THE EFFECTS OF PROBIOTIC AND *EIMERIA* ON GUT MORPHOLOGY

AND HUMORAL IMMUNITY IN BROILERS

A Thesis

by

SADIE LYN HORROCKS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2010

Major Subject: Poultry Science

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Approved by:

Chair of Committee,	Morgan Farnell
Committee Members,	James Byrd
	David Caldwell
	Jason Lee
Head of Department,	John Carey

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ABSTRACT

The Effects of Probiotic and *Eimeria* on Gut Morphology and Humoral Immunity

in Broilers.

(December 2010)

Sadie Lyn Dunn Horrocks, B.S., Texas A&M Universtiy

Chair of Advisory Committee: Dr. Morgan B. Farnell

Coccidiosis has a negative economic impact on the commercial poultry industry, and probiotics are beneficial bacteria that aid in maintaining healthy gut microflora. We hypothesized that probiotic administration would positively affect gut morphology and increase IgG secretion during an *Eimeria* challenge, which was evaluated by measuring total chicken IgG and gut morphology (villus height, villus width, villus surface area, crypt depth, villus height to crypt depth ratio and lamina propria thickness).

On day-of-hatch, broilers were placed into floor pens with 50% pine shavings and 50% used litter. The broilers were exposed to *Eimeria* oocysts via the feed on day 14 and challenged on day 36. On days 6, 22, 36, and 43, tissue samples from the intestine were collected for morphological evaluation, and blood samples were taken to quantify chicken IgG from serum. Data were measured using a factorial ANOVA and main effect means were deemed significant at $P \leq 0.05$. In cases where significant interactions were observed, data was subjected to a one-way ANOVA. All means were separated using a Duncan's Multiple Range Test.

On day 6 in the duodenum, a significant interaction was observed regarding vaccination and probiotic administration (Coccivac®-B, Intervet/Schlering-Plough Animal Health/Merck and Co., Inc., Whitehouse Station, NJ). Villus height to crypt depth ratio decreased in ionophore treated birds compared to control birds in the duodenum and lower ileum on day 6, 36, and 43. Villus crypt depth in vaccinated birds decreased in the duodenum after the challenge. On day 43, the ionophore treated birds had less villus height and surface area compared to control and vaccinated birds, while lamina propria thickness increased in the duodenum, and non probiotic birds had longer villi than probiotic birds.

On day 22, vaccinated birds had significantly increased chicken IgG levels compared to the control and ionophore birds, and the non probiotic birds had significantly increased IgG secretion compared to probiotic fed birds. On day 36, the ionophore birds had significantly increased levels of IgG compared to the control birds, which could also support that the ionophore delayed exposure to the parasite.

These results suggest that gut morphology and humoral immunity are affected by probiotic administration, coccidiosis vaccination, ionophore application and *Eimeria* challenge. Both the day 43 morphology results and day 36 chicken IgG results for the ionophore treated birds demonstrates that ionophore administration delays exposure of the avian gut to invasive coccidia. More research is necessary to evaluate how probiotics influence coccidiosis vaccination and humoral immunity, so that probiotics may be used to improve the effectiveness of coccidiosis vaccination and to evaluate if probiotics aid in ameliorating the effects of an *Eimeria* infection.

DEDICATION

My thesis is dedicated to my husband and family. Thank you, Shane, for all of your emotional and spiritual support during school. You have the most amazing ability to maintain perspective and you never let me forget the big picture. I feel blessed that God chose a husband for me that challenges and motivates me to be a better scientist and woman. I would also like to thank my parents for their support and for always believing in my ability to succeed. My parents inspired my love for science and nurtured my desire to learn from an early age and I am so blessed to have grown up in a home and family that valued education. Thank you for always having the highest expectations for me and for loving me unconditionally. I would also like to thank my family for always setting high standards for success, for joyously celebrating my achievements, and for always keeping me humble.

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CHAPTER I

INTRODUCTION

Coccidiosis is an intestinal disease of poultry caused by the protozoan parasite *Eimeria* (Williams, 1998). The disease has been estimated to cost the U.S. poultry industry approximately $3 billion U.S. dollars annually, which is attributed to the cost of in-feed anticoccidial drugs and production losses due to morbidity (decreased feed efficiency and body weight gain) and mortality (Dalloul and Lillehoj, 2006). There are eight species of *Eimeria* known to parasitize chickens: *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. mivati*, *E. necatrix*, *E. praecox*, and *E. tenella* (Conway and McKenzie, 2007). *Eimeria* species are diverse and a challenge exists to control all species with one method (Lillehoj, 1988; Lillehoj et al, 1989). Current *Eimeria* control methods include the use of in-feed anticoccidial drugs, such as ionophores. Anticoccidial drugs have been used in poultry feeds as a means of controlling coccidiosis infections; however, drug resistance to anticoccidials exists throughout the poultry industry and though the mechanisms of drug resistance have been studied, they are not yet fully understood (Jeffers, 1974; McDougald, 1981; Chapman, 1982; Williams, 2006).

Recent research has focused on developing vaccines to provide improved protection from *Eimeria* (Williams, 2002; Mathis and Broussard, 2006; Williams, 2006). Live oocyst vaccination is a viable alternative to anticoccidial drug use, because the vaccines have been shown to stimulate immunity to the parasite early in production,

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This thesis follows the style of Poultry Science.

conferring protection (Williams, 1998). Unfortunately, *Eimeria* vaccines are laborious and costly to produce because immunity to a single species of *Eimeria* does not protect the host against other species, so vaccines must include all species known to parasitize chickens in order to be completely effective (Dalloul and Lillehoj, 2005). As drug resistance to anticoccidial drugs and reluctance to use *Eimeria* vaccines due to concerns for production losses continues, researchers must explore new, economical ways to improve current *Eimeria* control methods.

Probiotics are defined as a live microbial feed supplement that benefits the host by improving the normal flora of the gut (Fuller, 1989). The health of the gut is facilitated by normal bacterial flora, and probiotics have been shown to facilitate their mechanisms (Dalloul and Lillehoj, 2005). Probiotics not only compete with pathogens for nutrients and attachment sites on the intestinal epithelium, but probiotics also secrete antibacterial factors, like volatile fatty acids, that can inhibit the growth and efficacy of pathogens (Nurmi and Rantala, 1973). Probiotic supplementation has been shown to impact *Eimeria* infection by reducing oocyst shedding, increasing body weights, and up regulating secretion of *Eimeria* specific antibodies in broilers infected with *E. acervulina* and *E. tenella* (Dalloul et al., 2003; Lee et al., 2007). However, the effect of probiotics on coccidiosis vaccination in broilers has not been reported.

Probiotics have been shown to improve gut morphology and performance in broilers. Research has shown that probiotics can increase surface area in the intestine available for nutrient absorption, while other research has shown improved feed efficiency, body weight gain and longer villus heights in the ileum with dietary inclusion of probiotics

(Samanya and Yamauchi, 2002; Awad et al., 2009; Eckert et al., 2010). Longer villi indicate increased surface area for nutrient absorption and greater digestive capacity (Yang et al., 2007). Villus height is also a morphological indicator of the integrity of the gut tissue, because longer villi are also indicative of active cell mitosis and enterocyte turnover (Samanya and Yamuchi, 2002). Coccidiosis infection decreases absorption of nutrients in the small intestine when the parasite creates lesions in the wall of the epithelium and causes epithelial cell sloughing; this impairs growth and feed utilization (Dalloul and Lillehoj, 2005). If probiotics can increase digestive capacity in the intestine, perhaps they could counteract some of the production losses that result from reduced gut surface area due to tissue destruction during a coccidiosis infection.

Mountzouris and colleagues (2010) investigated the efficacy of *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus*, and *Pediococcus* by evaluating digestive function, intestinal environment, and broiler health by measuring plasma antibody levels in addition to nutrient utilization, cecal microflora composition and performance parameters. The use of probiotics may modulate the systemic immune system by increasing the total levels of serum IgG in broilers and be indicative of the overall humoral immune status of the bird (Mountzouris et al., 2010). Koenen and colleagues (2002) also explored the effects of probiotics in the systemic humoral immune response, and found that different *Lactobacillus* spp. increase the levels of IgG in laying hens. The role of humoral immunity during coccidiosis, however, has yet to be clearly defined (Dalloul and Lillehoj, 2005).

The objectives of the current research was to compare current coccidiosis control methods, like in-feed ionophore application and live oocyst coccidiosis vaccination, to

probiotics, to examine how each of these treatments behaved when applied together, and to observe how each treatment was affected by a field strain *Eimeria* challenge. The investigators measured the effects of each treatment on the architecture of the gut tissues by evaluating gut morphology, and also measured the effects of each treatment on the humoral status of the bird by quantifying IgG levels in serum.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Coccidiosis is an intestinal disease of poultry caused by the protozoan parasites of the genus *Eimeria*, in the phylum Apicomplexa (Levine, 1982). These parasites are obligate intracellular parasites that are transmitted via the fecal-oral route, and are especially important in commercial broilers due to the intense rearing strategies and environmental conditions in poultry houses (Shirley et al., 2005). High stocking densities, typical of commercial poultry rearing barns, and the warm, moist environment created in poultry litter are conducive to the propagation of *Eimeria* (Williams, 2002). The presence of *Eimeria* is an important economic issue for producers not only because clinical coccidiosis can cause weight loss and mortality, but also because the cost of disease prevention and treatment is high (Shirley et al., 2005). In fact, the cost of coccidiosis to the poultry industry worldwide is estimated to be approximately 3 billion U.S. dollars annually (Dalloul and Lillehoj, 2006). Protecting poultry flocks from coccidiosis depends largely on the development of protective immunity to the resident *Eimeria* species present in a location. Since commercial broiler chickens only live for six weeks before slaughter, it is necessary to develop and explore new, effective methods of control for coccidiosis in order to protect producers from production losses before the birds are able to fully develop protective immunity (Shirley et al., 2005).

***Eimeria* Life Cycle**

The coccidia parasite in birds was first reported by Fantham in 1910 (Chapman, 2003). *Eimeria* spp. has complex life cycles that include three phases: sporogony, merogony, and gametogony (Long, 1982). Depending on species, the endogenous phase in the intestine (which includes merogony and gametogony) consists of multiple stages of asexual reproduction, also called schizogony, which is followed by sexual differentiation, fertilization, and shedding of unsporulated oocysts (Lal et al., 2009).

The exogenous phase (sporogony) occurs in the environment, where excreted oocysts are stable and eventually sporulate to become infective (Lal et al., 2009). The infective oocyst is stable in the environment for several months due to its thick wall, making eradication of the parasite with disinfectant nearly impossible (Shirley, 1993). The oocysts contain a diploid single cell called a sporont, which undergoes a reduction division in the presence of oxygen which allows it to "throw off" its polar body and begin sporogeny (Levine, 1982). Infection begins after the mature oocyst is ingested and excysts in response to conditions in the host (Levine, 1982). In the gizzard, mechanical grinding releases the sporocysts into the lumen. Then, bile and trypsin stimulate the release of the sporozoites from the sporocysts via the operculum into the lumen of the duodenum (Levine, 1982). The sporozoite is the infective stage of the parasite and after release from the sporocysts they move to the base of the intestinal epithelial cells lining the villi, where the sporozoite will use proteolytic enzymes to penetrate the host cell. Sporozoites are first observed in intraepithelial lymphocytes (IELs) and then develop inside epithelial cells

because host IELs have been shown to transport the sporozoites from the villi to the intestinal crypts (Fernando et al., 1987; Trout and Lillehoj, 1996). While in these cells, the sporozoite develops into a rounded body called a first generation trophozoite, then it grows into a first generation schizont, the asexually reproducing stage of the parasite. *Eimeria brunetti* and *E. praecox* undergo the entire endogenous phase (both merogony and gametogony) in these villus enterocytes while other *Eimeria* species develop in enterocytes located in crypts before infecting superficial enterocytes during successive stages of shizogony (Shirley et al., 2005). The first generation schizont divides into many first generation merozoites.

Merogony beings when one sporozoite releases approximately 1,000 first generation merozoites into the gut lumen, a cycle which repeats 2-4 generations depending on species (Yun et al., 2000). This rupture of intestinal epithelial cells creates extensive cell damage and inflammation in the host and is the basis for the pathologic signs of coccidiosis (Yun et al., 2000). Once in the lumen, merozoites penetrate other epithelial cells and develop into second generation trophozoites, which develop into second generation schizonts. The new and numerous schizonts release second generation merozoites which invade new epithelial cells. Each new generation of schizonts results in the production of more merozoites leading to widespread infection.

