2_R		CACCGACGCATTGAAGCA
TLR-2_F	NM_204278	GCGAGCCCCCACGAA
TLR-2_R		GGAGTCGTTCTCACTGTAGGAGACA
TLR-4_F	NM_001030693	CCACACACCTGCCTACATGAA
TLR-4_R		GGATGGCAAGAGGACATATCAAA

[1]Primers designed by Primer Express software (Applied Biosystems, Foster City, CA).

Table 4.2. Effect of in ovo and dietary probiotic supplementation on body weight (g).

Age	DOH	d4	d6	d8	d14	d19	d42
Main Effects							
In Ovo Treatment							
Neg[1]	45.3 ± 0.53	88.9 ± 0.85	135.6 ± 1.65	187.5 ± 4.11	460.1 ± 4.99	796.7 ± 11.64	3171.1 ± 31.06
Dry[2]	46.3 ± 0.53	89.8 ± 0.85	138.1 ± 1.64	192.4 ± 4.06	468.5 ± 4.96	788.8 ± 11.59	3156.8 ± 30.62
P1[3]	45.6 ± 0.53	89.4 ± 0.85	138.5 ± 1.64	200.9 ± 4.05	470.6 ± 4.95	762.4 ± 11.59	3061.7 ± 30.87
P2[4]	46.8 ± 0.53	90.0 ± 0.86	137.5 ± 1.66	193.6 ± 4.20	466.8 ± 5.03	790.9 ± 11.73	3120.9 ± 32.60
Dietary Treatment							
Not Supplemented	--	89.9 ± 0.61	138.0 ± 1.17	192.7 ± 2.91	469.0 ± 3.52	785.2 ± 8.23	3146.0 ± 22.19
Supplemented	--	89.1 ± 0.61	137.0 ± 1.17	194.5 ± 2.90	464.0 ± 3.52	784.2 ± 8.23	3109.1 ± 22.06
Interactions							
Neg/Not Supp[5]	--	89.0 ± 1.21	135.2 ± 2.32	186.4 ± 5.84	462.0 ± 7.06	784.7 ± 16.48	3195.4 ± 44.04
Dry/Not Supp	--	89.7 ± 1.21	138.6 ± 2.32	193.6 ± 5.73	469.0 ± 7.00	780.1 ± 16.38	3190.1 ± 43.42
P1/Not Supp	--	89.1 ± 1.20	137.7 ± 2.32	193.4 ± 5.75	469.3 ± 7.01	760.4 ± 16.40	3085.3 ± 43.96
P2/Not Supp	--	91.9 ± 1.22	140.5 ± 2.34	197.3 ± 5.94	475.7 ± 7.12	791.6 ± 16.57	3113.1 ± 46.02
Neg/Supp[6]	--	88.9 ± 1.21	136.1 ± 2.33	188.5 ± 5.80	458.2 ± 7.04	784.7 ± 16.45	3146.7 ± 43.81
Dry/Supp	--	90.0 ± 1.21	137.7 ± 2.33	191.2 ± 5.76	468.1 ± 7.02	797.5 ± 16.41	3123.4 ± 43.17
P1/Supp	--	89.7 ± 1.20	139.3 ± 2.32	208.5 ± 5.71	472.0 ± 6.99	764.5 ± 16.36	3037.8 ± 43.36
P2/Supp	--	88.0 ± 1.22	134.5 ± 2.35	189.9 ± 5.95	457.9 ± 7.12	790.2 ± 16.60	3128.7 ± 46.19
Statistical Effects (P-Value)							
In Ovo Treatment	0.06	0.83	0.62	0.15	0.48	0.18	0.07
Dietary Treatment	--	0.37	0.52	0.65	0.33	0.93	0.25
In Ovo * Diet	--	0.23	0.37	0.26	0.50	0.65	0.81

[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria
[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.3. Effect of in ovo and dietary probiotic supplementation on body weight gain (g).

Age	DOH -d4	d4 - d6	d6 - d8	d8 - d14	d14 - d19	d19 - d42	DOH - d42
Main Effects							
In Ovo Treatment							
Neg	44.0 ± 0.87	47.1 ± 0.97	52.5 ± 0.88[b]	274.4 ± 2.69	339.0 ± 11.44	2368.8 ± 32.04	3138.2 ± 31.92
Dry	43.8 ± 0.87	48.4 ± 0.97	54.3 ± 0.87[ab]	275.2 ± 2.65	320.2 ± 11.43	2365.6 ± 31.42	3107.3 ± 31.17
P1	44.1 ± 0.86	49.5 ± 0.97	56.7 ± 0.87[a]	278.3 ± 2.66	295.2 ± 11.43	2307.0 ± 31.84	3026.0 ± 31.63
P2	43.1 ± 0.87	47.6 ± 0.98	55.5 ± 0.88[a]	274.2 ± 2.70	325.4 ± 11.46	2328.9 ± 33.08	3074.5 ± 33.03
Dietary Treatment							
Not Supplemented	44.2 ± 0.61	48.3 ± 0.69	54.6 ± 0.62	277.5 ± 1.89	316.8 ± 8.09	2354.3 ± 22.73	3102.1 ± 22.64
Supplemented	43.3 ± 0.61	48.0 ± 0.69	55.0 ± 0.62	273.5 ± 1.89	323.1 ± 8.09	2330.9 ± 22.66	3070.9 ± 22.54
Interactions							
Neg/Not Supp	43.5 ± 1.23	46.3 ± 1.38	51.5 ± 1.24	276.5 ± 3.81	345.9 ± 16.19	2348.0 ± 45.35	3142.0 ± 46.26
Dry/Not Supp	44.2 ± 1.22	49.1 ± 1.37	54.9 ± 1.23	275.7 ± 3.74	310.9 ± 16.16	2410.2 ± 44.53	3147.2 ± 44.19
P1/Not Supp	44.1 ± 1.22	49.0 ± 1.37	56.2 ± 1.24	279.0 ± 3.77	295.1 ± 16.17	2336.4 ± 45.34	3052.3 ± 45.08
P2/Not Supp	45.2 ± 1.23	48.9 ± 1.38	55.7 ± 1.24	278.8 ± 3.81	315.5 ± 16.20	2322.5 ± 46.64	3066.8 ± 46.55
Neg/Supp	44.5 ± 1.23	47.1 ± 1.58	53.5 ± 1.24	272.3 ± 3.79	332.1 ± 16.18	2389.7 ± 45.27	3134.4 ± 45.02
Dry/Supp	43.5 ± 1.22	47.7 ± 1.37	53.7 ± 1.23	274.6 ± 3.75	329.5 ± 16.16	2321.0 ± 44.34	3067.4 ± 43.97
P1/Supp	44.1 ± 1.22	50.0 ± 1.37	57.3 ± 1.23	276.6 ± 3.75	295.4 ± 16.16	2277.6 ± 44.73	2999.8 ± 44.38
P2/Supp	41.1 ± 1.23	46.3 ± 1.38	55.3 ± 1.25	269.6 ± 3.83	335.2 ± 16.21	2335.4 ± 46.92	3082.1 ± 46.88
Statistical Effects (P-Value)							
In Ovo Treatment	0.87	0.32	0.01	0.68	0.07	0.46	0.09
Dietary Treatment	0.27	0.70	0.65	0.15	0.59	0.47	0.34
In Ovo * Diet	0.19	0.39	0.57	0.70	0.69	0.44	0.72

Different letters within a column indicate significant difference among treatment groups

[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria

[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.4. Effect of in ovo and dietary probiotic supplementation on feed intake (g/bird).

