The effects of dietary β-glucan supplementation on performance and immune response of broiler chicks during an *Eimeria* challenge

By

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ABSTRACT

Escalating consumer concerns have placed the poultry industry under mounting pressure to reduce the use of chemotherapeutic agents as feed additives. One possible alternative receiving increased attention is the use of immunomodulators such as β-glucan. A pilot study evaluated the effects of a yeast derived β-glucan (Auxoferm YGT) on growth performance and immune response of broiler chickens. Day-old chicks were fed a diet containing 0, 0.02, or 0.1% yeast β-glucan. On days 7 and 14 post-hatch, body weight and relative immune organ weights were measured, peripheral blood was collected to determine heterophil to lymphocyte (H:L) ratios, and small intestinal sections were sampled to evaluate relative gene expression. The addition of β-glucan had no influence on growth. Dietary β-glucan supplementation modulated the expression of interleukin (IL)-8, IL-18, interferon (IFN)-γ and inducible nitric oxide synthase (iNOS) in the small intestine. A subsequent study was conducted to investigate the effects of dietary β-glucan on broiler chick (1440 birds) performance and immune response during a mixed *Eimeria* infection (day 8 of age). Measurements were taken and samples collected on days 4, 7, 10, 14 and 21 post-hatch. The results from this study show that β-glucan supplementation did not negatively impact performance. The addition of β-glucan to the diet resulted in reduced gross lesion severity and increased H:L ratios. The gene expression results suggest that β-glucans are capable of skewing the host immune response toward aTh1 mediated response and consequently down-regulating the Th2 mediated response.

Keywords: β-glucan, chicken, performance, immunity, cytokines

Dedication

I would like to dedicate this thesis to my late grandmother and great grandmother, Linda Ann Smith and Bertha May Stevens. Thank you for teaching me how to be a strong and caring woman. I will love and miss you always.

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Table of Contents

List of tables

List of figures

List of abbreviations

APCs: Antigen presenting cells

BW: Body weight

BWG: Body weight gain

Chall: Challenged with *Eimeria*

CMI: Cell-mediated immunity

d: Day

DOH: Day of hatch

Duo: Duodenum

FCR: Feed conversion ratio

FI: Feed intake

GALT: Gut-associated lymphoid tissues

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

H:L: Heterophil to lyphocyte ratio

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

Ile: Ileum

iNOS: Inducible nitric oxide synthase

Jej: Jejunum

LPS: Lipopolysaccharide

LS Means: Least squares means

M cells: Microfold cells

MALT: Mucosa-associated lymphoid tissues

MHC: Major histocompatability complex

Muc: Mucin

NK: Natural killer

Not Chall: Not challenged

ODNs: Oligodeoxynucleotides

PAMPs: Pathogen associated molcular patterns

PP: Peyer's patches

PRRs: Pathogen recognition receptors

qRT-PCR: Quantitative real-time polymerase chain reaction

SE: Standard error

SRBC: Sheep red blood cell

Tc: Cytotoxic T cell

Th: Helper T cell

Th1: Type 1 helper T cell

Th2: Type 2 helper t cell

TNF: Tumor necrosis factor

Chapter I

Introduction

Coccidiosis is a parasitic disease of the intestinal tract brought about by intracellular protozoan parasites belonging to the genus *Eimeria* (Dalloul and Lillehoj, 2005). There are seven *Eimeria* species that are known to infect chickens: *E. acervulina*, *E. maxima*, *E. tenella*, *E. brunetti*, *E. necatrix*, *E. mitis* and *E. praecox*. *Eimeria* sp. invade the intestinal lining and disrupt the enterocyte layer resulting in gross lesions, nutrient malabsorption and reduced performance (Brake et al., 1997). Controlling coccidiosis has had a profound negative economic impact on the poultry industry. The damage caused by this disease is estimated at a devastating $3 billion annually worldwide (Dalloul and Lillehoj, 2006). The monetary losses related to coccidiosis are primarily due to the costs of prophylactic measures, treatments, decontamination, restocking and lost revenues due to decreased production.

Currently, the poultry industry relies heavily on chemotherapy to control coccidiosis outbreaks. Recently, consumers have become exceptionally concerned about chemical residues in meat products and pathogen resistance. These concerns have resulted in mounting pressure to ban chemotherapeutic agents from animal feeds. Sub-therapeutic doses of antibiotics have been included in the diets of livestock animals over the past several decades to improve growth, feed efficiency, and general health. The controversy and potential risk have resulted in the ban of antibiotics as poultry feed additives by the European Union as of January 1, 2006. Unfortunately, this ban has resulted in a decline of animal health and increased the variability in carcass size and meat characteristics (Casewell et al., 2003). Changes in consumer preference and the risk of the ban spreading overseas to the U.S. has created an impetus for finding a more "natural" alternative capable of maintaining animal health without impeding performance.