Gametogony occurs when merozoites develop into either microgamonts or macrogamonts and form a zygote encased by a thick wall that maintains the viability of the oocyst in harsh external environments (Yun et al., 2000). Once outside the host, oocysts remain viable in the environment for long periods of time before being ingested and

starting the life cycle again (Yun et al., 2000). Though gametogony can induce partial immunity, the early endogenous stages are considered the most immunogenic (Shirley et al., 2005). Currently, there are eight species of *Eimeria* that parasitize chickens: *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. mivati*, *E. necatrix*, *E. praecox*, and *E. tenella*; however, each species differs in its pathology and immunogenicity (Chapman, 2000; Conway and McKenzie, 2007). The *Eimeria* life cycle contributes to the complexities of host immunity to the parasite, which involves innate and acquired immune systems (Lillehoj, 1998).

Pathology and Site Specificity of *Eimeria*

Each species of *Eimeria* is characterized based on differences in biology, such as development, infection site specificity and life-cycle stages (Schnitzler and Shirley, 1999; Chapman, 2000; Shirley et al., 2005). Different species of *Eimeria* are site specific in the intestine. McDougald and Reid (1997) reviewed the localization of different *Eimeria* species. Infections of *E. acervulina* are the most common of commercial poultry in the U.S. and localize in the duodenum, as well as infections with *E. praecox* and *E. mivati*, though *E. mivati* can localize in the duodenum and upper ileum. *Eimeria maxima* and *E. necatrix* localize in the upper ileum, but small numbers of *E. necatrix* oocysts can be found in the lower ileum and ceca. Localizing in the lower ileum and large intestine, *E. brunetti* causes bloody enteritis, while E. mitis localizes in the lower ileum and lacks discrete lesions. Infections with *E. tenella* are localized in the ceca and are characterized by severe hemorrhaging and high morbidity in commercial broilers. Furthermore, *Eimeria* spp have been found to disseminate beyond the digestive tract in some cases, where investigators

suspect the parasite was able to reach and infect liver tissue via the biliary system
(Rodriguez et al., 2007).

All *Eimeria* species can contribute to production losses, and though they may not
all cause mortality, the morbidity associated with coccidiosis is relevant to poultry
producers and has a negative economic impact on the industry. Meronts, gamonts, and
oocysts can all induce histological alterations in host cells including distortion,
inflammation, rupture, and intestinal cell sloughing, which contribute to clinical
coccidiosis (Yun et al., 2000). Clinical signs of coccidiosis include diarrhea, dehydration,
malabsorption, rectal prolapse and mortality. These effects are deleterious to production
because they disrupt digestive processes which eventually cause weight loss and poor feed
efficiency (McDougald and Reid, 1997; Allen and Fetterer, 2002).

Each species's immunogenicity and pathogenicity also differ, for example, the
number of oocysts are required to generate an immune response varies between species.
Eimeria maxima is highly immunogenic and only a small number of oocysts are required
to achieve complete immunity, while less pathogenic species, like *E. praecox* or *E. mitis*,
require a higher number of parasites to be present in the environment to generate immunity
(Chapman, 1999; Chapman, 2000). Factors such as intensive rearing practices in broilers
also increase the incidence of more prevalent species, like *Eimeria maxima*, *E. acervulina*
and *E. tenella* (McDougald et al., 1997). In order to establish complete protective
immunity to any species of *Eimeria*, fecal-oral re-infection is critical, but once re-infection
occurs, post infection immunity is long lasting (Chapman and Cherry, 1997; Yun et al.,
2000). The early endogenous phases of the life cycle are considered to be the most

immunogenic and are critical to development of immunity. Rose and Hesketh (1976; 1987) determined that the second generation schizont stage of an *Eimeria maxima* infection was the most associated with inducing protective immunity, followed by *E. brunetti* and *E. praecox*. The ability to manipulate immunity to coccidiosis based on different stages of the life cycle illustrates the importance of understanding immunity to better control the parasite.

Mucosal Immune System

The immune system is the collection of cells, tissues, and molecules that coordinates reactions to mediate infection resistance and antigen elimination (Abbas and Lichtman, 2006). The immune system is separated into two major mechanistic groups: the innate immune system, which mediates the initial protection from infection, and the adaptive immune system, which develops more slowly but is specific and more effective in antigen elimination (Abbas and Lichtman, 2006). Both the innate and adaptive immune systems depend on the activity of leukocytes, and there is cooperation between the leukocytes of each system so they can eliminate pathogens (Janeway et al., 2001; Beutler, 2004).

The body has several secondary lymphoid tissues that serve as sites for leukocyte and pathogen interactions, like the mucosal lymphoid tissues (Abbas and Lichtman, 2006). The mucosal associated lymphoid tissues are comprised of lymphoid tissues located in the nasal passages, bronchial organs, genital tract, and gut associated lymphoid tissue (GALT), that house leukocytes; these leukocytes seek and eliminate pathogens at ports of entry to the host (Yun et al., 2000). The GALT is a multilayered tissue that is continuously

exposed to antigens from food, normal microbial flora, and ingested pathogens. The outer layer of the GALT consists of epithelial cells and lymphocytes above the basement membrane which maximize antigen/host cell exposure, and lamina propria lymphocytes below the basement membrane, which is a mucosal effector site (Mowat and Viney, 1997; Yun et al., 2000). Poultry have specialized aggregates of lymphoid tissue within the GALT like Peyer's patches, cecal tonsils and the bursa of Fabricius that contain effector cells to eliminate pathogens; these tissues and cells are the basis for the development of protective immunity (Yun et al., 2000).

Avian GALT and Immunity to *Eimeria*

Defined lymph nodes are absent in most birds, however birds do have a well defined lymphatic system comprised of nodular aggregates of lymphoid tissue, like the GALT, strategically positioned along lymphoid vessels to drain the skin, gut, and lung to provide an interface between antigens and immune cells. The GALT has evolved special features that reflect its role as the first line of defense on mucosal surfaces which include the presence of antigen presenting cells, immunoregulatory cells, and effector cell types distinct from their counterparts in the systemic immune system (Lillehoj and Trout 1996**;** Lillehoj and Lillehoj, 2000; Yun et al., 2000). In chickens, a variety of specialized lymphoid organs (thymus, Peyer's patches, cecal tonsils, and bursa of Fabricius) and cell types have developed in the GALT to defend against intestinal pathogens like *Eimeria* (Lillehoj and Trout, 1996). The avian GALT serves three functions in host defense against enteric pathogens: processing and presentation of antigens, production of intestinal antibodies, and activation of cell mediated immunity (Dalloul and Lillehoj, 2005). It takes

approximately 3-4 weeks for chickens to acquire immunity to coccidiosis and during that time the infection can extensively impact mortality in a poultry flock (Schnitzler and Shirley, 1999). The immunity acquired during infection with one species of *Eimeria* will not protect hosts against infection with any of the other species, so the immune system is a key mediator in parasite elimination (Williams, 1998; Yun et al., 2000). Both cellular and humoral immune mechanisms are involved in *Eimeria* immunity development, and the avian GALT is largely responsible for initiating these mechanims.

Janeway and colleagues described the maturation of thymus dervided lymphocytes (T cells) as a unidirectional migration of immature T cells that forces interactions between developing T cells and self and non self antigens in order to elicit T cell maturation (2001). Immature T cells migrate to the thymic cortex where the immature T cells first express a complete T Cell Receptor (TCR), which allows the cell to recognize foreign antigens. Poultry have proportionately greater numbers of $\gamma\delta$TCR than other animals, and the greatest numbers of these cells are found within the GALT (Lillehoj and Trout, 1996). Chicken $\gamma\delta$TCR appears in the thymus at 11 days post embryonic development, and they appear in the intestine by days 14-15 (Dunon et al., 1993). Development of TCR is followed by maturation into either CD4 or CD8 positive cells, which will determine their function. For example, CD4 T cells will aid in the activation of macrophages and B cells, while CD8 T cells are responsible for killing infected host cells and also activating macrophages (Abbas and Lichtman, 2006). Then, T cells are exposed to host major histocompatibility complex (MHC) molecules and are either selected or die depending on whether they are able to recognize MHC; T cells unable to recognize self MHC will be

unable to eliminate pathogens (Abbas and Lichtman, 2006). The process ensures that CD4 T cells will recognize MHC class I molecules and that CD8 T cells recognize MHC class II molecules (Abbas and Lichtman, 2006). The T cells that survive the maturation process will be able to successfully recognize foreign antigens, tolerate self antigens, and express surface markers to carry out effector functions. Mature T cells will interact with macrophages and other effector cells to secrete cytokines and pro-inflammatory molecules that direct the appropriate immune responses to antigens (Dalloul and Lillehoj, 2005).

Cell mediated immunity is highly effective against coccidiosis infection and is mediated mostly by intestinal intraepithelial and lamina propria lymphocytes (Yun et al., 2000). Research has demonstrated increased levels of CD4 and CD8 T cells during an *Eimeria* infection, accompanied by T helper cells producing interferon gamma (IFNγ), which is an important part of the avian immune system due to its role in activation of lymphocytes and expression MHC class II (Kaspers et al., 1994; Bessay et al., 1996). Trout and Lillehoj (1995; 1996) also reported the importance of CD8 intraepithelial lymphocytes during coccidiosis.   By using immunoflorescence, the investigators were able to observe these cytotoxic cells directly interacting with host cells infected with *Eimeria* in order to eliminate them. Additionally, the expression levels of gene transcripts encoding for pro-inflammatory cytokines are up regulated in epithelial lymphocytes in the GALT during coccidiosis, demonstrating that T cells are mediating the infection while recruiting the other cells responsible for inducing inflammation to the site of infection (Park et al. 2008; Hong et al., 2006).

The avian bursa of Fabricius is located dorsal to the diverticulum of the proctodeal region of the cloaca. Like T cells, B cells undergo a similar, stringent selection process during their maturation, though they are not MHC restricted. Only about 5% of the B cells in the bursa ever leave, but these selection mechanisms are not as thoroughly understood as the selection mechanisms in the thymus. Mature B cells, when activated, will secrete immunoglobulins (Ig). Immunoglobulin G (IgG) is absent in birds, however, a monomeric Ig protein similar to IgG with an extra carbohydrate domain in the Fc region called IgY is present. Chicken IgG (IgY) is stored in the yolk and is the only source of maternal antibodies for chicks. After initial exposure to commensal bacteria, Peyer's patches in the GALT act as the inductive site of the secretory antibody response (Pickard et al., 2004). Researchers believe that repeated exposure to microflora and, eventually, foreign antigen stimulates the class switch between antibody producing B cells (Kiyono et al., 1985). In the lumens of mucosal organs, secretory IgA (sIgA) can be produced by B cells in the GALT, while systemically chicken IgG and IgM mediate infection (Janeway et al., 2001; Bar-Shira et al., 2002; Abbas and Lichtman, 2006).

Upon exposure to *Eimeria*, chickens produce IgM, IgA, and chicken IgG (Dalloul and Lillehoj, 2005). Chicken IgG is concentrated in the yolk sac of the egg and is transported to the embryo late during development, and these maternal antibodies have been reported to provide some passive immunity to *Eimeria* (Lillehoj, 1987; Lillehoj et al., 2004). Rose and Long (1971) explored the protective effects of transferring immunity to chick embryos. The authors found that antibodies provided protection from *E. tenella* infection only when the "donors" (hens) were actively developing immunity to *E. tenella*

(Rose and Long, 1971). Though the investigators felt that the result of maternal transmission of protection to embryos was inconclusive due to testing methods used at the time, they did note oocyst output reduction in the progeny of immunized hens when chicks were challenged at 4 days of age with *E. tenella* oocysts (Rose and Long, 1971).

In the 1990s, several investigators (Wallach et al., 1992; Smith et al., 1994a; Smith et al., 1994b; Wallach et al., 1995) reported that although maternal immunity to *E. maxima* infections decreased gradually over time, which was show in decreased IgG titers in egg yolk and chick sera, maternal antibodies still play a significant role in protecting the chick at the time of hatch. Parasite specific IgG could be transferred to chicks in the yolk, and protect against infection for the first 2-3 weeks outside of the egg (Rose and Long, 1971; Rose, 1972). Early protection against infection could be beneficial, especially in broilers, because immunizing one breeder hen could provide protection to numerous broiler chicks (Smith et al., 1994b). Also, though maternal immunity is considered almost non-existent by 3 weeks of age, Smith and colleagues (1994b) asserted that these chicks would be protected long enough to prevent severe coccidiosis before slaughter. Investigators also found that oocyst output in hatchlings that were progeny of immunized breeding hens was reduced after a challenge with *E. maxima* oocysts (Smith et al., 1994a; Wallach et al., 1995). Wallach and colleagues (1995) also determined that chicks immunized with affinity purified gametocyte antigens from *Eimeria maxima* were not only immunized to *Eimeria maxima*, but also partially immunized to infections with *E. tenella* and *E. acervulina*. These parasite specific maternal antibodies serve a role in humoral immunity by reducing infectivity as a consequence of parasite neutralization, and the ability to

modulate these antibodies would provide a means of decreasing the invasive potential of

Eimerai (Lillehoj and Lillehoj, 2000).