Age	DOH -d4	d4 - d6	d6 - d8	d8 - d14	d14 - d19	d19 - d42	DOH - d42
Main Effects							
In Ovo Treatment							
Neg	54.3 ± 2.28	50.2 ± 0.80	59.6 ± 1.08	291.6 ± 5.48	546.8 ± 10.59[a]	2542.8 ± 79.67[a]	3545.2 ± 78.74[a]
Dry	56.8 ± 2.28	51.0 ± 0.80	60.6 ± 1.08	299.6 ± 5.48	522.0 ± 10.59[ab]	2607.5 ± 79.67[a]	3597.5 ± 78.74[a]
P1	58.3 ± 2.28	50.4 ± 0.80	58.9 ± 1.08	292.1 ± 5.48	497.6 ± 10.59[b]	2494.6 ± 79.67[ab]	3451.8 ± 78.74[ab]
P2	57.0 ± 2.28	50.9 ± 0.80	59.7 ± 1.08	288.4 ± 5.48	538.4 ± 10.59[a]	2268.0 ± 79.67[b]	3262.2 ± 78.74[b]
Dietary Treatment							
Not Supplemented	61.3 ± 1.61[a]	50.8 ± 0.57	59.7 ± 0.76	299.0 ± 3.87[a]	524.3 ± 7.49	2467.9 ± 56.33	3462.9 ± 55.67
Supplemented	51.8 ± 1.61[b]	50.4 ± 0.57	59.7 ± 0.76	286.9 ± 3.87[b]	528.1 ± 7.49	2488.5 ± 56.33	3465.5 ± 55.67
Interactions							
Neg/Not Supp	57.7 ± 3.22	49.4 ± 1.14	58.0 ± 1.53	298.7 ± 7.75	559.6 ± 14.98	2495.3 ± 112.67	3518.7 ± 111.35
Dry/Not Supp	61.7 ± 3.22	51.9 ± 1.14	61.1 ± 1.53	306.7 ± 7.75	513.3 ± 14.98	2626.3 ± 112.67	3620.9 ± 111.35
P1/Not Supp	61.5 ± 3.22	50.1 ± 1.14	59.0 ± 1.53	293.4 ± 7.75	496.1 ± 14.98	2475.7 ± 112.67	3438.5 ± 111.35
P2/Not Supp	61.5 ± 3.22	51.9 ± 1.14	60.6 ± 1.53	297.2 ± 7.75	528.1 ± 14.98	2274.4 ± 112.67	3273.7 ± 111.35
Neg/Supp	50.9 ± 3.22	51.0 ± 1.14	61.1 ± 1.53	284.4 ± 7.75	534.0 ± 14.98	2590.3 ± 112.67	3571.7 ± 111.35
Dry/Supp	51.9 ± 3.22	50.1 ± 1.14	60.0 ± 1.53	292.6 ± 7.75	530.7 ± 14.98	2588.8 ± 112.67	3574.1 ± 111.35
P1/Supp	52.3 ± 3.22	50.7 ± 1.14	58.9 ± 1.53	290.8 ± 7.75	499.2 ± 14.98	2513.5 ± 112.67	3465.2 ± 111.35
P2/Supp	52.5 ± 3.22	49.9 ± 1.14	58.7 ± 1.53	279.7 ± 7.75	548.7 ± 14.98	2261.6 ± 112.67	3251.1 ± 111.35
Statistical Effects (P-Value)							
In Ovo Treatment	0.66	0.86	0.75	0.53	0.01	0.03	0.02
Dietary Treatment	0.0002	0.65	0.99	0.03	0.72	0.80	0.97
In Ovo * Diet	0.88	0.30	0.39	0.79	0.41	0.94	0.97

Different letters within a column indicate significant difference among treatment groups

[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria

[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.5. Effect of in ovo and dietary probiotic supplementation on feed conversion ratios.

Age	DOH -d4	d4 - d6	d6 - d8	d8 - d14	d14 - d19	d19 - d42	DOH - d42
Main Effects							
In Ovo Treatment							
Neg	1.23 ± 0.05	1.07 ± 0.02	1.13 ± 0.01[a]	1.06 ± 0.02	1.62 ± 0.04	1.07 ± 0.03[a]	1.08 ± 0.03
Dry	1.30 ± 0.05	1.05 ± 0.02	1.11 ± 0.01[a]	1.09 ± 0.02	1.64 ± 0.04	1.10 ± 0.03[a]	1.14 ± 0.03
P1	1.33 ± 0.05	1.02 ± 0.02	1.04 ± 0.01[c]	1.06 ± 0.02	1.72 ± 0.04	1.08 ± 0.03[a]	1.16 ± 0.03
P2	1.32 ± 0.05	1.07 ± 0.02	1.07 ± 0.01[b]	1.05 ± 0.02	1.66 ± 0.04	0.97 ± 0.03[b]	1.11 ± 0.03
Dietary Treatment							
Not Supplemented	1.39 ± 0.04[a]	1.05 ± 0.01	1.09 ± 0.01	1.09 ± 0.01[a]	1.67 ± 0.03	1.05 ± 0.02	1.12 ± 0.02
Supplemented	1.20 ± 0.04[b]	1.05 ± 0.01	1.09 ± 0.01	1.04 ± 0.01[b]	1.65 ± 0.03	1.07 ± 0.02	1.13 ± 0.02
Interactions							
Neg/Not Supp	1.33 ± 0.08	1.07 ± 0.02	1.13 ± 0.02	1.09 ± 0.02	1.62 ± 0.06	1.06 ± 0.05	1.13 ± 0.04
Dry/Not Supp	1.40 ± 0.08	1.06 ± 0.02	1.11 ± 0.02	1.11 ± 0.02	1.66 ± 0.06	1.09 ± 0.05	1.15 ± 0.04
P1/Not Supp	1.46 ± 0.08	1.02 ± 0.02	1.05 ± 0.02	1.07 ± 0.02	1.71 ± 0.06	1.06 ± 0.05	1.13 ± 0.04
P2/Not Supp	1.37 ± 0.08	1.06 ± 0.02	1.09 ± 0.02	1.08 ± 0.02	1.68 ± 0.06	0.98 ± 0.05	1.06 ± 0.04
Neg/Supp	1.14 ± 0.08	1.07 ± 0.02	1.14 ± 0.02	1.03 ± 0.02	1.62 ± 0.06	1.08 ± 0.05	1.04 ± 0.04
Dry/Supp	1.20 ± 0.08	1.05 ± 0.02	1.12 ± 0.02	1.06 ± 0.02	1.62 ± 0.06	1.11 ± 0.05	1.12 ± 0.04
P1/Supp	1.19 ± 0.08	1.01 ± 0.02	1.03 ± 0.02	1.06 ± 0.02	1.72 ± 0.06	1.10 ± 0.05	1.19 ± 0.04
P2/Supp	1.28 ± 0.08	1.08 ± 0.02	1.06 ± 0.02	1.02 ± 0.02	1.64 ± 0.06	0.97 ± 0.05	1.15 ± 0.04
Statistical Effects (P-Value)							
In Ovo Treatment	0.6	0.08	< 0.0001	0.28	0.35	0.03	0.18
Dietary Treatment	0.001	0.95	0.46	0.01	0.63	0.55	0.77
In Ovo * Diet	0.70	0.94	0.53	0.59	0.94	0.94	0.08

Different letters within a column indicate significant difference among treatment groups

[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria

[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.6. Effect of in ovo and dietary probiotic supplementation on bursa weight (%BW).

Age	DOH	d4	d6	d8	d14	d20	d42
Main Effects							
In Ovo Treatment							
Neg	0.083 ± 0.008	0.148 ± 0.010	0.151 ± 0.013	0.161 ± 0.012	0.194 ± 0.012	0.194 ± 0.011	0.131 ± 0.011
Dry	0.089 ± 0.008	0.146 ± 0.010	0.150 ± 0.013	0.161 ± 0.012	0.183 ± 0.012	0.181 ± 0.011	0.135 ± 0.011
P1	0.085 ± 0.008	0.157 ± 0.010	0.150 ± 0.013	0.157 ± 0.012	0.164 ± 0.012	0.167 ± 0.011	0.126 ± 0.011
P2	0.094 ± 0.008	0.161 ± 0.010	0.153 ± 0.013	0.161 ± 0.012	0.161 ± 0.012	0.167 ± 0.011	0.124 ± 0.011
Dietary Treatment							
Not Supplemented	--	0.151 ± 0.007	0.153 ± 0.009	0.156 ± 0.008	0.172 ± 0.009	0.178 ± 0.008	0.135 ± 0.008
Supplemented	--	0.155 ± 0.007	0.149 ± 0.008	0.162 ± 0.008	0.179 ± 0.009	0.176 ± 0.008	0.124 ± 0.008
Interactions							
Neg/Not Supp	--	0.148 ± 0.014	0.152 ± 0.018	0.152 ± 0.016	0.191 ± 0.017	0.181 ± 0.015	0.133 ± 0.016
Dry/Not Supp	--	0.143 ± 0.014	0.139 ± 0.018	0.166 ± 0.016	0.194 ± 0.017	0.203 ± 0.015	0.142 ± 0.016
P1/Not Supp	--	0.164 ± 0.014	0.165 ± 0.018	0.152 ± 0.016	0.134 ± 0.017	0.157 ± 0.015	0.135 ± 0.016
P2/Not Supp	--	0.148 ± 0.014	0.156 ± 0.018	0.161 ± 0.016	0.171 ± 0.017	0.170 ± 0.015	0.128 ± 0.016
Neg/Supp	--	0.148 ± 0.014	0.150 ± 0.018	0.170 ± 0.016	0.196 ± 0.017	0.207 ± 0.015	0.130 ± 0.016
Dry/Supp	--	0.149 ± 0.014	0.162 ± 0.018	0.156 ± 0.016	0.173 ± 0.017	0.158 ± 0.015	0.128 ± 0.016
P1/Supp	--	0.149 ± 0.014	0.135 ± 0.018	0.162 ± 0.016	0.194 ± 0.017	0.177 ± 0.015	0.118 ± 0.016
P2/Supp	--	0.174 ± 0.014	0.150 ± 0.018	0.161 ± 0.016	0.151 ± 0.017	0.164 ± 0.015	0.120 ± 0.016
Statistical Effects (P-Value)							
In Ovo Treatment	0.69	0.78	0.94	0.99	0.21	0.29	0.86
Dietary Treatment	--	0.74	0.59	0.73	0.52	0.85	0.32
In Ovo * Diet	--	0.67	0.74	0.85	0.06	0.11	0.95

[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria
[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.7. Effect of in ovo and dietary probiotic supplementation on spleen weight (%BW).