One alternative would be an immunostimulant that is capable of enhancing the overall functionality and efficiency of the immune system, which is important for the health and well-being of all animals. Yeast derived products are currently a large focus of interest for many groups seeking an alternative to chemical treatments and antibiotics due to their higher acceptance by consumers. Beta-glucans belong to a group of physiologically active compounds termed biological response modifiers due to their ability to stimulate the immune system. Beta-glucans are structural components of the cell wall of many bacteria, fungi, algae and yeast as well as cereal grains such as oat and barley. Large variation exists in the structure of β-glucans from these different sources, which ultimately results in differences in their physiological functions (Volman et al., 2008). Those β-glucans derived from yeast and fungi are termed 1,3/1,6-β-D-glucans and consist of 1,3 β-linked glycopyranosyl backbone with variable frequency of 1,6 β-linked side chains (Harada and Ohno, 2008). Due to their complex structure, 1,3/1,6-β-D-glucans are considered the most effective type of β-glucans in terms of stimulating the immune system (Vetvicka and Vetvickova, 2007).

The effects of β-glucans as biologically active immunomodulators in mammals have been well noted for over 50 years (Novak and Vetvicka, 2008). Beta-glucans enhanced migration, phagocytic activity, and overall efficiency of innate immune cells such as neutrophils (Liang et al., 1998; Mucksova et al., 2001; Pelizon et al., 2005; LeBlanc et al., 2006; Vetvicka and Vetvickova, 2007). Not only do β-glucans enhance innate immunity, but also augment the adaptive immune system by increasing B and T lymphocyte proliferation and antibody production (Estrada et al., 1999; Krakowski et al., 1999; Li et al., 2005; Hahn et al., 2006; Wang et al., 2008).

Only recently has research been published concerning the effects of β-glucans in poultry. Exposure to β-glucan enhanced proliferation and phagocytizing efficiency of avian macrophages (Guo et al., 2003) and heterophils (Lowry et al., 2005). Similar to what has been found in mammals, birds supplemented with β-glucans had amplified humoral (Guo et al., 2003; Zhang et al., 2008) and cell-mediated immune responses (Chen et al., 2003; Chae et al., 2006). The immune enhancing capabilities of β-glucans have resulted in the clearance of several economically important pathogens such as *Salmonella enterica* and *Escherichia coli*, further asserting their potential use as an antibiotic alternative. To date, little is known about their exact mode of action on the avian immune response, specifically in the context of gut immunity and by inference their protective mechanisms against enteric pathogens.

The objective of this study was to determine the effects of dietary supplementation of a β-glucan derived from the yeast *Saccharomyces cerevisiae* on performance and immune response of broiler chicks with or without an *Eimeria* sp. challenge.

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Chapter II

Literature Review

Avian Immune System

The structure of the avian immune system is comparable to its mammalian counterpart in terms of organization and mechanisms of action. The avian immune system, like in all vertebrates, can be broken down into two key components: innate and adaptive immunity.

Innate Immunity

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

Physical and biological barriers

The epithelial layers, body secretions and mucous membranes of the body create physical barriers that are generally impermeable to most infectious agents. Most pathogens gain access through the respiratory and gastrointestinal tracts. If a pathogen is inhaled, ciliated epithelium and mucus found within the respiratory tract act as a filter to assist in keeping the airways clear. If a pathogen enters via the digestive tract, a thick mucosal layer will block the pathogen from penetrating the host's cells and protect the digestive tract from infection. The gut also maintains a dense microbial flora capable of preventing colonization of invading organisms through competitive exclusion and by producing toxic substances (Sharma, 2003). The lower pH found within the gastrointestinal tract also serves as a powerful chemical defense against ingested pathogens (Goldsby et al., 2000). In addition, high body temperature found in avian species is

capable of prohibiting many infectious agents from causing disease (Butcher and Miles, 2001). Entry of the majority of pathogens is prohibited by these physical and chemical barriers except in situations where these layers are damaged and the pathogens can gain access.

Cellular barriers

If pathogens are capable of breaching the physical barriers of the innate immune system, there are cellular checks in place to provide protection. The most widely known cells of the innate immune system are phagocytic cells that are capable of ingesting antigens. These cells include macrophages, heterophils (the avian equivalent to mammalian neutrophils) and dendritic cells. Another major cellular component of the innate immune system is natural killer (NK) cells. Natural killer cells are a type of cytotoxic lymphocytes that do not require activation in order to destroy cells deemed to be "non-self", and thus are not restricted by the major histocompatibility complex (MHC), unlike B and T lymphocytes. Natural killer cells function by releasing small granules from their cytoplasm that contain proteins such as perforin and proteases in order to kill virus-infected and tumor cells (Sharma, 2003).

The cells of the innate immune system are usually triggered when conserved microbial motifs known as pathogen associated molecular patterns (PAMPs) on microbes are recognized by pattern recognition receptors (PRRs) located on the surface of the immune cell. Toll-like receptors are an invaluable class of PRRs that recognize microbial pathogens and induce an immediate response by innate immune cells. Following activation and phagocytosis, the phagocyte will present a processed fragment of the pathogen to members of the adaptive immune system, mainly B and T lymphocytes, and stimulate a response to the pathogen. Because of this presenting function, these innate immune cells are also called antigen-presenting cells (APCs). Recognition of pathogens by the innate immune system triggers both immediate innate defenses

as well as the activation of the adaptive immune response. Dendritic cells and macrophages play a key role in detecting and processing antigens and dictating the differentiation of naïve lymphocytes into appropriate effector cells in order to defeat specific types of pathogens (Lee and Iwasaki, 2007).