Secretory IgA in the intestinal lumen can also induce stearic hindrance, reducing

parasite motility by changing the conformation of the parasite's host cell receptor

molecules, and/or inhibit intracellular parasite development (Yun et al., 2000). Bursa cells

begin producing parasite-specific antibodies shortly after infection, and, upon repeat

exposure to *Eimeria*, class switching to Immunoglobulin A (IgA) in effector B cells does

occur under the influence of Interleukin-5 (IL-5) from T helper cells (Lillehoj and Ruff,

1987; Rose and Hesketh 1987). Overall, the ability of antibodies to mitigate coccidiosis is

considered to be minimal, since investigators have reported bursectomized birds remain

resistant to coccidia re-infection (Lillehoj, 1987). Investigators have used immunoglobulin

(chicken IgG, IgA and IgM) levels in serum samples to collect information about the

humoral immune status of chickens (Mountzouris et al., 2010). Instead, chicken IgG can be

an indicator of the overall humoral status of the bird during a coccidiosis infection

(Mountzouris et al., 2010).

Peyer's patches (PP) are nodules of lymphoid tissue within the submucosa in the

ileum of the small intestine. They have a morphologically distinct lymphoepithelium with

follicles, B cell dependent subepithelial zones, and T cell dependent central zones, both

encapsulated in germinal centers, while the overlaying specialized epithelium has

thickened villi, and lack goblet cells (Befus et al., 1980; Burns, 1982). Modified microfold

cells (M cells) nestled within the intestinal epithelium are capable of uptaking antigens

from the lumen by endocytosis. Microfold cells lack a surface glycocalyx and do not

secrete mucus, so they are adapted to interact directly with molecules and particles within the lumen (Janeway et al., 2001). The basal membrane of M cells delivers antigens directly to lymphocytes and DC within the lymphoid compartment of PP, so when antigens are transported through M cells via transcytosis they are immediately directed to antigen presenting cells (APC) expressing MHC II that will activate T and B cells (Premier and Meeusen, 1998). Activated lymphocytes migrate from the PP to the lamina propria and intestinal epithelium where they act out their effector mechanisms.

The cecal tonsils are the largest aggregate of GALT in the chicken (Lillehoj and Lillehoj, 2000). Located at the ileocecal junction, the immune properties of the cecal tonsils develop post-hatch in response to antigenic stimulation (del Cacho et al., 1993). Vervelde and Jeurissen (1995) illustrated that during an *Eimeria tenella* infection, there are increased numbers of leukocytes in the cecal tonsils, but the mechanisms involved are not fully understood. The majority of lymphocytes present in the cecal tonsils are B cells for antibody production, which are predominately chicken IgG cells (Befus et al., 1980; Lillehoj and Trout, 1996). Mountzouris and colleagues demonstrated that measuring chicken IgG production in serum can be a useful tool in determining the overall humoral immune status of a bird from a systemic perspective during an infection, since different antibodies can be localized and produced within the GALT (Bowman et al., 2002; Mountzouris et al., 2010).

Intraepithelial lymphocytes are present in the epithelium and the lamina propria, and consist of mostly CD4 T cells and some IgA B cells (Befus et al., 1980). Intraepithelial lymphocytes have the highest percentage of γδTCR in the GALT, are able to

directly recognize intracellular pathogens and damaged cells (Bandeira et al., 1991; Yun et al., 2000). Janeway and colleagues discussed the $\gamma\delta$TCR population in the gut and explained that these cells do not bind to normal MHC peptide ligands, but instead, they use an activating C-type lectin NK receptor (NKG2D) that binds two MHC-like molecules (MIC-A and MIC-B) that are expressed on epithelial cells in response to cellular injury, infection and stress (2001). This subset of T cells patrols the body to eliminate these.

The lamina propria is an epithelial tissue that constitutes the basement membrane of the gut, and it contains lamina propria lymphocytes that are mostly activated CD8 T cells which lyse infected host cells (Lillehoj and Trout, 1996). The thickness of the lamina propria tissue can relate to pathogen resistance. Tellez and colleagues (1993) noted that *Salmonella enteritidis*-immune lymphokines conferred resistance to *S. enteritidis* infectivity. The investigators associated the increase in the lamina propria tissue thickness with inflammatory cell infiltration, and determined that the infiltration was initiated by mucosal invasion by the pathogen. Avian heterophils, specifically, are equipped to respond rapidly to intracellular invaders, and destroy infected cells. Therefore, the morphology of the lamina propria tissue is vital to facilitating pathogen elimination.

Non-specific factors that can prevent infection include physical barriers, phagocytes and leukocytes, and the complement system. *Eimeria* enters host cells by penetrating mucosal epithelial cells, significantly compromising the physical integrity of the gut (Yun et al., 2000). Yun and colleagues (2000) explained the importance of intestinal epithelial cells during a coccidiosis infection in three parts: 1) epithelial cells absorb nutrients from the digesta; 2) they are the "first line of defense" against ingested

pathogens since they are continuously exposed to environmental antigens; and 3) intestinal epithelial cells constantly experience cell death and regeneration, which means they can act as a selective barrier that can resist and eliminate pathogens. The gut morphology can be indicative of the integrity of the gut during an infection; more specifically, the villi are an indicator of the effectiveness of the digestive and absorptive processes, especially in the highly active small intestine (Aptekmann et al., 2001). Highly mitotic enterocytes replicate in the villus crypts, and as they multiply, enterocytes migrate toward the villus base, pushing other cells apically to the lumen; this creates a continuous supply of new, maturing absorptive cells (Aptekmann et al., 2001). Dunsford and colleagues (1989) showed that reduced villus height can indicate enterocyte destruction, especially during a pathogen invasion. Tellez and colleagues (1993) also examined gut tissues during infection and found that significant increases in lamina propria thickness are not only associated with pathogen resistance, but also the lamina propria tissue is the basement membrane for the intestinal villi, so it serves as structural support for the villi. Better villus integrity can also aid in counteracting the clinical effects of infection, like the weight loss and malabsorption seen during coccidiosis.

In poultry, some adaptive physiological mechanisms exist in the intestine during coccidiosis infection. Ruff and Wilkins (1980) noted an increase in absorptive capacity of the intestine, especially in the uptake of glucose and methionine, early during the recovery phase. Turk (1974) also noted increased weights in all segments of the small intestine during a coccidiosis infection. These physiological changes within the host during parasitism is attributed the host to compensating for decreased surface area in the intestine

(Hoste, 2001). These mechanisms illustrate the importance of intestinal morphology during a coccidiosis infection. If the integrity of the gut tissue can be improved, the intestine will not only be more resistant to pathogen invasion, but also it will increase the performance of the bird. Birds with longer villi and greater surface area have a greater absorptive capacity for nutrients which aids in offsetting the negative effects of coccidiosis (Awad et al., 2009; Yang et al., 2007).

Ionophores

Prophylactic drugs have been used as anticoccidial feed additives for over 50 years and are classified as either chemicals with specific modes of action against parasite metabolism or as polyether ionophores which act by altering ion transport and disrupting osmotic balance (Allen and Fetterer, 2002). Chemical anticoccidials generally control late stages of *Eimeria* development; for example, amprolium, which is chemically similar to thiamine, disrupts parasite development by blocking the transport of thiamine across the cell membrane (Chapman, 1993). Ionophores are compounds that form lipid-soluble complexes with polar cations (K^+, Na^+, Ca^{2+}, Mg^{2+}) of biological importance (Pressman, 1976). Monensin, which was introduced to the United States in 1971, is a carboxylic ionophore that behaves as an "exchange diffusion" carrier of sodium, which may indirectly affect the intracellular concentration of calcium, inducing exocytotic release of secretory products from cells (Pressman, 1976; McDougald, 1990). It was the first ionophore used in chickens and remains one of the most widely used ionophores today (McDougald, 1990; Chapman, 1993; Shirley et al., 2005). During a coccidiosis infection, an ionophore will significantly disrupt ion balance in the sporozoite and cause severe cell damage, but the

trophozoite can also be affected (McDougald, 1990). In 1983, Smith and Galloway examined the mechanism of monensin in extracellular sporozoites during an *E. tenella* infection and concluded that monensin resulted in a significant influx of sodium in to the sporozoite at a rate that exceeded the activity of the sodium potassium pump, leading to sodium accumulation in the cell. The condition would eventually cause cells to swell from passive water influx, and then lyse (Leaf, 1970; Smith and Galloway, 1983).

Drug resistance to anticoccidials exists throughout the poultry industry and though the mechanisms of drug resistance have been studied, they are not yet fully understood (Jeffers, 1974; McDougald, 1981; Chapman, 1982; Williams, 2006). Drug resistance to monensin has been acquired by *Eimeria* isolates dating back to the late 1970's (Chapman, 1982). Chapman conducted several studies in the 70's where field isolates of *E. maxima*, *E. tenella*, and *E. acervulina* from broiler and breeder farms were tested for sensitivity to monensin and found that monensin was significantly less effective against broiler isolates in reducing oocyst output and preventing weight loss (1976; 1979; 1982). Investigators have postulated that *Eimeria* strains resistant to ionophores have sporozoites that exhibit decreased sodium uptake, possibly due to fundamental changes in the biophysical properties in the cell wall of the parasite specific to trans-membrane cation transport (Chapman, 1993). Recently, investigators have explored the efficacy of using live, drug-sensitive anticoccidial vaccines in combination with drugs in order to slow the resistance of local coccidia populations by alternating the two control methods and have found that sensitivity to prophylactics can be increased following administration of a live vaccine (Chapman, 1994; Stephan et al., 1997; Chapman et al., 2002).

Historically, many investigators believed that the use of prophylactic drugs could interfere with immune development to *Eimeria*, and when sulfonamides were first introduced in the 1940s, many studies were conducted to evaluate this relationship, which was reviewed by Chapman (2000). The use of anticoccidial drugs may prevent sufficient numbers of the parasite from generating an immune response in the bird, preventing the bird from acquiring immunity, while vaccines should ensure the development of immunity (Chapman, 2000). Furthermore, immunity development in the presence of anticoccidial drugs has been shown to take up to seven weeks, which is longer than most broilers are raised (Chapman, 1999; Chapman et al., 2004). Because anticoccidial drugs must be withdrawn before slaughter, prevention of immune development creates a problem for producers in that birds may not have developed protective immunity before the anticoccidial is withdrawn, leaving the birds open to infection late in production. The likelihood that new, effective drugs will be introduced in the future is low, so new methods of control must be investigated (Shirley, 1993).

Coccidiosis Vaccination

Recent research has been driven toward developing vaccines to provide a wider array of protection from *Eimeria*, though live vaccines have been available for over 50 years (Shirley et al., 2005. Vaccination is a viable means of controlling coccidiosis because of the strong protective immunity induced by the parasite against future infection by the same species (Yun et al., 2000; Williams, 2002; Mathis and Broussard, 2006; Williams, 2006). Furthermore, live oocyst vaccination in broilers has resulted in performance equal to broilers fed anticoccidial drugs, which could provide an alternative to

drug use since resistance to traditional anticoccidial drugs is now widespread (Lee et al.,

2009). Because *Eimeria* are highly immunogenic in chickens, live vaccines containing

oocysts are the basis of coccidiosis vaccination (Chapman, 2000). Vaccines that are

genetically engineered, irradiated, or made from non-infective parasite derivatives are just

a few examples of different vaccination strategies that have been investigated, but proven

less effective at providing long lasting immunity compared to live oocyst vaccines (Rose

and Hesketh, 1987; Danforth et al., 1989; Shirley, 1989). After the vaccine is

administered, immunity is stimulated by the development of the parasite, and then

"boosted" by re-infection from the vaccine and from the resident *Eimeria* population in the

poultry litter, but for protection to occur, auto re-infection from oocyst exposure is

absolutely necessary (Chapman and Cherry, 1997; Chapman, 2000). During the vaccine

exposure, efficacy depends on the induction of both a humoral and cell mediated immune

response (Yun et al., 2000). Once these occur, immunity to the parasite will be permanent.

Non-attenuated vaccines are comprised of mixtures of wild-type strains of *Eimeria*

where the numbers of oocysts are calculated so that when administered at the correct dose

the vaccine is immunogenic but does not generate the a clinical coccidiosis infection

(Chapman, 2000). Non-attenuated vaccines, like Coccivac®-B, include wild-type strains

of *Eimeria* (*E. acervulina, E. maxima*, *E. mivati*, and E. *tenella*) which immunize against

all species present in the vaccine (Chapman, 2000; Intervet/Schering-Plough Animal

Health/Merck and Co., Inc., Whitehouse Station, NJ). Since immunity to *Eimeria* is

species specific, it is necessary to include all species in a vaccine for complete protection.