Age	DOH	d4	d6	d8	d14	d20	d42
Main Effects							
In Ovo Treatment							
Neg	0.038 ± 0.004	0.076 ± 0.007	0.072 ± 0.005	0.067 ± 0.011	0.068 ± 0.006	0.072 ± 0.007	0.072 ± 0.004
Dry	0.034 ± 0.004	0.077 ± 0.007	0.071 ± 0.005	0.088 ± 0.011	0.070 ± 0.006	0.074 ± 0.007	0.067 ± 0.004
P1	0.032 ± 0.004	0.080 ± 0.007	0.074 ± 0.005	0.077 ± 0.011	0.085 ± 0.006	0.083 ± 0.007	0.075 ± 0.004
P2	0.041 ± 0.004	0.079 ± 0.007	0.080 ± 0.005	0.081 ± 0.011	0.076 ± 0.006	0.071 ± 0.007	0.080 ± 0.004
Dietary Treatment							
Not Supplemented	--	0.076 ± 0.005	0.073 ± 0.003	0.075 ± 0.008	0.077 ± 0.004	0.069 ± 0.005	0.072 ± 0.003
Supplemented	--	0.080 ± 0.005	0.075 ± 0.003	0.081 ± 0.008	0.073 ± 0.004	0.081 ± 0.005	0.075 ± 0.003
Interactions							
Neg/Not Supp	--	0.069 ± 0.009	0.073 ± 0.006[b]	0.069 ± 0.016	0.064 ± 0.009	0.068 ± 0.009	0.065 ± 0.006
Dry/Not Supp	--	0.068 ± 0.009	0.069 ± 0.006[b]	0.061 ± 0.016	0.067 ± 0.009	0.064 ± 0.009	0.065 ± 0.006
P1/Not Supp	--	0.082 ± 0.009	0.083 ± 0.007[ab]	0.081 ± 0.016	0.102 ± 0.009	0.078 ± 0.009	0.077 ± 0.006
P2/Not Supp	--	0.084 ± 0.009	0.067 ± 0.006[b]	0.091 ± 0.016	0.074 ± 0.009	0.065 ± 0.009	0.080 ± 0.006
Neg/Supp	--	0.083 ± 0.009	0.071 ± 0.006[b]	0.065 ± 0.016	0.072 ± 0.009	0.076 ± 0.009	0.079 ± 0.006
Dry/Supp	--	0.086 ± 0.009	0.073 ± 0.006[b]	0.116 ± 0.016	0.072 ± 0.009	0.084 ± 0.009	0.070 ± 0.006
P1/Supp	--	0.078 ± 0.009	0.065 ± 0.006[b]	0.073 ± 0.016	0.069 ± 0.009	0.088 ± 0.009	0.073 ± 0.006
P2/Supp	--	0.075 ± 0.009	0.093 ± 0.007[a]	0.071 ± 0.016	0.078 ± 0.009	0.077 ± 0.009	0.080 ± 0.006
Statistical Effects (P-Value)							
In Ovo Treatment	0.29	0.95	0.57	0.54	0.22	0.44	0.22
Dietary Treatment	--	0.67	0.61	0.51	0.61	0.06	0.32
In Ovo * Diet	--	0.44	0.02	0.09	0.11	0.97	0.43

Different letters within a column indicate significant difference among treatment groups

[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria

[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.8. Effect of in ovo and dietary probiotic supplementation on ileal *TLR-2* gene expression (RQ).

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
In Ovo Treatment						
Neg	1.00	1.00	1.00	1.00	1.00	1.00
Dry	0.94	1.12	0.76	1.15	1.10	1.20
P1	1.29	1.06	1.28	1.26	1.07	1.73
P2	0.93	0.87	1.20	1.43	1.11	1.45
Dietary Treatment						
Not Supplemented	--	1.00	1.00	1.00	1.00	1.00
Supplemented	--	0.91	0.93	1.28	0.98	0.91
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00^{ab}	1.00
Dry/Not Supp	--	0.98	0.62	1.40	1.21^{a}	1.27
P1/Not Supp	--	1.37	1.35	1.59	0.81^{b}	2.12
P2/Not Supp	--	0.69	0.84	1.61	1.04^{ab}	1.08
Neg/Supp	--	0.86	0.72	1.68	0.87^{ab}	0.90
Dry/Supp	--	1.11	0.67	1.60	0.86^{ab}	1.02
P1/Supp	--	0.71	0.88	1.68	1.22^{a}	1.27
P2/Supp	--	0.95	1.23	2.15	1.04^{ab}	1.75
Statistical Effects (P-Value)						
In Ovo Treatment	0.59	0.74	0.12	0.34	0.85	0.10
Dietary Treatment		0.60	0.67	0.08	0.85	0.57
In Ovo * Diet		0.20	0.28	0.66	0.048	0.17

Different letters within a column indicate significant difference among treatment groups
[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria
[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.9. Effect of in ovo and dietary probiotic supplementation on ileal *TLR-4* gene expression (RQ).

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
In Ovo Treatment						
Neg	1.00	1.00	1.00	1.00^{bc}	1.00	1.00
Dry	1.14	0.95	0.90	0.87^{c}	1.14	1.10
P1	1.38	1.15	1.11	1.29^{ab}	1.12	1.37
P2	1.09	0.86	1.30	1.59^{a}	0.85	1.48
Dietary Treatment						
Not Supplemented	--	1.00	1.00	1.00^{b}	1.00^{a}	1.00
Supplemented	--	1.00	1.01	1.77^{a}	0.62^{b}	0.95
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00^{abc}	1.00
Dry/Not Supp	--	0.83	0.67	1.21	1.39^{a}	0.97
P1/Not Supp	--	1.34	1.12	1.27	0.90^{bcd}	1.18
P2/Not Supp	--	0.78	1.04	1.44	1.10^{ab}	1.14
Neg/Supp	--	0.97	0.78	1.98	0.69^{cd}	0.73
Dry/Supp	--	1.04	0.94	1.22	0.64^{de}	0.91
P1/Supp	--	0.96	0.87	2.59	0.97^{abc}	1.17
P2/Supp	--	0.91	1.28	3.46	0.46^{e}	1.40
Statistical Effects (P-Value)						
In Ovo Treatment	0.59	0.45	0.28	0.01	0.15	0.20
Dietary Treatment		0.98	0.94	< 0.001	< 0.001	0.75
In Ovo * Diet		0.42	0.33	0.08	0.01	0.66

Different letters within a column indicate significant difference among treatment groups

[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria

[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.10. Effect of in ovo and dietary probiotic supplementation on ileal *iNOS* gene expression (RQ).

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
In Ovo Treatment						
Neg	1.00	1.00	1.00	1.00	1.00	1.00
Dry	0.85	1.04	0.86	1.00	0.96	1.00
P1	1.01	0.85	1.10	0.94	1.16	1.06
P2	0.83	0.75	0.90	1.25	0.90	1.07
Dietary Treatment						
Not Supplemented	--	1.00	1.00	1.00	1.00	1.00[a]
Supplemented	--	0.97	0.94	0.99	0.99	0.79[b]
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00	1.00
Dry/Not Supp	--	0.98	0.77	1.20	0.89	1.19
P1/Not Supp	--	0.76	1.39	1.09	0.93	0.99
P2/Not Supp	--	0.58	0.83	1.33	0.98	1.04
Neg/Supp	--	0.78	0.95	1.21	0.89	0.83
Dry/Supp	--	0.86	0.92	1.01	0.93	0.69
P1/Supp	--	0.75	0.83	0.98	1.29	0.94
P2/Supp	--	0.76	0.93	1.42	0.75	0.91
Statistical Effects (P-Value)						
In Ovo Treatment	0.33	0.32	0.54	0.31	0.38	0.96
Dietary Treatment		0.85	0.62	0.94	0.96	0.04
In Ovo * Diet		0.56	0.23	0.66	0.22	0.40

Different letters within a column indicate significant difference among treatment groups
[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria
[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.11. Effect of in ovo and dietary probiotic supplementation on ileal *TFF-2* gene expression (RQ).

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
In Ovo Treatment						
Neg	1.00	1.00	1.00	1.00	1.00	1.00
Dry	1.23	1.01	0.93	0.70	1.05	1.33
P1	0.89	1.31	1.00	0.76	1.15	1.23
P2	0.52	0.82	1.40	1.16	1.07	1.68
Dietary Treatment						
Not Supplemented	--	1.00	1.00	1.00	1.00	1.00
Supplemented	--	0.87	0.82	0.72	0.99	0.86
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00	1.00
Dry/Not Supp	--	1.06	0.64	1.08	1.32	1.38
P1/Not Supp	--	1.61	0.63	1.21	0.99	1.07
P2/Not Supp	--	0.75	0.88	1.61	1.48	1.29
Neg/Supp	--	0.94	0.42	1.32	1.21	0.71
Dry/Supp	--	0.91	0.58	0.60	1.01	0.92
P1/Supp	--	1.00	0.68	0.63	1.62	1.02
P2/Supp	--	0.85	0.95	1.11	0.93	1.56
Statistical Effects (P-Value)						
In Ovo Treatment	0.10	0.40	0.37	0.38	0.95	0.45
Dietary Treatment		0.47	0.28	0.16	0.95	0.51
In Ovo * Diet		0.72	0.29	0.49	0.24	0.78

[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria
[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.12. Effect of in ovo and dietary probiotic supplementation on ileal *Muc-2* gene expression (RQ).