Adaptive Immunity

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

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

Humoral immune system

The humoral immune system is primarily mediated by B lymphocytes. B cells produce specific immunoglobulins (Ig) or antibodies when stimulated by microbial exposure or other antigens. Antibodies defend the host against these invaders by three mechanisms: 1)

Opsonization: antibodies will bind to receptors and coat the surface of the pathogen in order to be more readily and efficiently engulfed by phagocytes; 2) Neutralization: antibodies will react with epitopes on an infectious agent and inhibit its ability to infect the host; and 3) Complement activation: the complement system is activated when antibodies bind to the surface of invading pathogens, which aid in phagocytosis (Sharma, 2003).

Unlike mammals who carry five main types of immunoglobulins, birds only have three main classes: IgM, IgG (also known as IgY), and IgA. IgM is found on the surface of B cells and is the first antibody found in circulation during a primary immune response. IgG is the most abundant immunoglobulin found in avian as well as mammalian blood, and is the primary antibody produced during a secondary immune response. IgA plays a critical role in mucosal immunity. Secretory IgA exists as a dimer and is most concentrated in mucosal surfaces as well as in tears and saliva (Sharma, 2003).

Cell-mediated immune system

Cell mediated immunity is characterized and controlled by T lymphocytes. As found in mammals, chicken T lymphocytes can be categorized into CD4+ (helper T cells or Th cells) and CD8+ (cytotoxic T cells) subpopulations (Viertlboeck and Gobel, 2008). Helper T cells are activated by recognition of a class II MHC coupled with processed antigen on an APC. Once activated, Th cells divide and produce a variety of cytokines in order to activate B and T lymphocytes as well as other immune cells. Depending on the cytokine profile present in the environment, proliferating Th cells will differentiate into one of two major subtypes known as Type 1 and Type 2 helper T cells (Th1 and Th2, respectively). The Th1 cells are primarily responsible for producing cytokines that encourage inflammation and activate B and T lymphocytes and macrophages while inhibiting the function of Th2 cells in order to generate

immunity to intracellular pathogens. Cytokines secreted by Th2 cells stimulate B lymphocyte proliferation and antibody production while inhibiting Th1 cell function to enhance immunity to extracellular pathogens (Tizard, 2009). A third and more recently discovered Th cell lineage has been identified as Th17 due to the production of its signature pro-inflammatory cytokine IL-17. Cytotoxic T (Tc) cells, on the other hand, are responsible for the recognition and lysis of cells infected with endogenous pathogens in association with a class I MHC molecule (Dalloul and Lillehoj, 2006).

Gut-Associated Lymphoid Tissues (GALT)

The primary function of the gastrointestinal tract is to digest and absorb nutrients in order to meet metabolic demands for normal growth and development, but it also acts as a vital barrier preventing the entry of several antigens and potentially harmful pathogens from the external environment (Beal et al., 2006). The GALT make up the largest component of the mucosa-associated lymphoid tissues (MALT) and are a significant source of immune cells that monitor and protect the mucosal layers of the intestine. The GALT are continuously being exposed to food antigens, microflora and ingested pathogens (Yun et al., 2000). Protection of the gut is achieved through use of both the innate and adaptive immune systems.

The mucosal layer of the gut is comprised of the epithelium and the lamina propria. The epithelial layer is marked by the presence of mostly T lymphocytes while the lamina propria is populated by a variety of immune cells including antibody-producing B lymphocytes (Lillehoj and Trout, 1996). Unlike the mammalian GALT, chickens do not possess lymph nodes, instead they have scattered lymphoid aggregates as well as organized lymphoid structures such as the bursa of Fabricius, cecal tonsils, Meckel's diverticulum and Peyer's patches (PP). The epithelium of the PP, as well as the other lymphoid structures of the GALT, contain areas

occupied by phagocytic antigen sampling cells known as microfold (M) cells (Muir et al., 2000). These M cells are responsible for taking up antigens from the lumen and delivering them to APCs. Upon encountering the antigen in the PP, B and T cells will mount their specific immune response in order to combat the invading pathogen (Beal et al., 2006).

Coccidiosis

Coccidiosis is an economically devastating parasitic disease of the poultry industry caused by the development and reproduction of several species of the *Eimeria* protozoan within the intestine resulting in an estimated loss of $3 billion annually worldwide (Dalloul and Lillehoj, 2006). The chicken is host to seven species of *Eimeria*, including the most common *E. acervulina*, *E. maxima* and *E. tenella*, that each infects a specific area of the gut and invades the intestinal epithelial cells resulting in varying levels of tissue damage and morbidity (McDonald and Shirley, 2009). The disruption of the intestinal epithelial layer naturally leads to the diminished ability of the intestine to absorb nutrients, resulting in reduced performance and higher susceptibility to other diseases such as necrotic enteritis (Yegani and Korver, 2008).

Eimeria Life Cycle

Birds become infected through the ingestion of sporulated *Eimeria* oocysts from contaminated feed, water or litter. The oocyst wall is crushed by the gizzard releasing the sporocysts. Once the sporocysts are broken down with the aid of trypsin and bile in the duodenum, sporozoites are released. The sporozoites then invade the intestinal epithelial cells where they develop into schizonts containing many offspring called merozoites. This developmental stage is capable of breaking out of the epithelial cells, invading other cells and asexually replicating even more. Due to this cyclic reproduction, many of the epithelial cells are destroyed. Once asexual reproduction ceases, the later generation merozoites develop into

sexual male and female gametocytes, identified as microgametes and macrogametes, respectively. The microgamete subsequently fertilizes the macrogamete to produce a zygote. The zygote matures into an oocyst, ruptures the intestinal cell, and is passed with the feces. Once outside the host, the oocysts will sporulate when environmental conditions such as temperature, humidity, and oxygen become conducive to growth and once again become infective. The entire lifecycle from ingestion to release may take 4-6 days to complete (McDougald, 1998; Allen and Fetterer, 2002).