There is particular concern with live vaccines, produced with non-attenuated strains, that

the vaccine could introduce species into a poultry flock that are not currently present in the environment, but using limited species in vaccines could counter this problem (Chapman, 2000; Williams, 2002). Historically, coccidiosis vaccines have been used in breeder type birds, however, Coccivac®-B has been tailored for use in broiler chickens to induce immunity earlier since their life span is shorter (Williams, 2002).

Attenuated vaccines are comprised of strains that have been selected for reduced pathogenicity (Chapman, 2000). Called "precocious lines", attenuated strains have reproductive potentials less than their parent strain, making them less pathogenic (Shirley, 1993). Precocious lines of parasites have shortened endogenous cycles where generations of schizogeny are deleted or depleted (Jeffers, 1974). The early stages of the life cycle are the most immunogenic, so they are present in the vaccine, but the deletion of endogenous cycles reduces pathogenicity for the host (Rose and Hesketh, 1976; Rose and Hesketh, 1987). These vaccines are not widely used in the United States.

Coccidiosis vaccines expose the host to low numbers of the parasite to stimulate protective immunity, but even light infections result in production losses (McDoulgald and Reid, 1997). Because vaccination can cause production losses, commercial producers are hesitant to use them on a wide scale, compared to the use of in-feed anticoccidial drugs. Many studies have compared birds receiving vaccination to birds receiving anticoccidial drugs and found that vaccinated birds saw decreased body weight and poor feed conversion (Allen and Fetterer, 2002). Other studies have demonstrated that vaccinated birds performed equally to medicated birds because once immunity was established the birds had a phase of compensatory gain (Lee et al., 2009). Though these studies exist,

continued research is necessary find ways to improve broiler performance during coccidiosis infection.

Probiotics

Oral Tolerance

The introduction of "commensal" or normal bacterial flora to the gastrointestinal (GI) tract is critical to the development of the GALT. Interactions between the gut microflora, the host GI tract, and the associated immune tissues are necessary for the complete and healthy development of the GI system (Dibner et al., 2008). Abundant evidence dating back to the 1950's indicates commensal microflora has a significant impact on the structure and function of the digestive tract (Coates, et al., 1955). Through the study of germ-free animals, Coates and colleagues (1955) demonstrated that animals without commensal microflora had smaller gut sizes, including thinner intestinal villi and a thinner total gut wall, when compared to normally exposed animals. During and immediately after parturition, or post hatch in chickens, exposure to non-pathogenic commensal bacteria occurs, then the gut is exposed to environmental antigens which stimulates the immune cells of the GALT (Pickard et al., 2004). Gut colonization in chicks is rapid (Barrow et al., 1988). Bar-Shira and colleagues (2002) described the events leading to GALT maturation in chickens occurring in this manner: the first wave happens after exposure to environmental antigens together with feed initially activates lymphocytes and natural killer (NK) cells residing mainly in the "intraepithelial lymphocyte (IEL) compartment" of the newly hatched chicks. Then, new T lymphocytes arrive in the lamina propria as it matures. Commensal bacteria continue to play a role in protecting the host,

even after the GALT has developed. The bacteria occupy "niches" in the gut which provides protection from pathogenic bacteria (Janeway et al., 2001). Nurmi and Rantala (1974) first described the theory of competitive exclusion as beneficial bacteria competing with foreign antigens for nutrients and attachment sites in the chicken small intestine. The commensal bacteria also, in some cases, secrete soluble factors capable of inhibiting the growth and development of pathogens in the gut. The benefits of "good bacteria" during a coccidiosis infection have been well documented in poultry, and indicate that the use of probiotics could help ameliorate coccidiosis (Dalloul et al., 2003; Awad, 2009).

Probiotic Administration

Because anticoccidial drugs are becoming less effective and coccidiosis vaccination is not widely used in broilers, investigators have begun exploring microbial supplements in an attempt to influence the host immune system (Dalloul et al., 2003; Lee et al., 2007). Probiotics are defined as live microbial supplements that when fed to an animal can confer a health benefit by improving intestinal microbial balance (Fuller, 1989). The natural bacteria population in the intestine is capable of competitively excluding pathogens, and probiotics have been shown to facilitate these mechanisms (Dalloul and Lillehoj, 2005). The health and maintenance of the digestive tract is facilitated by the normal bacteria microflora present in the digestive tract, along with its gut associated lymphoid tissue, or "GALT" (Dalloul and Lillehoj, 2005). Probiotics not only compete with foreign microbes for nutrients and attachment sites along the intestinal epithelium, but also probiotics secrete soluble factors that can inhibit the growth and efficacy of pathogens (Nurmi and Rantala, 1973).

Probiotics have been used to stimulate the immune system in poultry, and, more recent, to modulate the immune stystem during an *Eimeria* infection (Dalloul et al., 2003; Farnell et al., 2006). Probiotic supplementation has been shown ameliorate *Eimeria* infection by reducing oocyst shedding, increasing body weights, and increased secretion of *Eimeria* specific antibodies in broilers infected with *E. acervulina* and *E. tenella* (Dalloul et al., 2003; Lee et al., 2007). Also, probiotics have been reported to prevent co-infections from opportunistic pathogens normally found in the gut because probiotics help maintain a healthy microbial balance (Dalloul and Lillehoj, 2005). For example, probiotics could be especially helpful during an infection with *E. maxima*, which has been reported to promote necrotic enteritis when a host is suffering a co-infection with *E. maxima* and *Clostridum perfringens* (Park et al., 2008).

Probiotics have also been shown to improve performance in broilers. Eckert and colleagues (2010) administered a probiotic (*Lactobacillus*-based product also containing *Enterococcus*, *Pediococcus* and *Bifidobacterium*) intermittently via drinking water with and without monensin and found that birds not receiving monensin but receiving probiotic had increased growth compared to birds not receiving probiotic. Other research has credited better performance seen in probiotic treated birds to increases in gut surface area, which enlarges the capacity for absorption of nutirents in the intestine. Studies have shown that improved feed efficiency and body weight gain can correspond with increases in villus height and surface area in the small intestine (Samanya and Yamauchi, 2002; Awad et al., 2009). In fact, longer villi not only indicate increased surface area for nutrient absorption, but also can be indicative of overall gut health because longer villi suggest

there is active cell mitosis and significant enterocyte turnover occurring (Samanya and

Yamuchi, 2002; Yang et al., 2007). Coccidiosis negatively effects nutrient absorption in

the small intestine when the parasite creates lesions in the wall of the epithelium and

causes epithelial cell sloughing. The loss of these absorptive enterocytes impairs growth

and feed utilization (Dalloul and Lillehoj, 2006). If probiotics can increase digestive

capacity in the intestine, perhaps they could offset some of the production losses from

inhibited nutrient absorption during a coccidiosis infection.

Dalloul and colleagues (2003; 2005) explored the efficacy of *Lactobacillus*-based

in-feed probiotic on stimulating a local immune response to an infection with *E. acervulina*

in an effort to identify some of the mechanisms involved in pathogen protection. It was

demonstrated that probiotic fed birds had a significant reduction in oocyst shedding

following infection in two studies, indicating the probiotic fed birds were less susceptible

to infection. Also, observed increases in intestinal intraepithelial lymphocytes in probiotic

fed birds were suspected to be the result of probiotic antigens nonspecifically stimulating

the local immune system, which is supported by the well documented activity of T

lymphocytes during a coccidiosis infection (Lillehoj and Trout, 1996). In fact,

investigators noted an increase in CD 4 and CD 8 cells in probiotic fed birds. Probiotics

also significantly up regulated the production of IFN-γ early during the *E. acervulina*

infection, which has been shown to hinder intracellular parasite development (Lillehoj and

Choi, 1998). Chichlowski and colleagues (2007) demonstrated that feeding a

heterogeneous probiotic (*Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium*

thermophilum, and *Enterococcus faecium*) modified the innate intestinal immune response

by decreasing the level of a proinflammatory cytokine (IL 6) and increasing the level of an antiinflammatory cytokines (IL 10) in the intestine. The authors concluded that the probiotics had an antiinflammatory effect on the gut.

Probiotics have also been shown to improve the physical barriers in the gut: intestinal epithelium. Awad and colleagues (2009) investigated the effects of feeding a probiotic on the histomorphology of small intestinal mucosa using a probiotic product with *Lactobacillus* spp. The authors found that probiotic increased the duodenal and ileal villus height and the villus height:crypt depth ratio, which was associated with increased body weights. Sun and colleagues (2005) challenged birds with 3 species of *Eimeria* (*E. maxima*, *E. acervulina*, and *E. tenella*) and concurrently fed a probiotic containing *Lactobacillus*, *Enterococcus*, and *Pediococcus*. Researchers measured lamina propria thickness and found that in probiotic treated birds the lamina propria was thinner compared to control birds, due to a lower pathogen load in the lower intestine; these data also corresponded with improved cumulative body weight gain. Improving the gut architecture has multiple benefits. It not only increases the ability of the chicken to resist pathogen invasion, but also it increase the functional capacity of the gut. Greater functional capacity in the gut, indicated by increased surface area or length in the intestinal villi result in more enterocytes available for nutrient absorption.

Conclusion

Currently, the poultry industry employs in-feed anticoccidial drugs and coccidiosis vaccinations; however, neither control method is without flaw. The literature indicates that drug resistant *Eimeria* are widespread, and that future research and development on

anticoccidial drugs is unlikely. The use of live oocyst vaccines confers protection from

Eimeria, however, vaccines administration results in some production losses for producers

(McDoulgald and Reid, 1997). For a vaccine to fully protect against *Eimeria*, it must

contain multiple species, and still the efficacy of the vaccine is questionable due to

antigenic variations in strains and differences in resident *Eimeria* populations in different

locations. In previous research, probiotics have been shown to be viable means to

improving bird performance during coccidiosis vaccination or challenge. However, the

ability of probiotics at mediating a coccidiosis infection and the interaction between

probiotics and vaccination is still being explored. If probiotics can improve the efficacy of

coccidiosis vaccination or ionophores, probiotics could provide another tool for producers

to combat coccidiosis. Probiotics may improve gut morphology which could not only aid

in pathogen resistance during an infection, but also could counteract the negative effects

caused by *Eimeria* in the gut. Also, research has evaluated how the immunomodulatroy

effects of probiotics locally in the gut, but little research has explored how probiotics affect

the systemic humoral status of the bird during a coccidiosis infection. The goal of the

present research was to investigate how current coccidia control methods, like ionophore

application and vaccination, compare to and are affected by probiotic administration.

CHAPTER III

INFLUENCE OF PROBIOTIC ADMINISTRATION, COCCIDIOSIS

VACCINATION, *EIMERIA* CHALLENGE, OR IONOPHORE

ADMINISTRATION ON GUT MORPHOLOGY IN BROILERS

Introduction

Coccidiosis is an intestinal disease of poultry caused by the protozoan

parasite *Eimeria* (Williams, 1998). The disease has a severe economic impact on the U.S.

poultry industry (approximately $3 billion dollars annually) not only because of the cost of

disease prevention and control (in-feed anticoccidial drugs and vaccinations), but also

because of production losses from morbidity and mortality (Dalloul and Lillehoj, 2005).

Eight species of *Eimeria* parasitize chickens, including: *Eimeria acervulina*, *E. brunetti*, *E.*

maxima, *E. mitis*, *E. mivati*, *E. necatrix*, *E. praecox*, and *E. tenella* (Conway and

McKenzie, 2007). Each *Eimeria* species differs biologically, so controlling all *Eimeria*

species with one control method is challenging and not always effective (Lillehoj, 1988;

Lillehoj et al., 1989).

Current *Eimeria* control methods include the use of different in-feed anticoccidial

drugs, including ionophores. Ionophores have been used in poultry feeds as a means of

controlling coccidiosis infections for decades; however, drug resistance to ionophores in

resident *Eimeria* populations is well documented (Jeffers, 1974; McDougald, 1981;

Chapman, 1982; Williams, 2006). Recent research has focused on developing vaccines to

provide more protection from *Eimeria* (Williams, 2002; Mathis and Broussard, 2006;

Williams, 2006). Unfortunately, *Eimeria* vaccine administration results in some

production losses, so poultry producers are hesitant to use them (McDoulgald and Reid, 1997). As drug resistance becomes more widespread and reports of vaccine negatively affecting performance continue, researchers must explore new, economical ways to improve current *Eimeria* control methods for poultry producers.