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
In Ovo Treatment						
Neg	1.00	1.00	1.00[b]	1.00	1.00	1.00
Dry	1.22	1.31	0.85[b]	0.98	1.06	1.09
P1	0.94	0.84	1.79[a]	0.66	1.37	1.03
P2	1.65	1.15	1.22[ab]	0.64	1.17	0.69
Dietary Treatment						
Not Supplemented	--	1.00[b]	1.00	1.00	1.00[b]	1.00[b]
Supplemented	--	2.05[a]	1.22	1.08	1.42[a]	1.92[a]
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00	1.00
Dry/Not Supp	--	1.52	0.64	0.95	1.24	0.97
P1/Not Supp	--	0.95	1.45	0.85	1.68	0.98
P2/Not Supp	--	1.43	0.83	1.28	1.55	0.76
Neg/Supp	--	2.62	0.79	1.58	1.96	1.85
Dry/Supp	--	2.95	0.89	1.62	1.77	2.25
P1/Supp	--	1.94	1.74	1.13	2.20	2.00
P2/Supp	--	2.44	1.41	0.71	1.73	1.17
Statistical Effects (P-Value)						
In Ovo Treatment	0.10	0.18	0.01	0.45	0.22	0.12
Dietary Treatment		< 0.0001	0.19	0.29	0.003	< 0.0001
In Ovo * Diet		0.81	0.29	0.06	0.33	0.77

Different letters within a column indicate significant difference among treatment groups
[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria
[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.13. Effect of in ovo and dietary probiotic supplementation on ileal *IFN-γ* gene expression (RQ).

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
In Ovo Treatment						
Neg	1.00	1.00	1.00	1.00	1.00[a]	1.00
Dry	1.12	1.05	0.90	0.98	0.91[a]	0.83
P1	1.22	0.83	1.30	1.01	0.95[a]	1.00
P2	1.01	0.91	1.37	1.21	0.62[b]	0.94
Dietary Treatment						
Not Supplemented	--	1.00[a]	1.00	1.00	1.00	1.00
Supplemented	--	0.55[b]	1.26	0.94	0.86	1.07
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00[a]	1.00
Dry/Not Supp	--	1.67	0.68	1.03	0.96[a]	0.88
P1/Not Supp	--	1.02	1.32	1.07	0.54[b]	1.25
P2/Not Supp	--	1.09	1.68	1.33	0.40[b]	0.93
Neg/Supp	--	0.83	1.22	1.04	0.53[b]	1.34
Dry/Supp	--	0.55	1.46	0.98	0.45[b]	1.04
P1/Supp	--	0.57	1.57	0.99	0.89[a]	0.91
P2/Supp	--	0.63	1.37	1.15	0.52[b]	1.03
Statistical Effects (P-Value)						
In Ovo Treatment	0.58	0.66	0.20	0.79	0.01	0.61
Dietary Treatment		0.0002	0.15	0.72	0.18	0.60
In Ovo * Diet		0.19	0.22	0.98	0.0002	0.26

Different letters within a column indicate significant difference among treatment groups
[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria
[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.14. Effect of in ovo and dietary probiotic supplementation on ileal *LITAF* gene expression (RQ).

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
In Ovo Treatment						
Neg	1.00	1.00	1.00	1.00	1.00	1.00
Dry	0.88	0.91	0.94	0.97	1.10	1.04
P1	0.97	1.06	1.00	0.91	1.11	1.00
P2	1.04	0.98	0.88	0.90	1.11	0.90
Dietary Treatment						
Not Supplemented	--	1.00	1.00	1.00	1.00	1.00^{a}
Supplemented	--	0.90	0.95	1.08	0.89	0.80^{b}
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00	1.00^{a}
Dry/Not Supp	--	0.80	1.05	1.07	1.08	0.83^{abc}
P1/Not Supp	--	1.11	1.08	0.90	1.14	0.89^{ab}
P2/Not Supp	--	0.87	1.02	0.91	1.04	0.66^{cd}
Neg/Supp	--	0.81	1.13	1.00	0.87	0.58^{d}
Dry/Supp	--	0.85	0.94	1.14	0.98	0.75^{abcd}
P1/Supp	--	0.83	1.04	1.04	0.93	0.65^{cd}
P2/Supp	--	0.89	0.86	1.02	1.03	0.72^{bcd}
Statistical Effects (P-Value)						
In Ovo Treatment	0.78	0.55	0.60	0.73	0.66	0.56
Dietary Treatment		0.15	0.53	0.29	0.11	0.001
In Ovo * Diet		0.30	0.55	0.70	0.79	0.02

Different letters within a column indicate significant difference among treatment groups
[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^{6}$ probiotic bacteria; [4]P2 = $1x10^{7}$ probiotic bacteria
[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.15. Effect of in ovo and dietary probiotic supplementation on ileal *IL-4* gene expression (RQ).

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
In Ovo Treatment						
Neg	1.00	1.00	1.00	1.00	1.00	1.00
Dry	1.17	1.18	0.96	0.93	1.26	1.11
P1	1.12	0.82	1.36	1.12	1.20	1.02
P2	1.33	0.75	1.04	1.02	1.13	1.35
Dietary Treatment						
Not Supplemented	--	1.00[a]	1.00	1.00	1.00	1.00[a]
Supplemented	--	0.65[b]	1.13	1.07	1.19	0.71[b]
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00	1.00
Dry/Not Supp	--	1.47	1.00	1.19	1.47	0.97
P1/Not Supp	--	0.86	1.60	1.58	1.04	1.02
P2/Not Supp	--	0.80	1.40	1.45	1.04	1.57
Neg/Supp	--	0.76	1.44	1.71	1.15	0.72
Dry/Supp	--	0.72	1.35	1.25	1.23	0.91
P1/Supp	--	0.60	1.66	1.36	1.60	0.73
P2/Supp	--	0.54	1.12	1.23	1.40	0.83
Statistical Effects (P-Value)						
In Ovo Treatment	0.40	0.05	0.11	0.56	0.53	0.44
Dietary Treatment		0.001	0.26	0.48	0.15	0.02
In Ovo * Diet		0.62	0.20	0.07	0.31	0.55

Different letters within a column indicate significant difference among treatment groups
[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria
[5]Not supp = not supplemented; [6]Supp = supplemented

Table 4.16. Effect of in ovo and dietary probiotic supplementation on ileal *IL-13* gene expression (RQ).

Age	DOH	d4	d6	d8	d14	d20
Main Effects						
In Ovo Treatment						
Neg	1.00	1.00	1.00	1.00	1.00	1.00
Dry	1.37	1.72	0.58	1.03	1.00	1.39
P1	1.83	1.26	1.05	0.83	0.99	1.05
P2	1.06	1.09	1.15	1.67	1.01	1.27
Dietary Treatment						
Not Supplemented	--	1.00	1.00	1.00	1.00	1.00
Supplemented	--	1.02	1.21	1.01	1.08	1.05
Interactions						
Neg/Not Supp	--	1.00	1.00	1.00	1.00	1.00
Dry/Not Supp	--	1.74	0.38	1.82	1.26	1.67
P1/Not Supp	--	2.03	1.12	1.58	1.01	1.13
P2/Not Supp	--	0.86	0.68	3.42	0.87	1.26
Neg/Supp	--	1.15	0.78	2.66	1.13	1.19
Dry/Supp	--	1.96	0.69	1.56	0.90	1.37
P1/Supp	--	0.90	0.77	1.15	1.10	1.16
P2/Supp	--	1.60	1.52	2.18	1.34	1.53
Statistical Effects (P-Value)						
In Ovo Treatment	0.43	0.53	0.08	0.21	0.99	0.24
Dietary Treatment		0.95	0.34	0.96	0.56	0.70
In Ovo * Diet		0.29	0.11	0.19	0.23	0.69

[1]Neg = negative control; [2]Dry = dry punch; [3]P1 = $1x10^6$ probiotic bacteria; [4]P2 = $1x10^7$ probiotic bacteria
[5]Not supp = not supplemented; [6]Supp = supplemented