Coccidiosis Prevention and Treatment

Currently, most poultry producers rely on prophylactic measures such as anticoccidial drugs and vaccines to prevent coccidiosis in their flocks. There are two classes of anticoccidials; coccidiostats and coccidiocidals. Coccidiostats are dietary additives fed continuously at low levels and act by arresting the growth of intracellular coccidia. Coccidiocidals, on the other hand, act by actively destroying the parasites during their development (Mcdougald, 2003). However, growing consumer concerns about chemical residues in poultry products and microbial resistance has caused producers to shift to other methods of control, including vaccination. It has been established that natural immunity is acquired after an initial coccidial infection; this knowledge has resulted in the development of several anticoccidial vaccines in order to abate coccidiosis. Unfortunately, live, non-attenuated vaccines tend to reduce performance due to the initial infection and attenuated vaccines do not appear to be as effective as their virulent counterparts (Williams, 2002). Even though these methods are generally considered to be successful, due to the issues related to the use of anticoccidial drugs and vaccines, as well as the impending ban on animal feed additives, research has recently focused on more natural means of controlling and managing coccidiosis.

Host Immune Response to Eimeria

The complex life cycle of coccidia elicits a number of immunological responses involving both the innate and adaptive immune systems, with each response being specific to the *Eimeria* species involved. Prior to the activation of an adaptive immune response, the innate immune system of a naive host will attempt to prohibit the *Eimeria* infection through various pathways such as competitive exclusion by commensal microflora, gastric secretions, phagocytosis, and complement components. The GALT play an invaluable role in protecting the host and bridging the innate and adaptive immune systems. The GALT provide protection to the host by performing three main functions: processing and presenting antigens, producing intestinal antibodies by activating the humoral immune system, and activating cell mediated immunity (Yun et al., 2000; Dalloul and Lillehoj, 2006).

Humoral immune response

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

Cell-mediated immune response

Cell-mediated immune responses are thought to be the most effective against intracellular parasites such as *Eimeria*. Following an exposure to *Eimeria,* proliferation and infiltration of T lymphocytes, especially CD8+ T lymphocytes, are boosted at the site of infection. Elimination of CD8+ T lymphocytes results in aggravated severity of coccidiosis and greater oocyst shedding demonstrating their importance in providing immunity to the disease. Suppression of T lymphocyte function also resulted in impaired immunity (Dalloul and Lillehoj, 2006). Higher levels of CD8+ T lymphocytes were also found to be related to reduced oocyst shedding (Bessay et al., 1996). Furthermore, in chickens, splenocytes and peripheral blood lymphocytes from immune birds can transfer resistance to infection to naïve birds (Lillehoj, 1998b).

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

The response of T lymphocytes against an *Eimeria* infection is predominantly controlled and regulated by cytokines. Following primary *E. maxima* infection, mRNA transcripts of the Th1 cytokines, interferon (IFN)-γ, IL-1β, IL-6, IL-12, IL-15, IL-17 and IL-18 are up-regulated as well as the Th2 cytokines, IL-3, IL-10, IL-13 and granulocyte macrophage colony stimulating

factor (GM-CSF) (Hong et al., 2006b). In a similar study by the same group, an initial infection of *E. acervulina* resulted in the up-regulation of IFN-γ, IL-2, IL-12, IL-15, IL-16, IL-18, IL-3, GM-CSF and down-regulation of IL-4 and IL-13 mRNAs (Hong et al., 2006a). Similarly, Cornelissen et al. (2009) reported that *E. acervulina* or *E. tenella* infection up-regulated mRNA expression of the Th1 cytokines IL-2, IL-18 and IFN-γ. However, they observed an increase in Th2 cytokine gene expression of IL-4 and IL-10 as well as the chemokine IL-8 (Cornelissen et al., 2009). These data further confirm the importance of both the Th1 and Th2 subsets in controlling *Eimeria* infections.

Alternate Methods of Controlling Eimeria Infections

Prophylactic use of anti-coccidial feed additives is currently the most common method of controlling coccidiosis. However, due to increasing concerns regarding drug use and high costs of vaccines, alternative control methods have taken the forefront in the research community. The use of an immunomodulator to manipulate the immune system is currently the most promising alternative. Possible immunomodulators include, but are not limited to, vitamins, probiotics, synthetic oligodeoxynucleotides, and yeast cell wall components such as β-glucans.

The development and function of the avian immune system relies heavily on nutrition. Essential nutrients such as vitamin A have been shown to affect humoral as well as cell-mediated immunity. Vitamin A aids in the differentiation of epithelial cells and is indispensible for maintaining the integrity of mucosal surfaces (Chew and Park, 2004). Vitamin A plays a vital role in the maintenance of the immune system and its deficiency leads to heightened susceptibility of the host to enteric diseases such as coccidiosis. Vitamin A deficiency has been shown to impair local immune defenses within the GALT of broiler chickens. The deficit of vitamin A reduces $CD4^+$ T lymphocyte populations resulting in decreased resistance of broilers

to an *E. acervulina* infection and larger oocyst output (Dalloul et al., 2002). Insuring the proper levels of nutrients such as vitamin A will help maintain gut immunity and reduce immunosuppression and susceptibility to coccidiosis (Dalloul and Lillehoj, 2005).