The natural bacterial population in the intestine is capable of competitively excluding pathogenic organisms, and probiotics have been shown to facilitate these mechanisms (Dalloul and Lillehoj, 2005). A healthy digestive tract is maintained by the normal bacteria microflora present in the digestive tract, along with its gut associated lymphoid tissue (GALT) (Dalloul and Lillehoj, 2005). Probiotics aid natural flora by competing with pathogens for nutrients and attachment sites along the gut epithelium, and by secreting soluble factors that can inhibit the growth of pathogens (Nurmi and Rantala, 1973). Probiotic supplementation has been shown to impact *Eimeria* infection by reducing oocyst shedding, increasing body weights, and increased secretion of *Eimeria* specific antibodies in broilers infected with *E. acervulina* and E. *tenella* (Dalloul et al., 2003; Lee et al., 2007). Probiotics have also been shown to competitively exclude opportunistic pathogens known to co-infect chickens during *Eimeria* infections, like *Clostridium perfringens* (Park et al., 2008). However, the interaction between probiotics and coccidiosis vaccination or ionophore application is still being investigated.

Probiotics have also been shown to improve performance in broilers. Eckert and colleagues (2010) administered a probiotic (*Lactobacillus*-based product also containing *Enterococcus*, *Pediococcus* and *Bifidobacterium*) intermittently via drinking water and found that receiving probiotic had increased growth compared to birds not receiving

probiotic. Other research has shown that improved feed efficiency and body weight gain can correspond with increases in villus height and surface area in the small intestine, which is due to increased digestive and absorptive capacity in the gut (Samanya and Yamauchi, 2002; Awad et al., 2009). Longer villi indicate increased surface area for nutrient absorption and can also be indicative of overall gut health because longer villi signify there is active cell mitosis occurring (Samanya and Yamuchi, 2002; Yang et al., 2007). Increased lamina propria thickness indicates an influx of immune cells to the intestinal mucosa, which previous research has associated with increased pathogen resistance (Tellez et al., 1993). Crypt depth and villus height to crypt depth ratio are both associated with enterocyte regeneration and proliferation, as well as the functional capacity of the villi (Hampson, 1986; Solis de los Santos et al., 2005; Yang et al., 2007). Coccidiosis inhibits nutrient absorption in the small intestine because the parasite creates lesions in the wall of the epithelium and causes epithelial cell sloughing; subsequently impairing feed utilization and growth (Dalloul and Lillehoj, 2005). Probiotics have the potential to benefit gut morphology, which could aid in resisting pathogens, *Eimeria* as well as opportunistic pathogens, and increase the absorptive capacity of the intestinal villi. Increasing the digestive and absorptive capacity of the small intestine during a coccidiosis infection could help offset malabsorption and weight loss caused by the parasite, which would benefit the poultry producer. We hypothesized that probiotics would benefit gut morphology by increasing surface area in the intestine. Our objective was to evaluate the effects of administering probiotics compared to and when administered with coccidiosis vaccination and ionophore treatement by measuring changes in gut morphology (villus height, villus

width, villus surface area, crypt depth, villus height to crypt depth ratio and lamina propria thickness).

Materials and Methods

Birds and Experimental Design

On day-of-hatch, Cobb 500 male chickens were obtained from a local commercial hatchery, placed into floor pens with 50% fresh pine shavings and 50% built up litter, and provided supplemental heat to simulate industry rearing conditions. The birds were provided water via a nipple drinker system, which also dispensed the probiotic, and were fed a diet shown to enhance performance of birds administered a coccidiosis vaccination using the following feeding program: starter phase (D1-15), grower phase (D15-29), finisher phase (D29-32) and withdrawal phase (D33-42) (Lee et al., 2009). The ionophore and challenge (below) were administered via the feed. The experiment was conducted in a broiler rearing facility at the Texas A&M University Poultry Science Teaching, Research, and Extension Center, and animal care and husbandry were provided according to an approved Texas A&M Institutional Animal Care and Use protocol.

The experimental design used a 3x2 factorial ANOVA design with 3 coccidia control methods (control, ionophore and coccidiosis vaccination) and 2 probiotic groups (probiotic and no probiotic) to determine how the administration of probiotic and coccidia control methods affect avian gut morphology. Individual treatment groups were negative control, probiotic only, vaccination only, vaccination with probiotic, ionophore only, and ionophore with probiotic.

Eimeria Challenge

Field strain *Eimeria* oocysts derived from a local broiler production facility was used to challenge the birds. Species present included: *E. acervulina*, *E. mivati*, *E. maxima*, and *E. tenella*. Feed challenges were administered on day 14 (50,000 oocysts per bird) and on day 36 (750,000 oocysts per bird).

Probiotic Administration

A commercially available probiotic (Poultry Star[®], Biomin, GmbH, Herzogenburg, Austria) was administered at a concentration in accordance with the manufacturer's recommendations (20 grams per 1,000 broilers) to the appropriate treatment pens in drinking water by utilizing an independent watering system. Species present in the probiotic were *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, and *Lactobacillus reuteri*. Probiotic was administered intermittently from day of placement through day 2, from days 9-10, 13-15, 25-27, and 32-34, which correspond with each feed change.

Ionophore Administration

The ionophore monensin (Coban-90, Elanco Animal Health, Indianapolis, IN) was added to the feed per manufacturer's recommendations (90 grams per ton) according to the treatment group. On day 33 the ionophore was removed from the feed to create a withdrawal ration to simulate industry methods.

Vaccination

Vaccinated birds received Coccivac®-B (Intervet/Schering-Plough Animal Health/Merck and Co., Inc., Whitehouse Station, NJ), a non-attenuated live oocyst

coccidiosis vaccine for use in broiler chickens. The vaccine was applied using a Spraycox® II cabinet (Intervet/Schering-Plough Animal Health/Merck and Co., Inc., Whitehouse Station, NJ). Before placement, the birds were allowed to preen for an hour under bright light.

Histological Sampling

On days 6, 22, 36, and 43, a 2-3 cM sample was excised from the midpoint of the duodenum and lower ileum from each broiler and flushed with ice cold saline. The samples were stored in 50 mL of 10% neutral buffered formalin in a plastic tissue sample container. The samples were prepared for morphological evaluation by cutting approximately 2-3 mM of each sample with a razor blade and placing the tissue in a tissue cassette for future tissue embedding. The sample cassettes were labeled per section of intestine, day and treatment group, then stored in 10% neutral buffered formalin and shipped to a commercial service laboratory (Histo-Scientific Research Laboratory, Mount Jackson, VA) for tissue embedding, slide fixing and hemotoxylin and eosin staining.

Morphological Measurements

Sample slides were scanned into the Adobe Photoshop CS4 Extended program (Adobe, San Jose, CA) using an Epson Perfection 4990 Photo (Epson America, Inc., Long Beach, CA) scanner at 4800 dpi (pixels per inch) on a fixed scale. In Photoshop, the measurement tool was used to measure the number of pixels to determine the height and width of the villi, the depths of the crypts and the thickness of the lamina propria tissue. The pixels were converted to millimeters (mM) using the dpi from the original scan. The measurements from Adobe were entered into Microsoft Excel (Microsoft Office

Proffessional 2007, Redmond, WA) and a formula was used to calculate surface area (mM^2) and villus height to crypt depth ratio.

Each section on a slide represented 1 bird, and 5 measurements were recorded per bird in each group. The replicate measurements for each bird were averaged to yield a mean villus height and width (used to calculate mean surface area), mean crypt depth, (used to calculate mean villus height to crypt depth ratio) and lamina propria thickness for each bird. Villus height was measured from the top of the villus to the top of the lamina propria (Solis de los Santos et al., 2005). Villus width was measured at the base of the villus (Solis de los Santos et al., 2005). Surface area was calculated using the formula: $(2\Pi)*$(villus width/2)*(villus height) (Sakamoto et al., 2000; Solis de los Santos et al., 2005). Crypt depth was measured from the base of the villus upward to the region of transition between the crypt and villus. The crypt is defined as the depth of invagination between adjacent villi. Villus height to crypt depth ratio was calculated by dividing each bird's villus height by its crypt depth (Aptekmann et al., 2001; Solis de los Santos et al., 2005; Awad et al., 2009). Lamina propria thickness was measured from the basement membrane of the epithelium to the muscularis mucosa (Sun et al., 2005).

Statistical Analysis

Data were measured using a factorial ANOVA and main effect means were deemed significant at $P \leq 0.05$. In cases where significant interactions were observed, data was subjected to a one-way ANOVA. All means were separated using a Duncan's Multiple Range Test. Statistical analysis was completed with the SPSS statistical software package (Chicago, IL).

Results

Duodenum

On day 6, no significant differences were observed in intestinal crypt depth (Table 3-1). The main effect means in villus height to crypt depth ratio were significantly different. Control and vaccinated birds had a significantly higher ratio compared to medicated birds, and there was no significant difference in probiotic and non probiotic birds (Table 3-2). There were significant interactions observed in the duodenum in villus height, width, surface area and lamina propria thickness (Table 3-3, 3-4, 3-5, and 3-6). The vaccinated only group had significantly longer villi, while the vaccine with probiotic group had significantly shorter villi compared to the control and probiotic only group (Table 3-3). The ionophore only and ionophore with probiotic groups also had significantly shorter villi compared to the control and probiotic only group. The vaccinated only, probiotic only and control birds had significantly wider villi and greater surface area than the vaccine with probiotic, ionophore only, and ionophore with probiotic birds (Table 3-4 and 3-5). The probiotic only and vaccinated only birds had the thickest lamina propria (Table 3-6). Control birds had signigicantly thicker lamina propria than the ionophore with probiotic birds, but not the vaccine with probiotic or medicated only birds.

On day 22, there were no significant differences in main effect means in villus height to crypt depth ratio. Vaccinated and medicated birds had significantly deeper crypts compared to control birds, while there was no difference in probiotic versus non probiotic birds (Table 3-1). Vaccinated birds had significantly thicker lamina propria compared to control and medicated birds, and there was no difference in probiotic versus non probiotic

Table 3-1. Villus crypt depth (mM) in the duodenum and lower ileum for all experimental groups on each sample collection day.

Treatment		Duodenum				Lower Ileum			
Control Measure	**Probiotic**	**Day 6**	**Day 22**	**Day 36**	**Day 43**	**Day 6**	**Day 22**	**Day 36**	**Day 43**
Control	Control	0.10	0.16	0.28	0.44[a]	0.06	0.16	0.19[a]	0.22
Control	Probiotic	0.10	0.17	0.29	0.35[b]	0.06	0.15	0.17[bc]	0.24
Vaccine	Control	0.11	0.19	0.30	0.31[bc]	0.08	0.17	0.15[c]	0.23
Vaccine	Probiotic	0.09	0.20	0.30	0.32[bc]	0.07	0.16	0.19[ab]	0.23
Ionophore	Control	0.09	0.20	0.28	0.26[c]	0.07	0.18	0.21[a]	0.23
Ionophore	Probiotic	0.10	0.21	0.30	0.34[b]	0.07	0.17	0.20[a]	0.25
Main Effects									
Control		0.10	0.16[b]	0.29	0.40	0.06[c]	0.15[c]	0.18	0.23
Vaccine		0.10	0.19[a]	0.30	0.31	0.07[a]	0.16[ab]	0.18	0.23
Ionophore		0.09	0.20[a]	0.29	0.29	0.07[b]	0.17[a]	0.21	0.24
	Probiotic	0.10	0.19	0.30	0.34	0.07	0.16	0.19	0.24
	Non Probiotic	0.10	0.18	0.29	0.34	0.07	0.17	0.19	0.23

[a-c] Means of individual experimental groups with different subscripts differ significantly at $P \leq 0.05$
[a-c] Means of main effects with different subscripts differ significantly at $P \leq 0.05$

Table 3-2. Villus height to crypt depth ratio in the duodenum and lower ileum for all experimental groups on each sample collection day.

Treatment		Duodenum				Lower Ileum			
Control Measure	Probiotic	Day 6	Day 22	Day 36	Day 43	Day 6	Day 22	Day 36	Day 43
Control	Control	9.52	12.73	9.16	5.59	7.52	5.54	5.24	5.03
Control	Probiotic	11.29	11.41	8.28	5.93	8.22	6.32	5.84	4.34
Vaccine	Control	10.55	9.91	7.77	7.05	5.75	4.97	5.67	4.52
Vaccine	Probiotic	10.54	10.97	7.99	7.48	6.07	5.48	4.64	5.15
Ionophore	Control	8.09	12.04	7.32	7.79	6.56	5.16	4.90	4.01
Ionophore	Probiotic	7.49	11.53	6.82	5.09	5.92	5.48	4.71	3.53
Main Effects									
Control		10.22^a	12.07	8.72^a	5.75^b	7.85^a	5.91	5.54	4.68^a
Vaccine		10.54^a	10.44	7.87^{ab}	7.27^a	5.91^b	5.24	5.19	4.81^a
Ionophore		7.73^b	11.75	7.07^b	6.74^{ab}	6.23^b	5.31	4.81	3.80^b
	Probiotic	9.24	11.30	7.68	6.32	6.68	5.76	5.10	4.34
	Non Probiotic	9.40	11.52	8.08	6.80	6.63	5.25	5.27	4.52

[a,b] Means of main effects with different subscripts differ significantly at $P \leq 0.05$

Table 3-3. Villus height (mM) in the duodenum and lower ileum for all experimental

groups on each sample collection day.