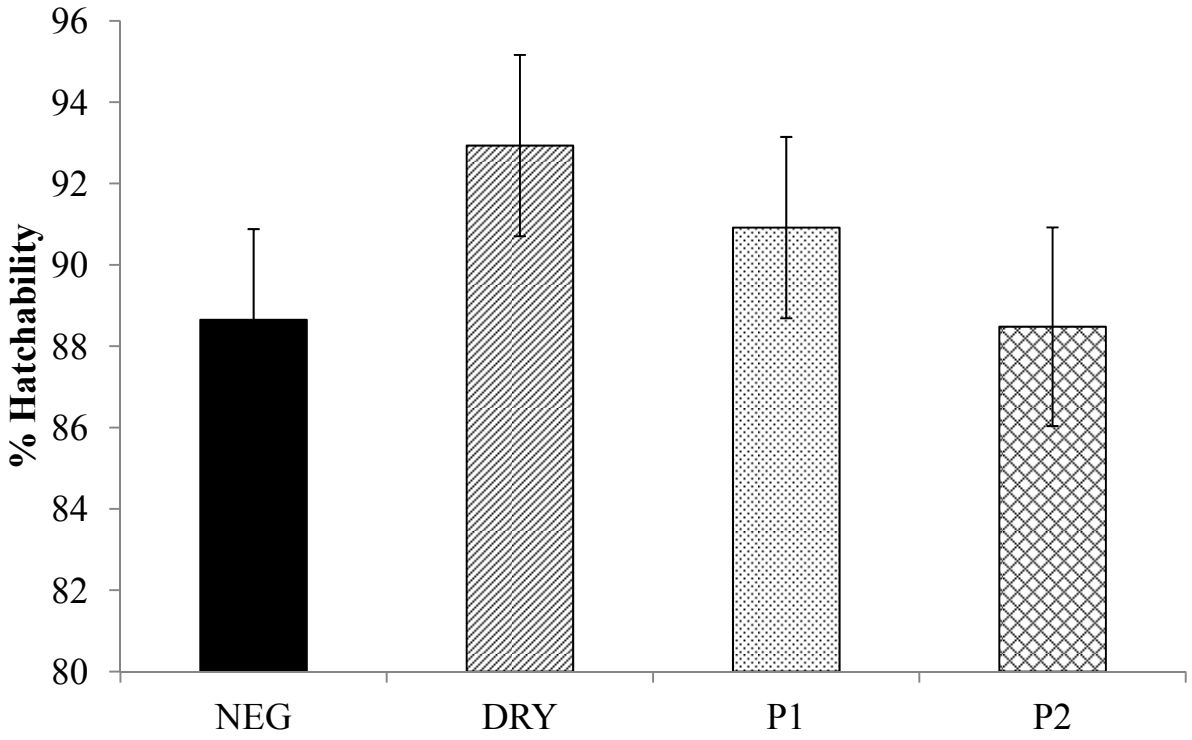

Figure 4.1. Effect of in ovo probiotic supplementation on hatchability of Cobb 500 chicks. Data are represented as least squares means ± SEM. Neg = negative control, Dry = dry punch, P1 = $1x10^6$ probiotic bacteria, P2 = $1x10^7$ probiotic bacteria. (P = 0.48).

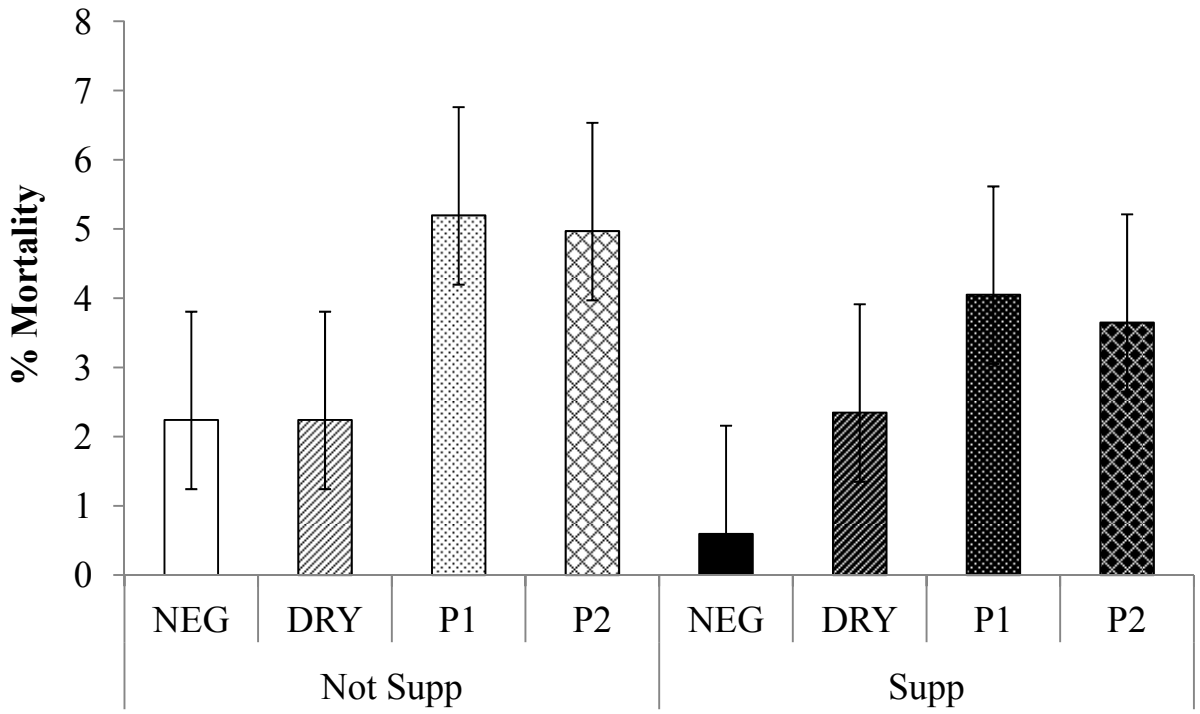

Figure 4.2. Effect of in ovo and dietary probiotic supplementation on mortality of Cobb 500 chicks. Data are represented as least squares means ± SEM. Neg = negative control, Dry = dry punch, P1 = $1x10^6$ probiotic bacteria, P2 = $1x10^7$ probiotic bacteria. (P = 0.38).

CHAPTER V

Effects of In Ovo Supplementation of Primalac on Performance and Immunocompetence of Broiler Chicks to an *Eimeria* Challenge

ABSTRACT: Coccidiosis is regarded as the parasitic disease with the greatest economic impact on the poultry industry due to reduced performance and increased mortality. A study was conducted to investigate the effects of in ovo administration of probiotics (Primalac W/S) on hatchability, performance, immune organ weights, and lesion scores in broiler chicks during a mixed *Eimeria* infection. At embryonic day 18, 210 eggs were injected with either sterile water (neg) or $1x10^6$ probiotic bacteria (Pro). On day (d) 3 post-hatch, half of the chicks from each treatment group were challenged with a mixed inoculum of *Eimeria acervulina* (50,000 oocysts/bird), *E. maxima* (10,000 oocysts/bird) and *E. tenella* (2,500 oocysts/bird). Measurements and tissue samples were taken on day of hatch (DOH) and d3, 9 and 15. On d9, 24 birds per treatment were scored for intestinal *Eimeria* lesions. No differences were seen among groups for hatchability as well as for body weight (BW), BW gain (BWG), or immune organ weights prior to the *Eimeria* challenge. On d9, the non-challenged birds with probiotic supplementation had higher BW and BWG than the non-supplemented controls while no differences were seen among the challenged groups. On d15, probiotic supplemented birds had improved BW compared to the non-supplemented birds as well as increased BWG from d9 to d15. Bursa weight was not affected by treatment at any time point while spleen weight was increased in supplemented birds on d15. Birds receiving the probiotic had significantly lower mortality than non-treated birds. Additionally, gross lesion severity was reduced due to probiotic supplementation in all intestinal segments evaluated. These results suggest that in ovo

supplementation of Primalac can improve performance and provide protection against a mixed *Eimeria* infection.

INTRODUCTION

Parasitism of the intestinal tract is a major stress factor leading to decreased nutrient utilization and reduced performance in livestock and poultry. Coccidiosis is an intestinal infection by intracellular protozoan parasites belonging to the genus *Eimeria* (Dalloul and Lillehoj, 2005). There are seven *Eimeria* species that infect chickens: *E. acervulina*, *E. maxima*, *E. tenella*, *E. brunetti*, *E. necatrix*, *E. mitis* and *E. praecox*. *Eimeria* sp. invade the intestinal lining and disrupt the enterocyte layer resulting in gross lesions, nutrient malabsorption, reduced performance, and susceptibility to other diseases such as necrotic enteritis (Yegani and Korver, 2008). Coccidiosis is regarded as the parasitic disease with the greatest economic impact on the poultry industry, estimated at $3 billion annually worldwide (Dalloul and Lillehoj, 2006). The monetary loss related to coccidiosis is primarily due to the costs of prophylactic measures, mortality, inefficient feed utilization and lost revenues due to decreased production (Dalloul and Lillehoj, 2005).