Another possible alternative is the use of probiotics. The microflora found in the gut represents a critical component of the first lines of defense in humans and animals (Dalloul and Lillehoj, 2005). Probiotics are microorganisms known to have a positive effect on the host by improving the natural balance of pathogenic and beneficial bacteria present in the gut (Fuller, 1989). The use of probiotics is based on the understanding that the microflora in the gut is essential for resistance to enteric infections. Probiotics have been shown to aid in protection against a variety of pathogens including *Escherichia coli*, *Salmonella* spp., *Campylobacter jejuni*, as well as *Eimeria* spp. (Dalloul and Lillehoj, 2005). Chicks fed diets supplemented with probiotics and subsequently infected with *E. acervulina* or *E. tenella* had significantly reduced oocyst shedding (Dalloul et al., 2003; Dalloul et al., 2005; Lee et al., 2007a; Lee et al., 2007b). Also, *E. acervulina* infected birds fed probiotics produced more *Eimeria*-specific antibodies (Lee et al., 2007a; Lee et al., 2007b). Probiotics magnified the numbers of intestinal intraepithelial lymphocytes expressing the cell surface markers CD3, CD4 and CD8 (Dalloul et al., 2003). Furthermore, probiotics significantly increased IFN-γ and IL-2 production indicating modulation of cell-mediated immune responses (Dalloul et al., 2005). The addition of probiotics will help maintain a healthy balance of microflora in the gut and provide protection against several enteric pathogens through stimulation of the mucosal immune system.

Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs have also been shown to act as immune adjuvants in a variety of animals, including chickens. Oligodeoxynucleotides play an important role in host defense by enhancing innate immunity as well adaptive immunity

through stimulation of T cells and the production of predominantly Th1 cytokines (Klinman et al., 1999; Dalloul and Lillehoj, 2005). CpG ODNs have been shown to significantly improve disease resistance against *Salmonella* spp. and *Eimeria* infections in chickens by enhancing intestinal immunity (Dalloul and Lillehoj, 2005). CpG ODNs have strong immunostimulatory effects on chicken macrophages resulting in elevated IL-6 production, enhanced nitric oxide release as well as augmented intracellular bacterial killing (Xie et al., 2003). The effects of CpG ODNs on *E. acervulina* infections have been observed *in vivo* and *in ovo* and both have resulted in reduced oocyst shedding, further demonstrating their capabilities as immunomodulatory agents (Dalloul and Lillehoj, 2005).

Beta-Glucans

Beta-glucans are known as biological response modifiers due to their immune enhancing capabilities (Vetvicka and Vetvickova, 2007b). Beta-glucans are glucose polymers that are structural components of the cell wall of many bacteria, fungi, algae and yeast as well as cereal grains such as oat and barley. Large variation exists in the structure of β-glucans from these different sources that ultimately results in differences in their physiological functions (Volman et al., 2008). Those β-glucans derived from yeast and fungi are termed 1,3/1,6-β-D-glucans and consist of 1,3 β-linked glycopyranosyl backbone with variable frequency of 1,6 β-linked side chains (Harada and Ohno, 2008) (Figure 2.1). The effects of β-glucans as biologically active immunomodulators have been well noted for over 50 years. Due to their highly branched structure, 1,3/1,6-β-D-glucans are considered the most effective type of β-glucans in terms of stimulating the immune system (Vetvicka and Vetvickova, 2007). Beta-glucans have been found to primarily activate the members of the innate immune system and appear to be a suitable

candidate for a natural alternative to chemical treatments commonly used today to treat or prevent many diseases.

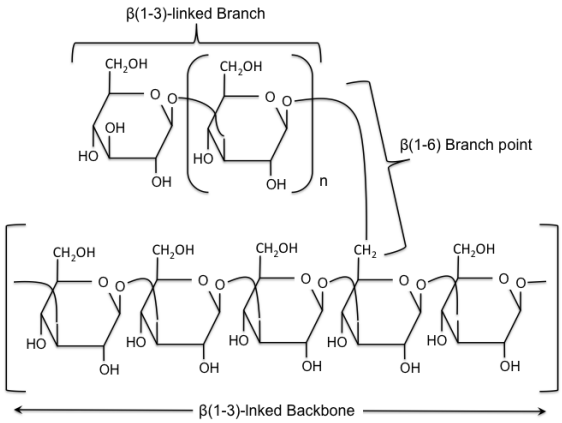

Figure 2.1. Molecular structure of 1,3/1,6-β-D-glucans

Immunomodulating effects of 1-3/1-6 β-glucans in mammals

Beta-glucans from both fungal and plant sources have been used as immunomodulators for at least the past five decades. Their effects have been well studied in a variety of animal species including pigs, sheep, cows, mice and humans, and to a lesser extent in non-mammalian species such as poultry, fish and crustaceans. Yeast β-glucans have been shown to stimulate the immune system through both innate and adaptive means. Understanding the effects of β-glucans in other species plays a pivotal role in discerning their effects in avian species.