Treatment		Duodenum				Lower Ileum			
Control Measure	Probiotic	Day 6	Day 22	Day 36	Day 43	Day 6	Day 22	Day 36	Day 43
Control	Control	0.93[b]	2.03[bc]	2.48[a]	2.40	0.47	0.86	1.03	1.09
Control	Probiotic	1.08[ab]	1.84[bc]	2.39[a]	2.11	0.52	0.96	0.97	1.03
Vaccine	Control	1.17[a]	1.81[c]	2.26[ab]	2.13	0.44	0.82	0.89	1.02
Vaccine	Probiotic	0.73[c]	2.12[ab]	2.36[a]	2.35	0.44	0.89	0.87	1.09
Ionophore	Control	0.70[c]	2.36[a]	2.03[b]	1.93	0.44	0.94	1.03	0.94
Ionophore	Probiotic	0.70[c]	2.39[a]	2.00[b]	1.77	0.42	0.93	0.95	0.86
Main Effects									
Control		0.99	1.94	2.44	2.25[a]	0.48[a]	0.91	.99[a]	1.06[a]
Vaccine		1.01	1.97	2.32	2.24[a]	0.44[ab]	0.86	.99[a]	1.04[a]
Ionophore		0.71	2.38	2.02	1.86[b]	0.42[b]	0.93	.87[b]	.89[b]
	Probiotic	0.82	2.12	2.25	2.10	0.46	0.93	0.93	0.98
	Non Probiotic	0.93	2.04	2.26	2.15	0.45	0.88	0.98	1.01

[a-c] Means of individual experimental groups with different subscripts differ significantly at $P \le 0.05$

[a,b] Means of main effects with different subscripts differ significantly at $P \le 0.05$

Table 3-4. Villus width (mM) in the duodenum and lower ileum for all experimental

groups on each sample collection day.

Treatment		Duodenum				Lower Ileum			
Control Measure	**Probiotic**	**Day 6**	**Day 22**	**Day 36**	**Day 43**	**Day 6**	**Day 22**	**Day 36**	**Day 43**
Control	Control	0.13^a	0.27^a	0.35	0.44	0.16	0.29	0.23	0.26
Control	Probiotic	0.14^a	0.26^a	0.36	0.45	0.16	0.29	0.27	0.26
Vaccine	Control	0.13^a	0.25^{ab}	0.35	0.35	0.13	0.25	0.26	0.23
Vaccine	Probiotic	0.10^b	0.26^a	0.39	0.36	0.17	0.22	0.24	0.27
Ionophore	Control	0.09^b	0.26^a	0.36	0.29	0.14	0.19	0.29	0.24
Ionophore	Probiotic	0.10^b	0.21^b	0.37	0.41	0.17	0.19	0.26	0.24
Main Effects									
Control		0.14	0.27	0.35	0.44^a	0.16	0.29^a	0.25	0.26
Vaccine		0.13	0.26	0.37	0.35^b	0.15	0.23^b	0.25	0.25
Ionophore		0.10	0.24	0.36	0.34^b	0.15	0.19^c	0.27	0.24
	Probiotic	0.12	0.25	0.37	0.41	0.17	0.23	0.26	0.26
	Non Probiotic	0.12	0.26	0.35	0.36	0.14	0.25	0.26	0.24

[a,b] Means of individual experimental groups with different subscripts differ significantly at $P \leq 0.05$
[a-c] Means of main effects with different subscripts differ significantly at $P \leq 0.05$

Table 3-5. Villus surface area (mM^2) in the duodenum and lower ileum for all experimental groups on each sample collection day.

Treatment		Duodenum				Lower Ileum			
Control Measure	Probiotic	Day 6	Day 22	Day 36	Day 43	Day 6	Day 22	Day 36	Day 43
Control	Control	0.41^a	1.76^{ab}	2.72	3.32	0.24	0.80	0.75	0.90
Control	Probiotic	0.48^a	1.53^b	2.67	3.01	0.26	0.88	0.80	0.86
Vaccine	Control	0.45^a	1.43^b	2.49	2.33	0.19	0.65	0.72	0.75
Vaccine	Probiotic	0.24^b	1.75^{ab}	2.92	2.54	0.24	0.62	0.64	0.88
Ionophore	Control	0.21^b	1.96^a	2.32	1.81	0.18	0.56	0.93	0.70
Ionophore	Probiotic	0.23^b	1.64^{ab}	2.35	2.32	0.22	0.53	0.78	0.65
Main Effects									
Control		0.44	1.65	2.70	3.17^a	0.24^a	0.83^a	0.77^a	0.87^a
Vaccine		0.37	1.60	2.70	2.43^b	0.21^{ab}	0.63^b	0.68^b	0.81^a
Ionophore		0.22	1.79	2.34	2.02^b	0.20^b	0.54^b	0.67^b	0.67^b
	Probiotic	0.31	1.64	2.65	2.63	0.24	0.68	0.74	0.80
	Non Probiotic	0.35	1.70	2.51	2.49	0.20	0.67	0.80	0.79

[a,b] Means of individual experimental groups with different subscripts differ significantly at $P \leq 0.05$
[a,b] Means of main effects with different subscripts differ significantly at $P \leq 0.05$

Table 3-6. Lamina propria thickness (mM) in the duodenum and lower ileum for all experimental groups on each sample collection day.

Treatment		Duodenum				Lower Ileum			
Control Measure	**Probiotic**	**Day 6**	**Day 22**	**Day 36**	**Day 43**	**Day 6**	**Day 22**	**Day 36**	**Day 43**
Control	Control	0.13^b	0.28	0.35	0.33	0.15	0.23	0.23	0.27
Control	Probiotic	0.17^a	0.24	0.34	0.39	0.15	0.22	0.25	0.27
Vaccine	Control	0.18^a	0.33	0.31	0.35	0.16	0.23	0.22	0.23
Vaccine	Probiotic	0.12^{bc}	0.33	0.32	0.35	0.16	0.21	0.22	0.27
Ionophore	Control	0.12^{bc}	0.27	0.38	0.38	0.17	0.23	0.25	0.23
Ionophore	Probiotic	0.10^c	0.23	0.36	0.36	0.16	0.25	0.23	0.25
Main Effects									
Control		0.15	0.26^b	0.34^{ab}	0.36	0.14^b	0.22	0.23^{ab}	0.26^a
Vaccine		0.16	0.32^a	0.31^b	0.35	0.16^{ab}	0.22	0.21^b	0.24^{ab}
Ionophore		0.12	0.25^b	0.37^a	0.37	0.16^a	0.24	0.24^a	0.24^b
	Probiotic	0.14	0.27	0.34	0.37	0.16	0.22	0.23	0.26^a
	Non Probiotic	0.15	0.29	0.35	0.36	0.16	0.23	0.23	0.24^b

[a-c] Means of individual experimental groups with different subscripts differ significantly at $P \leq 0.05$
[a,b] Means of main effects with different subscripts differ significantly at $P \leq 0.05$

birds (Table 3-6). There were significant interactions observed in villus height, width and surface area. The ionophore only and ionophore with probiotic birds had significantly longer villi than the vaccinated only, control and probiotic only birds (Table 3-3). The vaccine with probiotic birds had significantly longer villi than the vaccinated only birds. The ionophore with probiotic group had significantly thinner villi compared to all other groups except the vaccinated only group, which was not significantly different from any group (Table 3-4). Medicated only birds had significantly greater surface area compared to the vaccinated only and probiotic only birds (Table 3-5).

On day 36, significant interactions were only observed in villus height, and there were no significant differences observed in main effect means in villus width, surface area, or crypt depth. Control birds had significantly increased villus height to crypt depth ratio compared to ionophore treated birds and there was no difference in probiotic administration (Table 3-2). Ionophore treated birds had significantly thicker lamina propria compared to vaccinated birds and there was no difference in probiotic administration (Table 3-6). There was a significant interaction between probiotic administration and control method in regard to villus height (Table 3-3). The vaccine with probiotic, probiotic only, and control groups had significantly longer villi compared to the medicated only and ionophore with probiotic group.

On day 43, there were no significant differences in lamina propria thickness. Ionophore treated birds had significantly shorter villi compared to the vaccinated and control birds (Table 3-3). The control birds had significantly wider villi, greater surface area, and deeper crypts than vaccinated and medicated birds, while vaccinated birds had a significantly higher villus height to crypt depth ratio than control birds (Table 3-4, 3-5, and 3-1). No differences were observed with regard to probiotic administration.

Lower Ileum

On day 6, control birds had significantly longer villi, greater surface area, and higher ratio than medicated birds, but not vaccinated birds (Table 3-3, 3-5, and 3-2). There were no significant differences in villus width. Vaccinated birds had significantly deeper crypts compared to the ionophore treated and control birds, while control birds had the shallowest crypts (Table 3-1). The ionophore fed birds had significantly thicker lamina

propria compared to the control birds, while vaccinated birds were intermediate (Table 3-6). There were no observed differences in probiotic administration for any measurement.

On day 22, significant differences were observed in main effect means in villus width, surface area, and crypt depth. Control birds had significantly wider villi than ionophore and vaccinated birds, and ionophore birds had significantly thinner villi than vaccinated and control birds (Table 3-4). Control birds had significantly greater surface area compared to vaccinated and ionophore treated birds (Table 3-5). Ionophore birds had significantly deeper crypts compared to control birds (Table 3-4). There were no significant differences in probiotic administration for any measurement.

On day 36, there was a significant interaction in crypt depth, and significant differences in main effect means in villus height and surface area. Control and vaccinated birds had significantly longer villi compared to ionophore birds (Table 3-3). Ionophore treated birds had significantly greater surface area compared to vaccinated birds, and significantly increased lamina propria thickness compared to vaccinated birds (Table 3-5 and 3-6). There were no observed differences in probiotic administration. There was an observed interaction between the control measure and probiotic administration, with the probiotic group having significantly deeper crypts than the vaccinated only group (Table 3-1). The control, ionophore, and ionophore with probiotic group had significantly deeper crypts than the probiotic only, vaccine only and vaccine with probiotic groups.

On day 43, differences in main effect means were observed in villus height, surface area, ratio, and lamina propria thickness. Control and vaccinated birds had significantly longer villi, greater surface area, and deeper crypts compared to ionophore treated birds (Table 3-

3, 3-5, and 3-1). Control birds also had significantly thicker lamina propria compared to ionophore treated birds (Table 3-6). Non probiotic birds had significantly longer villi compared to probiotic birds (Table 3-3).

Discussion

Vaccine and Probiotic Interactions

The addition of probiotics to the vaccine caused several significant interactions in the duodenum on days 6 and 22, which corresponded to timepoints before and after the first feed challenge on day 14. On day 6, the vaccine with probiotic group had significantly decreased villus height, villus width, villus surface area, and lamina propria thickness compared to the vaccinated only birds. Probiotics adhere to the intestinal mucosa, preventing pathogens from binding to epithelial receptors in the intestine and limiting exposure (Koenan et al., 2002). These data suggest that the probiotic, when added to the vaccine, may have prevented cell damage caused by the invasion of *Eimeria*; however, the probiotic may not have been able to prevent the destruction of all the enterocytes, which could explain why there was some decreases in villus integrity. In one study, Dalloul and colleagues found (2005) that during an *Eimeria* challenge splenic lymphocytes from non probiotic birds secreted more IFNγ when compared to probiotic fed birds and concluded that the probiotic may enhance the mucosal immune response and provide better local protection from infection when compared to non probiotic fed birds. These data may suggest that probiotics given with a coccidiosis vaccination delays the onset of activity by systemic immune cells. Mayer (1997) reported that the mucosal immune system can have an immunosuppressive effect on the systemic immune system,

though the mechanism is not fully understood. Since probiotics have been shown to modulate the mucosal immune system, they could contribute to the suppression of the systemic immune system. When comparing vaccinated birds to ionophore treated birds on day 6, vaccinated birds had a significantly higher villus height to crypt depth ratio, though there was no difference in probiotic administration. Increased villus height to crypt depth ratios have been associated with improved performance, while shallow crypts can be an indicator of low enterocyte turnover; therefore, these data could suggest that the vaccinated birds had less cell damage resulting from parasite infection, and did not need to compensate for decreased numbers of enterocytes (Yang et al., 2007; Awad et al., 2009). This result does not indicate a decrease in performance, since in the vaccinated only group, measurements of villus height, villus width and villus surface area were still significantly better than both ionophore treated groups, and comparable to the control birds. These birds should have had increased functional capacity of their villi for absorption of nutrients from the lumen (Aptekmann et al., 2001). In the lower ileum, probiotic administration had no effect on intestinal morphology on day 6, and morphology in vaccinated birds was not significantly different from control birds except in crypt depth and ratio. Vaccinated birds had significantly deeper crypts and lower villus height to crypt depth ratio, which indicates that there could have been increased enterocyte proliferation and generation, though this did not increase villus height, villus width, or villus surface area. These data could indicate that there was some cell damage occurring in the vaccinated groups compared to the control groups, and perhaps the enterocytes are compensating for cell damage by regenerating cells that had been damaged by the parasite (Ruff and Wilkins, 1980; Yun et

al., 2000). Also, these data suggest that the vaccinated birds are being affected differently by the vaccine in lower ileum versus the duodenum, possibly due to the *Eimeria* species localized in each section of the intestine. In the lower ileum, *E. tenella* can cause severe cell damage, which could explain why there is increased enterocyte generation in the lower ileum compared to the duodenum in the vaccinated birds.