Currently, the poultry industry relies heavily on the use of anticoccidial feed additives to prevent coccidiosis outbreaks. Recently, consumers have become exceedingly concerned about chemical residues in poultry products and increased pathogen resistance. These concerns have resulted in mounting pressure to ban chemotherapeutic agents from animal feeds. The European and U.S. bans of antibiotic growth promoters as prophylactic measures against disease are forcing the industry to cease this practice, thus prompting an intense search for alternatives capable of maintaining animal health without negatively affecting performance (Dalloul and Lillehoj, 2006). Probiotics have received considerable interest due to their immunomodulating

capabilities and higher acceptance by consumers. A 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). A well-balanced gut microflora is essential for animal health and performance and probiotics maintain this normal flora through a number of mechanisms including competitive exclusion. For poultry, supplementation of probiotics enhances growth and improves feed conversion in chickens and turkeys (Kabir et al., 2004). Manipulation of intestinal microflora through the use of probiotics may also influence the immune response. Probiotics may help maintain a healthy balance of microflora in the gut and provide protection against several enteric pathogens through stimulation of the mucosal immune system (Nava et al., 2005). As for application, in ovo technology represents one means to administer probiotics and promote early colonization of beneficial bacteria in order to stimulate intestinal and immune system development.

The objective of this study was to determine the effects of in ovo supplementation of the probiotic product Primalac on hatchability, post-hatch performance, immune organ weights, and intestinal lesion scores in broiler chicks with or without an *Eimeria* sp. challenge.

MATERIALS AND METHODS

Birds and treatments

This project was approved and conducted under the guidelines of the Virginia Tech Institutional Animal Care and Use Committee. At embryonic day (d) 18, 210 fertile Cobb 500 eggs were obtained from a commercial hatchery (Pilgrim's Pride, Broadway, VA) and transported to the Virginia Tech Turkey Research Center. Prior to injection, all eggs were candled to determine position of the air cell. Eggs were sanitized by swabbing the large end (outside of the air cell) with 0.5% sodium hypochlorite and once dried, they were sprayed with

70% isopropyl alcohol. To create a guide and avoid cracking, a hole was made in the center of the air cell using an 18-gauge needle fitted with a rubber stopper to prevent the needle from piercing the air cell membrane. Needles were disinfected in between each injection by dipping in 0.5% sodium hypochlorite. Next, half of the eggs were injected with $1x10^6$ probiotic bacteria (Primalac W/S, Star-Labs Inc.) dissolved in sterile water. The remaining eggs were injected with sterile water and served as a control. Injections were performed using a 1 mL syringe equipped with a 22-gauge, 1-inch short bevel needle. A new syringe and needle were used for each injection. Eggs were placed into one of 3 replicate hatching trays (35 eggs/tray). On day of hatch (DOH), birds from each treatment group were divided in half, individually tagged and placed into one of 4 floor pens relative to treatment group (~47 birds/pen). Birds were placed on clean shavings and provided a standard unmedicated broiler starter feed in crumble form and water ad libitum. On d3 post-hatch, one of the two pens from each in ovo treatment group was orally gavaged with1 mL of a mixed inoculum containing 50,000 *Eimeria acervulina*, 10,000 *E. maxima*, and 2,500 *E. tenella* sporulated oocysts.

Hatchability and post-hatch performance parameters

On DOH, percent hatchability was recorded, and birds were weighed prior to placement and on d3, 9, and 15. Mean body weight (BW) for each treatment group was calculated from the individual bird replicates for each weigh day. Average body weight gains (BWG) were calculated for each period and cumulatively based on individual bird weights. Mortality was recorded on a daily basis throughout the trial.

Immune organ weights

On DOH, d3, 9, and 15, 6 birds per treatment were randomly selected and euthanized by cervical dislocation. The bursa of Fabricius and spleen were excised and weighed, and relative organ weights were expressed as a percentage of live BW.

Coccidial lesion scoring

On d9 (6 days post infection), 24 birds per treatment were randomly selected and euthanized for scoring of lesions from intestinal *Eimeria* infection. Lesions in the duodenum, jejunum and ceca were scored by the method of Johnson and Reid (1970) by personnel blinded to the treatment based on scores ranging from 0 (no gross lesion) to 4 (most severe lesion).

Statistical analysis

Data were analyzed using the Fit Model platform in JMP 9.0 (SAS Institute Inc., Cary, NC). For measurements taken on DOH and d3, in ovo treatment was placed in the model. On d9 and d15, in ovo treatment, challenge, and their 2-way interaction were placed in the model. Percent hatchability data were arc sine transformed prior to analysis. Differences among groups were compared with Student *t*-test following analysis. Mortality data were analyzed using the chi-square test. Values were considered statistically different at $P \leq 0.05$. Results are reported as least squares means (LS Means) with standard errors (SE).

RESULTS

Hatchability and post-hatch performance

In ovo supplementation of Primalac did not significantly affect hatchability in this study (96.2% versus 98.1%) ($P = 0.26$). Prior to the *Eimeria* challenge, no differences in BW or BWG were seen between the groups (Tables 5.1 and 5.2 respectively). On d9, in ovo treatment and

challenge resulted in a 2-way interaction ($P < 0.0001$) with non-challenged birds that received probiotic supplementation having higher BW than the non-treated controls. Additionally, there was an expected decrease in BW due to challenge, but no differences were discerned among the in ovo treatment groups. The same pattern was also seen for BWG from d3 to d9 ($P < 0.0001$). There was a main effect of in ovo treatment for BW on d15 ($P = 0.048$) and BWG from d9 to d15 where performance was augmented for probiotic supplemented birds. Again, challenged birds had an anticipated decrease in BW ($P < 0.0001$) and BWG ($P < 0.0001$). This same pattern was observed for the duration of the challenge period (d3 to d15), with probiotic supplementation resulting in increased BWG ($P = 0.045$) and *Eimeria* challenge demonstrating decreased BWG ($P < 0.0001$). There was a main effect of in ovo treatment on mortality where probiotic supplemented birds had a significantly lower mortality than the non-supplemented controls (4.1% versus 20.4%) ($P < 0.0001$) (Figure 5.1). Additionally, there was a main effect of *Eimeria* challenge with non-challenged birds having lower mortality than challenged birds (7.6% versus 16.8%) ($P = 0.005$) (Figure 5.1).

Immune organ weights

Neither in ovo supplementation nor *Eimeri*a challenge had an effect on bursa weight at any time point during the study (Table 5.3). Prior to the challenge, probiotic administration had no effect on spleen weights (Table 5.4). On d9, however, in ovo treatment and *Eimeria* challenge presented a 2-way interaction for spleen weight ($P = 0.04$) though no differences were seen among groups. There was a main effect of in ovo treatment for spleen weight on d15 with probiotic supplemented birds having larger spleens than non-treated birds ($P = 0.01$).

Coccidial lesion scoring

In ovo treatment and *Eimeria* challenge presented a 2-way interaction for lesion scores in the duodenum ($P = 0.01$), jejunum ($P < 0.0001$), and ceca ($P = 0.005$) on d9 (Figure 5.2). Gross lesion severity was significantly reduced in all intestinal segments of challenged birds supplemented with probiotics when compared to the challenged controls. Furthermore, lesion severity in the probiotic supplemented groups was reduced to levels comparable to non-challenged, probiotic supplemented group in the jejunum, and to both non-challenged groups in the ceca.

DISCUSSION

In this study, the influence of in ovo probiotic supplementation on broiler chicks with and without an *Eimeria* challenge was investigated. In terms of hatchability, no significant differences were found among treatments, corroborating our previous studies (unpublished data). Correlating with our findings, Edens et al. (1997) compared the hatchability of broiler embryos injected with *Lactobacillus reuteri* either in the air cell or the amniotic fluid to uninocculated controls and found no differences among the treatment groups. Similar results were also seen in turkey embryos (Edens et al., 1997). In reference to post-hatch performance, we observed augmented BW and BWG after d3 and enhanced survivability due to probiotic supplementation. Edens and colleagues (1997) found that broiler chicks inoculated with *L. reuteri* and subsequently challenged with *Salmonella* displayed reduced mortality and increased BW when compared to the challenge only birds. Studies utilizing probiotics in the diet have found varying results debating the effects of probiotics on performance in poultry. Some studies suggest dietary probiotics improve BWG and FCR 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)

while others observed no benefits (Rahimi et al., 2011; Seifert et al., 2011; Wolfenden et al., 2011). These discrepancies could be due to a variety of factors including, but not limited to, strain(s) of bacteria utilized, composition and viability of the probiotic, preparation method, dosage, application method, frequency of application, overall diet, drug interactions, and condition of the animal (Huang et al., 2004; Mountzouris et al., 2007). In contrast to our results, some studies have shown negative consequences in terms of hatchability, post-hatch performance and mortality due to in ovo probiotic supplementation, but these results could be due to the probiotic strain used and injection site (Cox et al., 1992; Meijerhof and Hulet, 1997)

The reduction in BW, BWG, and survivability due to the *Eimeria* inoculation were not surprising because coccidial infections are known to cause significant damage to the intestinal mucosa and enterocytes during the progression of their lifecycle, thus causing nutrient malabsorption and reduced performance. Moreover, parasitic infections elicit nutrient-demanding immune responses, which may have also contributed to the impaired growth (Allen and Fetterer, 2002; Dalloul and Lillehoj, 2005).