Beta-glucans and Innate Immunity

The innate immune system is commonly referred to as the primary line of defense protecting the host from invading pathogens through both cellular and chemical pathways. Beta-glucans derived from the yeast *Saccharomyces cerevisiae* have demonstrated their ability to activate and enhance the function of phagocytic innate immune cells. Le Blanc et al. (2006) found that systemic treatment of poly-[1-6]-D-glucopyranosyl-[1-3]-D-glucopyranose glucan

(PGG), a soluble form of yeast β-glucan, increased the migration of neutrophils to areas of inflammation in rats with experimentally induced acute bacterial pneumonia. These results were consistent with previous work by Liang and colleagues (1998), where monocyte and neutrophil numbers were magnified in β-glucan treated rats when exposed to *Staphylococcus aureus.* Furthermore, neutrophils obtained from β-glucan treated rats and mice show heightened levels of respiratory burst resulting in enhanced clearance of pathogens (Liang et al., 1998; Mucksova et al., 2001; LeBlanc et al., 2006). Liang and colleagues (1998) noted no differences among treatment groups prior to the bacterial challenge, suggesting that β-glucans may act by priming the innate immune system leading to its amplified functionality in times of pathogen exposure. Beta-glucan treatment also resulted in enhanced phagocytic activity and destructive capability of neutrophils isolated from mice (Vetvicka and Vetvickova, 2007a), as well as in foals from treated mares even before drinking colostrum (Krakowski et al., 1999). The pretreatment of C57BL/6 mice with β-glucan also generated a surge in natural killer (NK) cell activity (Pelizon et al., 2005).

The activation of innate immune cells leads to their production of various cytokines that orchestrate the attack of the immune system on the invading pathogen by further stimulating more innate immune cells as well as members of the adaptive immune system. Following β-glucan supplementation, IL-6 and tumor necrosis factor (TNF)-α levels diminished in pigs exposed to lipopolysaccharide (LPS) when compared to the control fed group while IL-10 levels were enhanced (Li et al., 2005). Similar results were observed in a later study where intramuscular injection of β-glucan in Wistar rats blocked the elevation of TNF-α, IL-1β and IL-6 that was seen in the control group following sepsis-induced lung injury (Bedirli et al., 2007). These results suggest that β-glucans act by mitigating the elevation of pro-inflammatory

cytokines and enhance the production of anti-inflammatory cytokines during a challenge. Contradicting studies have noted greater relative abundance of TNF-α mRNA in spleens from pigs fed β-glucan as well as increased IL-1β gene expression in the intestine (Eicher et al., 2006). Pelizon et al. (2005) also found that splenocytes from mice previously treated with β-glucan produced higher IL-12p40, IL-12p70 and TNF-α following stimulation with *S. aureus* suggesting more of a pro-inflammatory response. These varying results may be due to the array of sources, extraction and purification procedures, and administration methods of β-glucans. More needs to be understood about the molecular structure of β-glucans and its diverse effects on the immune system in order to comprehend these contradictions.

Beta-glucans and Adaptive Immunity

Humoral immune response

The adaptive immune response functions through the collaboration of the innate immune response with the humoral and cell-mediated immune responses. Beta-glucans have demonstrated their ability to improve humoral immunity in several studies. Wang et al. (2008) reported that oral administration of β-glucans significantly increased B cell proliferation and antibodies specific for the classical swine fever virus vaccine showing that supplementing β-glucans did have a beneficial effect on humoral immunity. Antibody responses to ovalbumin have also been shown to increase in pigs and immunosuppressed beef steers (Estrada et al., 1999; Li et al., 2005). Furthermore, Krakowski et al. (1999) found that colostrum from mares given intramuscular injections of β-glucans during pregnancy had increased levels of IgG and IgM. These enhancing properties of β-glucans on the humoral immune response should provide stronger resistance against current and subsequent infections.

Cell-mediated immune response

Following stimulation of the innate immune response, activation of the cell-mediated response is important for the clearance of pathogens as well as for protection during future encounters with the same pathogen. Dietary supplementation of β-glucans has been shown to enhance proliferation of T lymphocytes (Wang et al., 2008). Hahn et al. (2006) observed an increase in the $CD4^+$ T lymphocyte subpopulation in β-glucan fed piglets. In a similar study; however, Wang and colleagues (2008) did not observe an increase in the $CD4^+$ subpopulation but instead found an increase in the $CD8^+$ T lymphocyte subpopulation resulting in an overall decline in the $CD4^+$ to $CD8^+$ ratio. These varying results could be due to the effect of vaccination in the Wang study considering differences were only seen following vaccination and no vaccination was noted in the study by the other group. The increase in IL-12 production by splenocytes exposed to β-glucan seen by Pelizon et al. (2005), suggests that β-glucans may lead to the differentiation of Th1 cells, which are ultimately responsible for controlling intracellular pathogens.

Anti-infective Properties of β-glucans

Due to varying results, much confusion has arisen on the exact nature of how β-glucans function. Despite these inconsistencies, β-glucans have been shown to protect against infections of fungal, bacterial, viral and protozoal pathogens (Brown and Gordon, 2003). In an ex vivo study by Pelizon et al. (2005), peritoneal macrophages collected from mice treated with β-glucan showed higher fungicidal activity when exposed to the fungal pathogen *Paracoccidioides brasiliensis*. The protective effect of β-glucans has also been observed in cases of bacterial infections including *Escherichia coli, Staphylococcus aureus*, and the bacteria responsible for Anthrax, *Bacillus anthracis* (Liang et al., 1998; Kournikakis et al., 2003; Stuyven et al., 2009).