After the day 14 feed challenge, the addition of probiotic to the vaccine significantly increased villus height in the duodenum, and vaccinated birds had significantly increased lamina propria thickness compared to medicated and control birds. The increase in villus height supports that in response to a pathogen, probiotics could benefit villus morphology (Awad et al, 2006). In the lower ileum, vaccinated birds had significantly decreased surface area and increased crypt depth compared to the control birds post-challenge. Reduced villus integrity indicates damage from a pathogen, and the decrease in morphology seen here could be associated with second peak cycling from the vaccine (Dunsford et al., 1989).

Ionophore

Ionophore birds had significantly decreased villus height and surface area compared to control birds on day 6 in the duodenum and lower ileum; also, in the lower ileum decreased surface area and increased crypt depth was observed in ionophore treated birds compared to the control birds after the day 14 feed challenge, but in the duodenum the ionophore only birds had significantly longer villi than the control birds and greater surface area than the vaccinated birds. There was significantly decreased villus height at day 36 and 43 in the duodenum and at day 43 in the ileum, with a corresponding increase

in lamina propria thickness at day 36 in the duodenum. These observations suggest that the ionophore could have been protecting the birds from the *Eimeria* until the monensin was removed from the feed on day 33. Increased lamina propria thickness could indicate that there was an influx of immune cells into the tissue by day 43 due to the ionophore removal and day 36 feed challenge, while the decreased villus height could have been caused by the destruction of enterocytes by the parasite (Tellez et al, 1993; Dunsford et al., 1989). These data support that the use of ionophore in the feed delayed exposure to the parasite until the ionophore was removed, leaving the birds open to infection late in production. Also, poor villus integrity in ionophore birds demonstrated throughout the experiment suggests that while the ionophore may have been protecting the birds from the lower level day 14 exposure in the duodenum, enterocyte destruction was still occurring in the lower ileum. The ionophore may have prevented the birds from generating protective immunity to the *Eimeria*, which explains why after the removal of the ionophore on day 33, lamina propria tissue in the duodenum and lower ileum significantly increased compared to vaccinated birds. Ionophore birds also had a decreased ratio compared to control birds on day 36, which suggests that the ionophore birds had increased enterocyte regeneration, possibly in compensation for the cell damage that was occurring as a result of the delayed infection (Ruff and Wilkins, 1980).

Probiotic

In the lower ileum on day 43, probiotic birds had thicker lamina propria compared to non probiotic birds. These data suggest that probiotic birds had increased immune cell activity in the mucosa late during production.

Conclusions

The data demonstrate that feeding of probiotics, coccidiastats, and coccidia vaccination influence gut morphology and maturation. At select timepoints the addition of probiotic to a control method improved gut morphology by increasing villus height or surface area, which indicates that those birds had improved digestive and absorptive capacity. Furthermore, there were instances in both the vaccinated and ionophore groups where the gut demonstrated what could have been compensatory cell production in response to cell damage from the *Eimeria*, however, in the ionophore treated birds this occurred in the last phase of production, indicating decreased functional capacity. Further investigation is necessary to evaluate the compensatory mechanisms of the intestine in response to a coccidiosis infection, and to evaluate if ionophores negatively affect gut morphology. More research is needed to evaluate the interaction of probiotics and coccidiosis vaccination, since the addition of probiotic to the vaccine did not always produce the same result. If probiotics could be used to improve the activity of coccidiosis vaccines by achieving immunity earlier, or ameliorating production losses caused by vaccine administration, that knowledge could be used to benefit poultry producers.

CHAPTER IV

INFLUENCE OF PROBIOTIC ADMINISTRATION, COCCIDIOSIS

VACCINATION, *EIMERIA* CHALLENGE, OR IONOPHORE

ADMINISTRATION ON BROILER IgG SECRETION

Introduction

The intestinal disease coccidiosis is economically burdensome to the commercial poultry industry, with the costs of the disease per annum totaling 3 billion U.S. dollars (Dalloul and Lillehoj, 2006). Eight species of *Eimeria* have been identified to parasitize chickens, such as: *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. mivati*, *E. necatrix*, *E. praecox*, and *E. tenella* (Chapman 2000; Conway and McKenzie, 2007). Because each *Eimeria* species is immunogenically unique, a challenge exists to control all species with a single method (Lillehoj, 1989).

Current *Eimeria* control methods include the use of in-feed anticoccidial drugs, like ionophores, however, drug resistance to ionophores is now widespread (Jeffers, 1974; McDougald, 1981; Chapman, 1982; Williams, 2006). Recent research has focused on developing vaccines to provide more protection against *Eimeria* (Williams, 2002; Mathis and Broussard, 2006; Williams, 2006). Poultry producers are hesitant to use coccidiosis vaccines because of the production losses incurred during immunity development in response to the vaccine. Many studies have compared birds receiving vaccination to birds receiving anticoccidial drugs and found that vaccinated birds saw decreased body weight and poor feed conversion (Allen and Fetterer, 2002). As resistance to anticoccidial drugs

and the reluctance to use vaccines continue, new methods to improve coccidiosis control must be investigated further.

Upon exposure to *Eimeria*, chickens produce IgM, IgA, and chicken IgG (Dalloul and Lillehoj, 2005). Chicken IgG is concentrated in the yolk sac of the egg and is transported to the embryo late during development, and these maternal antibodies have been reported to provide some passive immunity to *Eimeria* (Lillehoj, 1987; Lillehoj et al., 2004). Rose and Long (1971) explored the protective effects of transferring immunity to chick embryos. The authors found that antibodies provided protection from *E. tenella* infection only when the "donors" (hens) were actively developing immunity to *E. tenella* (Rose and Long, 1971). Though the investigators felt that the result of maternal transmission of protection to embryos was inconclusive due to testing methods used at the time, they did note oocyst output reduction in the progeny of immunized hens when chicks were challenged at 4 days of age with *E. tenella* oocysts (Rose and Long, 1971).

In the 1990s, several investigators (Wallach et al., 1992; Smith et al., 1994a; Smith et al., 1994b; Wallach et al., 1995) reported that although maternal immunity to *E. maxima* infections decreased gradually over time, which was show in decreased IgG titers in egg yolk and chick sera, maternal antibodies still play a significant role in protecting the chick at the time of hatch. Parasite specific IgG could be transferred to chicks in the yolk, and protect against infection for the first 2-3 weeks outside of the egg (Rose and Long, 1971; Rose, 1972). Early protection against infection could be beneficial, especially in broilers, because immunizing one breeder hen could provide protection to numerous broiler chicks (Smith et al., 1994b). Also, though maternal immunity is considered almost non-existent

by 3 weeks of age, Smith and colleagues (1994b) asserted that these chicks would be protected long enough to prevent severe coccidiosis before slaughter. Investigators also found that oocyst output in hatchlings that were progeny of immunized breeding hens was reduced after a challenge with *E. maxima* oocysts (Smith et al., 1994a; Wallach et al., 1995). Wallach and colleagues (1995) also determined that chicks immunized with affinity purified gametocyte antigens from *Eimeria maxima* were not only immunized to *Eimeria maxima*, but also partially immunized to infections with *E. tenella* and *E. acervulina*. These parasite specific maternal antibodies serve a role in humoral immunity by reducing infectivity as a consequence of parasite neutralization, and the ability to modulate these antibodies would provide a means of decreasing the invasive potential of *Eimerai* (Lillehoj and Lillehoj, 2000).

The natural microflora present in the gut is capable of competitively excluding pathogenic organisms and facilitating gut health (Dalloul and Lillehoj, 2005). Previous studies have shown that probiotics can prevent disease by competing with pathogens for nutrients and attachment sites along the intestinal epithelium, but also probiotics secrete soluble factors that can inhibit the growth and efficacy of pathogens (Nurmi and Rantala, 1973; Koenen, 2004). Probiotics were reported to protect against bacterial pathogens, but recently have been shown to protect poultry from *Eimeria* (Dalloul et al., 2003; Dalloul and Lillehoj, 2005; Lee et al., 2007). Probiotic supplementation has been shown to impact *Eimeria* infection by reducing oocyst shedding, increasing body weights, and increasing secretion of *Eimeria* specific antibodies in broilers infected with *E. acervulina* and E. *tenella*, however, the role of humoral immunity during a coccidiosis infection is still being

defined (Dalloul and Lillehoj, 2005). We hypothesized that probiotic administration would increase IgG secretion systemically in response to coccidiosis. The objective of this study was to evaluate how probiotic administration affects the humoral immune status of the bird compared to and when administered with ionophores and coccidiosis vaccination during an *Eimeria* infection.

Materials and Methods

Birds and Experimental Design

On day-of-hatch, Cobb 500 male chickens were obtained from a local commercial hatchery, placed into floor pens with 50% fresh pine shavings and 50% built up litter, and provided supplemental heat to simulate industry rearing conditions. The birds were provided water via a nipple drinker system, which dispensed the probiotic, and were fed a diet shown to enhance performance of birds administered a coccidiosis vaccination using the following feeding program: starter phase (D1-15), grower phase (D15-29), finisher phase (D29-32) and withdrawal phase (D33-42) (Lee et al., 2009). The ionophore and challenge were administered via the feed. The experiment was conducted in a broiler rearing facility at the Texas A&M University Poultry Science Teaching, Research, and Extension Center, and animal care and husbandry were provided according to an approved Texas A&M Institutional Animal Care and Use protocol.

The experimental design used a 3x2 factorial ANOVA design with 3 coccidia control methods (control, ionophore and coccidiosis vaccination) and 2 probiotic groups (probiotic and no probiotic) to determine how the administration of probiotic and coccidia control methods affect avian gut morphology. Individual treatment groups were negative

control, probiotic only, vaccination only, vaccination with probiotic, ionophore only, and ionophore with probiotic.

Eimeria Challenge

A field strain of *Eimeria* oocysts derived from a local broiler production facility was used to challenge the birds. Species present included: *E. acervulina*, *E. mivati*, *E. maxima*, and *E. tenella*. Two feed challenges were administered: on day 14 (50,000 oocysts per bird) and on day 36 (750,000 oocysts per bird).

Probiotic Administration

A commercially available probiotic (Poultry Star®, Biomin, GmbH, Herzogenburg, Austria) was administered at a concentration in accordance with the manufacturer's recommendations (20 grams per 1,000 broilers) to the appropriate treatment pens in drinking water by utilizing an independent watering system. Species present in the probiotic were *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, and *Lactobacillus reuteri*. Probiotic was administered intermittently from day of placement through day 2, days 9-10, 13-15, 25-27, and 32-33, which corresponded with each feed change.

Ionophore Administration

The ionophore, monensin (Coban-90, Elanco Animal Health, Indianapolis, IN), was added to the feed per manufacturer's recommendations (90 grams per ton) according to the treatment group. On day 33 the ionophore was removed from the feed to create a withdrawal ration to simulate industry methods.

Vaccination

Vaccinated birds received Coccivac®-B (Intervet/Schering-Plough Animal Health/Merck and Co., Inc., Whitehouse Station, NJ), a non-attenuated live oocyst coccidiosis vaccine for use in broiler chickens. The vaccine was applied using a Spraycox® II cabinet (Intervet/Schering-Plough Animal Health/Merck and Co., Inc., Whitehouse Station, NJ). Before placement, the birds were allowed to preen for an hour under bright light.

Blood Collection and Processing

On days 6, 22, 36 and 43, blood samples were taken using venipuncture in the wing vein of 8 broilers per treatment group and deposited into 2 mL snap-cap microcentrifuge tubes (Eppendorf North America, Hauppauge, NY). The blood samples were allowed to clot overnight at 4ºC. The tubes were centrifuged at 2,000 x g for 2 minutes, the serum was removed, alliquotted into fresh microcentrifuge tubes, and stored at -80ºC for later antibody analysis.