In ovo supplementation of Primalac reduced lesion severity in all intestinal segments evaluated. There are numerous reports regarding the ability of probiotics to enhance the mucosal immune system during an *Eimeria* infection. Probiotic supplementation has been shown to ameliorate *Eimeria* infection by reducing oocyst shedding and lesion severity, as well as increasing body weights and secretion of *Eimeria* specific antibodies (Dalloul et al., 2003, 2005; Lee et al., 2007a&b, 2010; Santosh and Gupta, 2011). These results indicate that probiotics administered in ovo exhibit immunoprotective properties and enhance host defenses against *E. acervulina*, *E. maxima* and *E. tenella*.

In this study, the relative size of the bursa was unaffected by probiotic treatment or *Eimeria* challenge, which supports our previous work where in ovo supplementation of probiotics did not affect immune organ weights (unpublished data). In ovo treatment and the *Eimeria* challenge did present a 2-way interaction for relative spleen weight on d9; however, no differences among groups were observed. On d15, however, there was a main effect of in ovo treatment where probiotic supplementation resulted in larger spleens. Similar results have also been noted in studies where probiotics have been added to the diets of broilers (Kabir et al., 2004; Ahmadi, 2011). However, there are conflicting reports suggesting that probiotics do not affect immune organ weights (Al-Barwary et al., 2012; Naseem et al., 2012).

In conclusion, our data suggest that in ovo administration of Primalac has a positive impact on performance and provides protection from an *Eimeria* challenge. Though research in the areas of probiotic supplementation and in ovo feeding is quickly growing in popularity, little has been done to extend the concept of in ovo administration of probiotics. To our knowledge, this is the first study to evaluate the effects of in ovo administration of the probiotic product Primalac W/S on performance and disease status during an *Eimeria* challenge in broiler chicks. This study provides new evidence for the potential use of in ovo administered probiotics for the prevention or amelioration of coccidial infections.

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Table 5.1. Effect of in ovo probiotic treatment and *Eimeria* challenge on body weight.

Age	DOH	d3	d9	d15
Main Effects				
Treatment				
Neg[1]	46.7 ± 0.33	70.3 ± 0.67	149.8 ± 2.60[b]	295.0 ± 10.16[b]
Pro[2]	46.3 ± 0.32	69.4 ± 0.66	157.6 ± 2.36[a]	320.2 ± 7.10[a]
***Eimeria* Challenge**				
Not Chall[3]	--	--	182.5 ± 2.44[a]	373.1 ± 8.21[a]
Chall[4]	--	--	125.0 ± 2.53[b]	242.0 ± 9.29[b]
Interactions				
Not Chall/ Neg	--	--	169.8 ± 3.57[b]	350.1 ± 13.18
Not Chall/ Pro	--	--	195.1 ± 3.32[a]	396.1 ± 9.78
Chall/ Neg	--	--	129.9 ± 3.78[c]	239.8 ± 15.46
Chall/ Pro	--	--	120.1 ± 3.36[c]	244.3 ± 10.31
Statistical Effects (P-Value)				
Treatment	0.48	0.33	0.03	0.05
Challenge	--	--	< 0.0001	< 0.0001
Treatment * Challenge	--	--	< 0.0001	0.10

[1]Neg = not supplemented with Primalac
[2]Pro = supplemented with Primalac
[3]Not Chall = not challenged
[4]Chall = challenged with *Eimeria*

Table 5.2. Effect of in ovo probiotic treatment and *Eimeria* challenge on body weight gain.

Age	DOH - D3	d3 - d9	d9 - d15	d3 - d15
Main Effects				
Dietary Treatment				
Neg[1]	23.3 ± 0.59	77.0 ± 2.50[b]	136.3 ± 7.37[b]	226.0 ± 10.02[b]
Pro[2]	22.9 ± 0.58	88.4 ± 2.32[a]	161.4 ± 5.25[a]	351.1 ± 7.01[a]
***Eimeria* Challenge**				
Not Chall[3]	--	110.1 ± 2.37[a]	179.6 ± 5.90[a]	301.0 ± 8.10[a]
Chall[4]	--	55.3 ± 2.44[b]	118.1 ± 6.86[b]	176.0 ± 9.16[b]
Interactions				
Not Chall/ Neg	--	97.4 ± 3.46[b]	159.5 ± 9.32	279.3 ± 13.00
Not Chall/ Pro	--	122.7 ± 3.26[a]	199.6 ± 7.22	322.8 ± 9.64
Chall/ Neg	--	56.5 ± 3.60[c]	113.0 ± 11.42	172.8 ± 15.25
Chall/ Pro	--	54.1 ± 3.29[c]	123.1 ± 7.61	179.3 ± 10.17
Statistical Effects (P-Value)				
Treatment	0.66	0.001	0.01	0.05
Challenge	--	< 0.0001	< 0.0001	< 0.0001
Treatment * Challenge	--	< 0.0001	0.10	0.14

[1]Neg = not supplemented with Primalac
[2]Pro = supplemented with Primalac
[3]Not Chall = not challenged
[4]Chall = challenged with *Eimeria*

Table 5.3. Effect of in ovo probiotic treatment and *Eimeria* challenge on relative bursa weight.

Age	DOH	d3	d9	d15
Main Effects				
Dietary Treatment				
Neg[1]	0.113 ± 0.010	0.145 ± 0.015	0.176 ± 0.008	0.193 ± 0.014
Pro[2]	0.100 ± 0.010	0.141 ± 0.015	0.156 ± 0.008	0.194 ± 0.014
***Eimeria* Challenge**				
Not Chall[3]	--	--	0.168 ± 0.008	0.195 ± 0.014
Chall[4]	--	--	0.164 ± 0.008	0.192 ± 0.014
Interactions				
Not Chall/ Neg	--	--	0.175 ± 0.011	0.201 ± 0.020
Not Chall/ Pro	--	--	0.161 ± 0.011	0.190 ± 0.020
Chall/ Neg	--	--	0.177 ± 0.011	0.185 ± 0.020
Chall/ Pro	--	--	0.151 ± 0.011	0.199 ± 0.020
Statistical Effects (P-Value)				
Treatment	0.36	0.90	0.09	0.91
Challenge	--	--	0.70	0.91
Treatment * Challenge	--	--	0.55	0.52

[1]Neg = not supplemented with Primalac
[2]Pro = supplemented with Primalac
[3]Not Chall = not challenged
[4]Chall = challenged with *Eimeria*

Table 5.4. Effect of in ovo probiotic treatment and *Eimeria* challenge on relative spleen weight.

Age	DOH	d3	d9	d15
Main Effects				
Dietary Treatment				
Neg[1]	0.034 ± 0.003	0.073 ± 0.007	0.083 ± 0.004	0.070 ± 0.006[b]
Pro[2]	0.041 ± 0.003	0.066 ± 0.007	0.074 ± 0.004	0.091 ± 0.006[a]
***Eimeria* Challenge**				
Not Chall[3]	--	--	0.080 ± 0.004	0.072 ± 0.006
Chall[4]	--	--	0.077 ± 0.004	0.088 ± 0.006
Interactions				
Not Chall/ Neg	--	--	0.078 ± 0.005	0.066 ± 0.008
Not Chall/ Pro	--	--	0.082 ± 0.005	0.078 ± 0.008
Chall/ Neg	--	--	0.087 ± 0.005	0.072 ± 0.008
Chall/ Pro	--	--	0.067 ± 0.005	0.104 ± 0.008
Statistical Effects (P-Value)				
Treatment	0.17	0.45	0.15	0.01
Challenge	--	--	0.55	0.05
Treatment * Challenge	--	--	0.04	0.29

[1]Neg = not supplemented with Primalac
[2]Pro = supplemented with Primalac
[3]Not Chall = not challenged
[4]Chall = challenged with *Eimeria*