The antiviral effects of yeast β-glucans were investigated using swine influenza virus in piglets. Dietary supplementation of β-glucan was capable of reducing pulmonary lesion scores and viral replication rate in the infected pigs suggesting a possible treatment for other influenza cases (Jung et al., 2004). In human trials, administration of β-glucans reduced infectious complications and death following high-risk abdominal or thoracic surgery as well as decreased the need for intravenous antibiotics and shorter stays in the intensive care units (Babineau et al., 1994a; Babineau et al., 1994b; Dellinger et al., 1999). These studies, as well as many others, demonstrate that β-glucans are capable of eliciting a broad spectrum of anti-infective effects and are promising treatment or prophylactic alternatives.

Immunomodulating Effects of 1-3/1-6 β-glucans in Poultry

Effects of beta-glucans on performance parameters

Since the ban of the sub-therapeutic use of antibiotics in food animals by the European Union on January 1, 2006, and the impending threat of prohibiting their use in the United States, animal producers have been actively searching for an alternative that offers the same growth promoting benefits while suppressing pathogenic threats. Due to their immunomodulating effects, β-glucans have received significant attention as a potential alternative source.

Chae et al. (2006) investigated the effect of β-glucan supplementation on growth in two feeding trials. Their first experiment consisted of feeding broiler chicks β-glucan supplemented in the diet at 0%, 0.02% and 0.04%. The second trial compared the use of antibiotics and 0.03% β-glucan supplementation to a control diet. An improvement in weight gain was observed due to the addition of β-glucan above 0.02% in the overall study but this may be attributed to the increased feed intake observed. During the second trial, weight gain was not altered by β-glucan, antibiotics, or their interactions. Feed intake was again raised with the β-glucan supplemented

diet in the starter phase and overall study over the non-supplemented diet (Chae et al., 2006). Similar results were reported by other studies where no deleterious effects were observed due to β-glucan supplementation (Cheng et al., 2004; Chen et al., 2006; Chen et al., 2008; Morales-Lopez et al., 2009).

In a related study, Zhang et al. (2008) fed male broilers one of six diets varying in level of β-glucans at the inclusion rates of 0, 25, 50, 75, 100, and 125 mg/kg. They observed that weight gain was significantly increased in birds fed diets including 50 and 75 mg/kg of β-glucan. Feed intake was also increased in these groups resulting in lower feed: weight gain ratios when compared to the control-fed birds (Zhang et al., 2008). Huff et al. (2006) reported contradictory results where they found that β-glucan supplementation resulted in the reduction of body weight. This lowering of body weight may be a negative consequence of stimulating an energy consuming immune response (Huff et al., 2006). Beta-glucans supplementation has resulted in varying results in performance. These data indicate that more research must be conducted to identify the proper dosage of β-glucan supplementation to optimize growth in order to better compete with the use of antibiotics.

Effects of beta-glucans on the immune response of broilers

Innate immune system

The innate immune system is vital to the immune response and represents the first line of defense for the host against pathogens. Guo et al. (2003) demonstrated the effects of β-glucan on innate immune cells by exposing the chicken macrophage cell line, MQ-NCSU, to varying levels of β-glucans from the yeast *S. cerevisiae*. Exposure to β-glucan enhanced the proliferating ability of macrophages in a dose dependent manner (Guo et al., 2003). The authors also employed an in vivo model. Chickens fed a β-glucan supplemented diet had improved

macrophage phagocytic abilities. When exposed to sheep red blood cells (SRBCs), the macrophages from β-glucan fed birds were more efficient in phagocytizing the foreign cells and engulfed more SRBCs per macrophage than those from the control fed birds (Guo et al., 2003).

The introduction of β-glucan induces the production of nitrite by macrophages once they have been activated. Nitrite is a product of nitric oxide synthase gene activity (Guo et al., 2003). Nitric oxide is produced by immune cells as a consequence of foreign antigen exposure and carries a toxic effect on bacteria and other potentially harmful pathogens (Ohno et al., 1996).

Another study by Chen et al. (2003) demonstrated that a mushroom-derived β-glucan called lentinan also enhanced the function of chicken splenocytes. Exposure to β-glucan induced greater splenocyte proliferation than that of the unexposed splenocytes (Chen et al., 2003). The inclusion of β-glucan as a feed additive also improved phagocytosis of live bacteria by heterophils isolated from chickens challenged with *Salmonella enterica*. Those heterophils were more efficient and capable of engulfing a significantly higher number of bacteria per cell than heterophils isolated from birds not supplemented with β-glucan (Lowry et al., 2005). The up-regulation of host innate immunity, including enhanced proliferation and phagocytosis, may result in the reduction and/or elimination of pathogenic invaders.

Adaptive immune system

Once an antigen is processed by the cells of the innate immune system, it is presented to and recognized by the key players of the adaptive immune system: B and T lymphocytes. Once activated, B lymphocytes produce antibodies to aid in the destruction of the pathogen. Supplementation with β-glucan significantly increased quantities of plasma globulins, serum IgG and intestinal secretory IgA (Zhang et al., 2008). Additionally, when exposed to SRBCs, broilers fed β-glucan supplemented feed had a much improved secondary immune response after

receiving a booster (Guo et al., 2003). This augmentation of immunoglobulins demonstrates the up-regulation of B lymphocytes and the humoral immune system by the immunomodulator.