ELISA

Serum IgG concentrations were determined using a chicken-specific IgG ELISA quantitation kit (Bethyl Laboratories Inc., Montgomery, Tx). The ELISA procedure was carried out according to manufacturer protocol and absorbance was measured at 450 nm. The concentration of IgG was determined using standard curves generated from IgG standards, run on the assay microtiter plate and were expressed as absorbance (Mountzouris et al., 2010).

Statistical Analysis

Data were measured using a factorial ANOVA and main effect means were deemed significant at $P \leq 0.05$. In cases where significant interactions were observed, data was subjected to a one-way ANOVA. All means were separated using a Duncan's Multiple Range Test. Statistical analysis was completed with the SPSS statistical software package (Chicago, IL).

Results and Discussion

Upon exposure on day 14, the vaccinated birds had significantly higher levels of IgG in serum compared to ionophore treated and control birds (Table 4-1). During vaccine administration, the vaccinated birds were exposed to a level of that should have been immunogenic. By day 22, the vaccinated birds had increased IgG levels systemically, which was most likely due to the vaccine. These data are supported by oocyst output data from this trial, which showed that vaccinated birds had two peaks of cycling before day 24 (Klein et al., 2008). Meanwhile, on day 36, the ionophore birds had significantly higher IgG levels compared to the control birds. Chicken IgG is the predominant antibody during antibody responses that occur after the first exposure to an antigen (secondary, tertiary, etc.) (Abbas and Lichtman, 2006). After the ionophore was removed from the feed on day 33, the birds could have been undergoing an immune response to the day 36 challenge. By day 36, this could have been a secondary antibody response, because the oocyst output data also shows a peak in oocyst cyling observed in ionophore treated birds after day 24 (Klein et al., 2008). The interference with immunity development from ionophore use has

been noted in previous research (Chapman, 1999; Chapman et al., 2004; Klein et al., 2008).

On day 22, non probiotic birds had greater IgG secretion compared to probiotic birds, which could indicate that the probiotic limited the exposure of the *Eimeria* parasite, inhibiting the development of a systemic humoral immune response (Table 4-1). When Dalloul and colleagues (2003) evaluated antibody secretion during a coccidiosis infection between control and *Lactobacillus*-based probiotic fed birds, they also found that non probiotic birds had significantly higher antibody levels in intestinal secretions when compared to probiotic birds, though their serum antibody levels were not different (2003). They concluded that after infection, permeability across the mucosal barrier would increase and the pathogen would easily penetrate the submucosa, reaching the lamina propria where most of the antibodies producing B cells are located. The data indicated that while higher antibody levels can indicate the effectiveness of antibody producing cells, they do not necessarily reflect resistance to infection.

These data support that coccidiosis vaccination does induce protective immunity earlier during production than other control methods. The late peak of chicken IgG in the ionophore treated birds also supports previous research that indicates that ionophores can delay exposure to *Eimeria*, leaving the birds more susceptible to infection during the withdrawal phase. These data also indicate that current coccidia control methods and probiotics can have an effect on the humoral immune status of the bird, and may not just focus locally in the intestine, though the specific mechanisms by which the treatments interact to affect humoral immunity need to be investigated further.

Table 4-1. Chicken IgG in serum shown as absorbance (450 nM) for each sample

collection day.

Treatment		Absorbance			
Control Measure	**Probiotic**	**Day 6**	**Day 22**	**Day 36**	**Day 43**
Control	Control	0.553	0.371	0.397	1.103
Control	Probiotic	0.664	0.224	0.443	1.039
Vaccine	Control	0.556	0.483	0.547	1.137
Vaccine	Probiotic	0.574	0.346	0.421	0.870
Ionophore	Control	0.496	0.300	0.593	0.986
Ionophore	Probiotic	0.605	0.341	0.514	1.103
Main Effects					
Control		0.609	0.298^b	0.420^b	1.071
Vaccine		0.565	0.415^a	0.484^{ab}	1.007
Ionophore		0.551	0.321^b	0.554^a	1.044
	Probiotic	0.614	0.304^b	0.459	1.007
	Non Probiotic	0.535	0.385^a	0.513	1.075

[a,b] Means of main effects with different subscripts differ significantly at $P \leq 0.05$

CHAPTER V

CONCLUSIONS

Coccidiosis continues to be a relevant disease to the commercial poultry industry due to the severe economic impact it has on producers. The parasite *Eimeria* is ubiquitous among commercial poultry rearing facilities, due the nature of poultry rearing. Confinement, high stocking density, and litter conditions all contribute to the efficacy of the parasite among poultry flocks (Williams, 2002). Consequently, the presence of *Eimeria* in the future is certain, so the development of prevention and control strategies are critical to facilitating the success of poultry producers.

Historically, coccidiosis control methods consisted of the use of in-feed anticoccidial drugs, like ionophores. These prophylactic treatments have been and still are widely used as a means of coccidia control in the commercial broiler industry, because the drugs can eliminate the parasite before it is able to invade the intestine and cause damage to the intestine that could translate into losses for the poultry producer (Allen and Fetterer, 2002). Parasite resistance to ionophores is now well documented and widespread, which limits their effectiveness (Shirley et al., 2005). Producers continue to use ionophores because little research is being done to produce new, more effective anticoccidial drugs.

Recent research has been geared toward developing effective coccidiosis vaccines to control the parasite. Several types of vaccines are available, but the most effective are live oocyst vaccines (Rose and Hesketh, 1987; Shirley, 1989). Live oocyst vaccines are either attenuated or non attenuated, and may contain different species of *Eimeria*, depending on the type of bird being vaccinated, and also on environmental factors, like

geographic location. Coccidiosis vaccinations have been shown to provide the bird with long lasting immunity early during production, but in order to protect a bird from *Eimeria*, the vaccine must induce an immune response to the species of *Eimeria* the bird will be exposed too (Chapman, 2000). Because immunity to one species of *Eimeria* does not protect against other species, vaccines must include multiple species. Commercial poultry producers are hesitant to use coccidiosis vaccines because bird performance has been shown to suffer during vaccination (Allen and Fetterer, 2002).

Probiotics are defined as direct fed microbials, and when ingested, probiotics can occupy niches in the gut, and competitively exclude pathogens (Fuller, 1989; Dalloul and Lillehoj, 2005). Probiotics have been shown to increase bird performance, digestive function by improving the integrity of structures in the gut, facilitate resistance to infection, and affect antibody secretion (Dalloul and Lillehoj, 2005; Eckert et al., 2010). Improvements in gut morphology, like increased surface area or deepened crypts, can be indicative of greater absorptive and digestive capacity, as well as enterocyte turnover (Samanya and Yamauchi, 2002; Awad et al., 2009). These effects could not only facilitate pathogen resistance, but also they could ameliorate the cell damage incurred during an *Eimeria* infection, which leads to weight loss due to malabsorption in birds. Probiotics are capable of excluding opportunistic pathogens because they promote a healthy balance of normal bacteria (Dalloul and Lillehoj, 2005). Probiotics could help prevent necrotic enteritis which often results from co-infections of *Clostridium perfringens* and *E. maxima* (Park et al., 2008).

Probiotics have been reported to modulate the immune system (Lillehoj and Choi, 1998; Dalloul et al., 2003; Chichlowski et al., 2007). However, little research exists to specifically evaluate how probiotics interact with current coccidia control methods, like ionophores and coccidiosis vaccination, to determine if probiotics could improve the effectiveness of ionophores or vaccines in mediating a coccidiosis infection, or if probiotics alone could mediate the infection. The current research focused on the effects of feeding probiotics, combined with current coccidia control methods, to evaluate the impact of these factors on gut morphology as well as the humoral immune status of the bird.

When examining gut morphology, we found that the addition of probiotics to the vaccine caused several significant interactions in the duodenum before and after the first feed challenge. Pre challenge data suggested that the probiotic, when added to the vaccine, may have limited enterocytes exposure to the *Eimeria* by adhering to the intestinal mucosa, thus limiting exposure of enterocytes to the parasite, and decreasing the influx of immune cells in the lamina propria in response to the vaccine. On day 6, vaccinated birds had a significantly higher villus height to crypt depth ratio when compared to ionophore treated birds, which is associated with improved performance (Yang et al., 2007; Awad et al., 2009). These data could suggest that the vaccine did not cause cell damage in the duodenum, and that vaccinated birds had increased gut functionality due to improved villus height, villus width and villus surface area (Aptekmann et al., 2001). In the lower ileum, vaccinated birds had significantly deeper crypts and lower villus height to crypt depth ratio, which indicates increased enterocyte production, however, villus height, width, and

surface area were not increased, suggesting there was some cell damage occurring in the vaccinated groups compared to the control groups, and perhaps the enterocytes are compensating for cell damage (Ruff and Wilkins, 1980; Yun et al., 2000).

Post challenge, the addition of probiotic to the vaccine significantly increased villus height in the duodenum, and vaccinated birds had significantly increased lamina propria thickness compared to ionophore and control birds. These data suggest that probiotics could benefit gut morphology by restoring absorptive capacity to the gut after an *Eimeria* challenge (Awad et al., 2006). In the lower ileum, vaccinated birds had decreased surface area and increased crypt depth compared to the control birds post challenge; similar to what was seen in the vaccinated birds pre challenge. The vaccine may not be fully protecting the birds from infection post challenge in the lower ileum, possibly due the difference in *Eimeria* localizing in the lower ileum. The morphology of the lower ileum suggests that the lower ileum had decreased capacity and greater enterocytes production when compared to the morphology of the duodenum. *Eimeria* in the lower ileum may be causing more cell damage, resulting in poor gut morphology (Dunsford et al., 1989).

Observations in the ionophore birds in the duodenum and lower ileum suggest that the ionophore could have been protecting the birds from the *Eimeria* until the feed was removed on day 33. The increase in lamina propria thickness could indicate an influx of immune cells by day 43, while the decreased villus height could have been caused by a parasite invasion (Dunsford et al., 1989; Tellez et al., 1993). The data support that ionophores can delay exposure of the birds to the parasite, and when the ionophore was removed the birds were open to infection late in production. After ionophore removal on

day 33, lamina propria tissue in the duodenum and lower ileum significantly increased compared to vaccinated birds. Also, ionophore birds showed decreased villus height to crypt depth ratio compared to control birds on day 36, which suggests that the ionophore birds had increased enterocyte production, possibly to regenerate damaged cells (Ruff and Wilkins, 1980). The ionophore treated birds also peaked in chicken IgG secretion on day 36, indicating there could have been an increase in the invasive potential of *Eimeria* at that time, which is supported by oocyst shedding data from this trial, which will be discussed below (Dalloul et al., 2003; Klein et al., 2008).

The ELISA data indicates that the increase levels of IgG in vaccinated birds on day 22 was due to the vaccine cycling. The histology data strongly indicates that the ionophore birds had delayed exposure to the parasite until the ionophore was removed from the feed. The chicken IgG secretion data supports the histology data that after the ionophore was removed from the feed on day 33, the birds could have undergone a secondary antibody response by day 36. The interference with immunity development from ionophore use has been reported, and previous research suggests that it could take as long as seven weeks to obtain complete immunity to some *Eimeria* species (Chapman, 1999; Chapman et al., 2004; Klein et al., 2008).

On day 22, non probiotic birds had greater IgG secretion compared to probiotic birds, which could indicate that the probiotic limited the exposure of the gut to the *Eimeria* parasite, delaying the development of a systemic humoral immune response. Dalloul and colleagues (2003) saw similar results when they evaluated antibody secretion during a coccidiosis infection between control and probiotic fed birds. They found that non

probiotic birds had significantly higher antibody levels in intestinal secretions when compared to probiotic birds, though their serum antibody levels were not different (Dalloul et al., 2003). In another experiment, Dalloul and colleagues (2005) found that splenic lymphocytes in non probiotics birds secreted more IFNγ compared to probiotics fed birds. The investigators concluded that non probiotics birds do have an increased systemic immune response, possibly due to a more severe infection. These data could suggest that the probiotics contribute to the "immuno-suppressed tone" of the gut by delaying a systemic immune response (Mayer, 1997).

Probiotics, coccidiastats, and coccida vaccination do affect gut morphology and chicken IgG secretion. The need for improved methods of control for coccidiosis will only increase in the future as commercial poultry production grows, and probiotics may be a tool to help alleviate the negative effects of coccidiosis. Probiotics could improve gut morphology, which increases the absorptive capacity of the gut, as well as maintains a healthy balance of normal microbial flora, which helps to prevent co-infection. Also, IgG secretion data suggests that the coccidiosis vaccination produced an immune response in broilers earlier than in ionophore treated birds, which means vaccinated birds were protected from the *Eimeria* challenge. These factors all present mechanisms by which producers could alleviate the negative effects of coccidiosis.

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