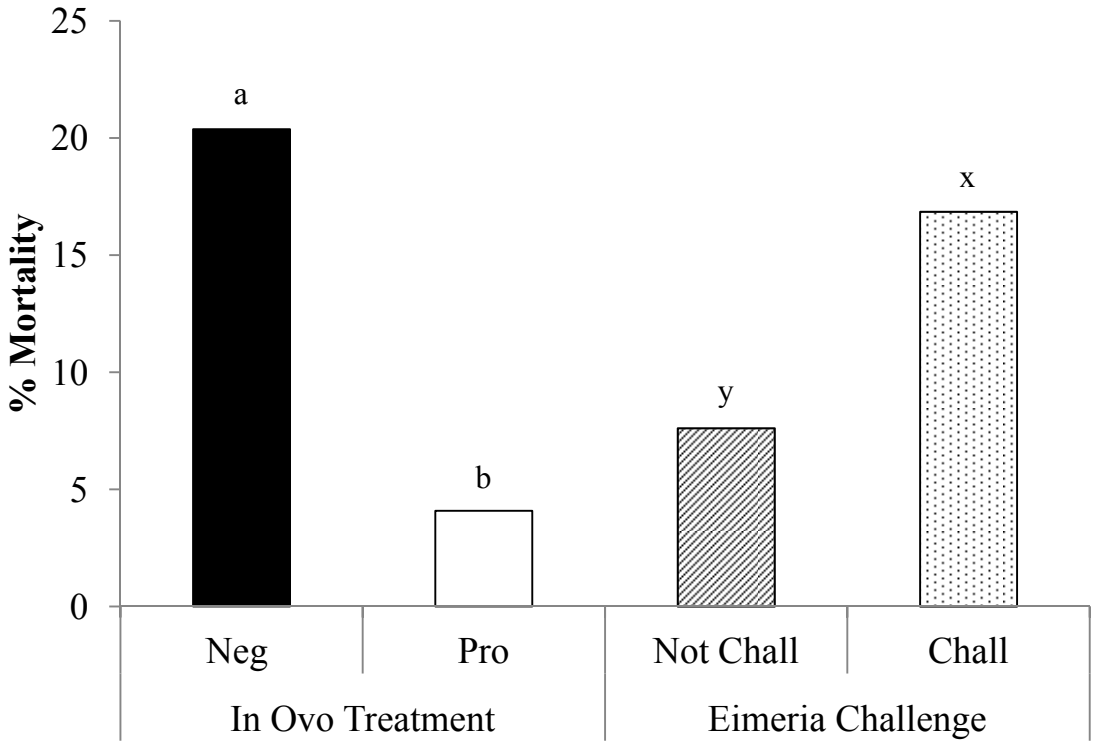

Figure 5.1. Main effects of in ovo probiotic supplementation and *Eimeria* challenge on mortality of Cobb 500 chicks. Neg = not supplemented; Pro = supplemented with Primalac; Not Chall = not challenged; Chall = challenge with *Eimeria*. There was a significant main effect of in ovo treatment ($P < 0.0001$) and *Eimeria* challenge ($P = 0.005$) on mortality.

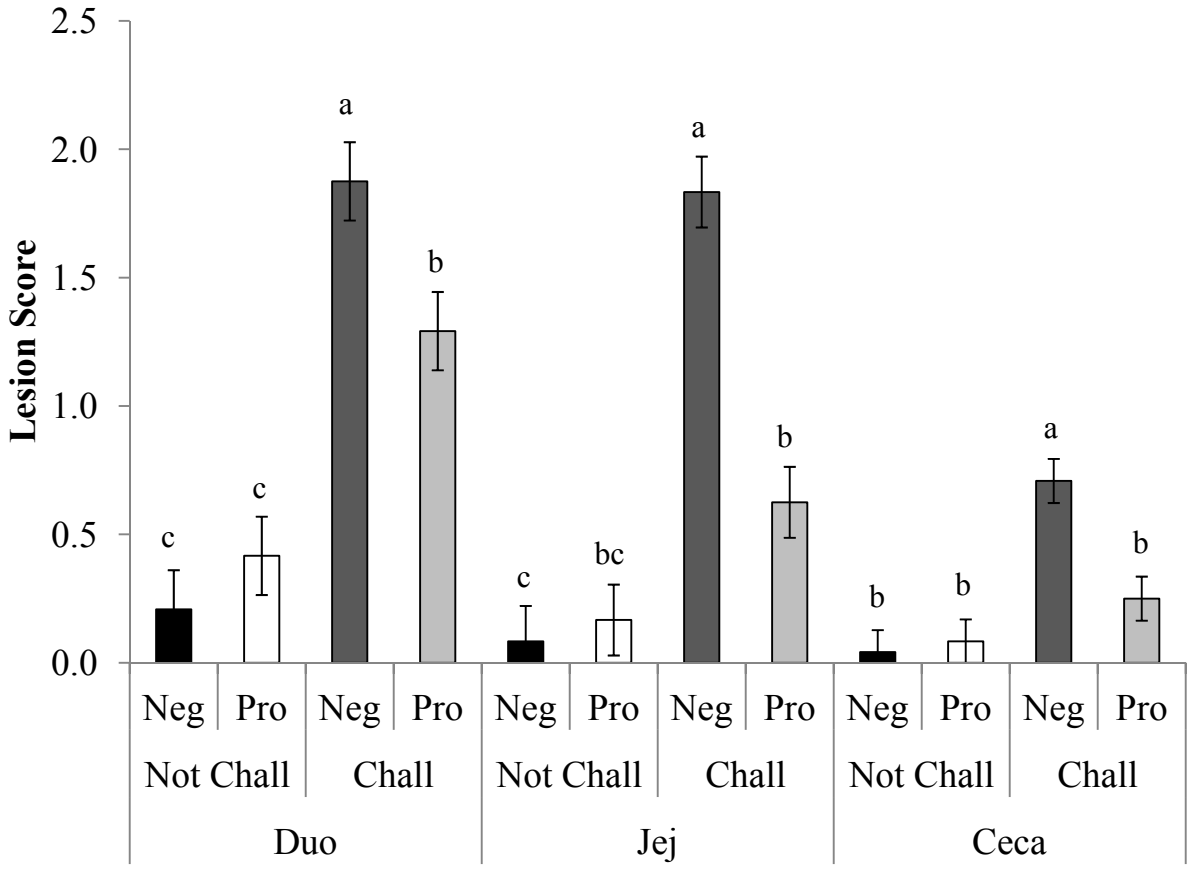

Figure 5.2. Effect of in ovo probiotic supplementation on gross intestinal lesions scores of Cobb 500 chicks. Data are represented as LS Means + SEM. Duo = duodenum; Jej = jejunum; Not Chall = not challenged; Chall = challenge with *Eimeria*; Neg = not supplemented; Pro = supplemented with Primalac. There was a significant two-way interaction of treatment and challenge in the duodenum ($P = 0.01$), jejunum ($P < 0.0001$) and ceca ($P = 0.005$).

Chapter VI

Epilogue

The small intestine is not only a vital component of the digestive system, but also the largest lymphoid organ. 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 presented in this dissertation indicate that in ovo supplementation of the probiotic product Primalac does not have a negative impact on hatchability as was previously found, can enhance performance, and modulate the immune system, specifically gut immune responses.

Probiotics are live microbial supplements that are administered to animals and humans to improve intestinal function by modifying the intestinal 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 immune system. Over the course of these three experiments, we have discovered that Primalac can be safely administered in ovo without affecting hatchability, which has been a cause for concern with other researchers attempting similar studies. Furthermore, our results indicate that in ovo application of Primalac has effects similar to those of dietary supplementation, thus propagating its endorsement as a means to promote early colonization of beneficial bacteria.

The first study conducted, however, did present results that were not expected. The sham inoculated group received an injection of sterile water at embryonic day 18 to act as a control.

Though only water was injected, we observed significant differences in gene expression. The only explanation I can offer is that the injection exposed the embryo to the external environment, leading to early colonization of the gut, or perhaps initiated some sort of stress reaction to such manipulation and exposure even in a clean laboratory setting.

These studies provide great potential for future research. Based on our results, in ovo supplementation of Primalac is capable of improving performance and reducing severity of gross intestinal lesions during an *Eimeria* infection, thus providing evidence that probiotics may be utilized as a tool to help alleviate the negative effects of coccidiosis. Though benefits were observed, the third study was rather small. I would like to see a larger study conducted to further elucidate the extent of potential benefits resulting from in ovo probiotic use. Additionally, it would be interesting to learn how probiotics may modulate the immune system in different challenge scenarios. For example, would in ovo probiotic administration also provide protection against extracellular pathogens such as *E. coli* or *Salmonella* sp.?

There has been much discussion about how probiotics could potentially serve as an alternative to the use of antibiotics and chemotherapeutic agents to promote growth and prevent disease. Though probiotics have, in some cases, been shown to perform similar to antibiotics, a study needs to be conducted to evaluate the credibility of in ovo probiotic usage as an alternative to the sub-therapeutic use of antibiotics.

During these studies, we examined the effects of in ovo and dietary administration of probiotics on immune-related gene expression in the intestine. Though identifying the gene expression patterns of these genes does assist with elucidating the underlying effects of probiotics on the immune system and their mode of action, it would have been advantageous to look at cytokine profiles in the blood. These profiles would assist in determining if the effects on

gene expression were translated into differences in protein levels, but unfortunately those capabilities are not yet available for poultry researchers.

In order to administer probiotics to the embryo, a manual injection technique was used during these experiments. In future studies, I suggest utilizing an automated injection system to help reduce labor, time spent outside the incubator and potential for human error. Furthermore, such application could prove very useful in commercial settings as broilers are routinely vaccinated in ovo, thus minimum labor is involved with little to no additional stress to the embryos. With this system already in place, it would be easy to implement a protocol that includes the addition of probiotics during the vaccination procedure. To do so, however, research needs to be done evaluating the interactive effects of probiotics with different vaccine applications.

Due to time constraints, gene expression analysis and gut microbial profiles for all three studies were not fully analyzed by the time this dissertation was defended and therefore only partial data were included, particularly for the last disease trial. Since probiotics tend to have more pronounced effects in a challenge situation, it would be interesting to see how the gene expression patterns and microbial profiles change especially due to *Eimeria* infection. Furthermore, much of the discussion regarding the underlying causes for the patterns of gene expression observed is speculative. 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.