Cell mediated immunity is characterized by the activation of T lymphocytes by innate immune cells and B lymphocytes and the release of various cytokines that mediate pro- or anti-inflammatory immune responses. Birds fed β-glucan exhibited heightened levels of $CD4^+$, $CD8^+$ and $CD4^+/CD8^+$ T lymphocytes suggesting that β-glucans have a role in activating the adaptive immune system (Chen et al., 2003). Those results were later confirmed by Chae and colleagues (2006) where they reported an increase in $CD8^+$ T lymphocytes due the addition of dietary β-glucan at 0.04%.

Secretion of IL-1, a proinflammatory cytokine that stimulates leukocytes to help fight potential infection, has been shown to be enhanced by β-glucan (Guo et al., 2003). TNF-α production by macrophages is induced by the increased IL-1 production resulting in a proinflammatory response (Zhang et al., 2008).

Interleukin-2 is instrumental in the response of the host to microbial infection by facilitating the production of antibodies by B lymphocytes and regulating the cell mediated immune response. An increase in circulating IL-2 levels was a result of β-glucan addition to broiler diets (Zhang et al., 2008). Similar data were observed by Chen and colleagues (2003) in a previous study using β-glucan derived from the edible mushroom *Lentinus edodes*. Another cytokine produced due to β-glucan exposure is IFN-γ. This cytokine is secreted by several cells including Th1 and Tc lymphocytes and is responsible for activating and enhancing antigen presentation by macrophages (Zhang et al., 2008).

Lymphoid organs

In birds, the bursa of Fabricius is a specialized immune organ responsible for B lymphocyte maturation and differentiation. The thymus, another primary immune organ, is responsible for T lymphocyte maturation. Once matured, lymphocytes migrate to the spleen, a secondary lymphoid organ, in order to interact with circulating antigens. Guo et al. (2003) revealed that β-glucan enlarges the size of the primary and secondary lymphoid organs over those from the control fed birds. These results were confirmed by a later study by Zhang et al. (2008) where they found a significant increase in all three lymphoid organs in birds supplemented with β-glucan.

Anti-infective Properties of β-glucans

Beta-glucan as a feed additive has been shown to enhance protection against bacteria such as *Salmonella enterica* serovar *Enteritidis*. Supplementation not only resulted in amplified phagocytic abilities in heterophils, but also led to enhanced bactericidal killing. The augmented production of nitric oxide and proinflammatory cytokines as well as enhanced bactericidal killing result in an overall reduction in bacterial invasion of the liver and spleen of challenged birds (Lowry et al., 2005).

When faced with an *E. coli* challenge, chicks fed seven days of β-glucan did not show a reduction in body weight due to the infection when compared to unchallenged controls. The β-glucan supplementation also improved the body weights and feed conversion ratios when compared to challenged, control fed birds. Liver and heart weights were heavier in challenged birds not supplemented with β-glucan. The bursa of Fabricius from challenged, treated birds was similar in size to the bursa found in the unchallenged, untreated birds implying that the immune organ had been protected. These results suggest that treatment with β-glucan may curtail

production losses when faced with an acute *E. coli* challenge; however, mortality was not found to be reduced by the treatment (Huff et al., 2006).

Yeast derived β-glucans have been proven to be effective immunomodulators in many animal species. They counteract the negative consequences of several diseases through innate immune cell activation and cytokine production. Unfortunately, little is known concerning the mechanistic actions of β-glucans and how they alter the immune response especially in poultry. The more we understand the capabilities of β-glucans and how they function, the better we can utilize them to our advantage. To this end, the studies described herein were conducted to shed some light on the mode of action of a yeast-derived β-glucan in poultry under healthy and challenge conditions.

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CHAPTER III

Effects of Dietary β-glucan on Immune-Related Gene Expression in Broiler Chicks

ABSTRACT: During the first week post hatch, the avian immune system is immature and inefficient at protecting chicks from invading pathogens. Among immunomodulators, β-glucans are known as biological response modifiers due to their ability to activate the immune system. Current research suggests that β-glucans may enhance avian immunity; however, very little is known about their influence on regulation of immune function. A pilot study was performed to evaluate the effects of dietary β-glucan on growth performance, immune organ weights, peripheral blood cell profiles, and immune related gene expression in the intestine. Day old chicks were fed a diet containing 0, 0.02, or 0.1% yeast β-glucan. On days 7 and 14 post-hatch, body and relative immune organ weights were measured and small intestinal sections were collected to evaluate gene expression by quantitative real-time PCR. Peripheral blood samples were also collected to determine heterophil to lymphocyte ratios. Supplementation of β-glucan did not significantly impact body weight gains. There were no significant differences observed among groups for relative immune organ weights or heterophil to lymphocyte ratios. Compared to controls, expression of interleukin (IL)-8 was down-regulated in the duodenum of both β-glucan treated groups on d7 and d14, and in the jejunum of the 0.1% β-glucan group on d7 and the 0.02% β-glucan group on d14. On d14, 0.1% β-glucan inclusion led to increased iNOS expression in the duodenum and ileum. Expression of IL-18 was up-regulated in the jejunum on d7 but reduced on d14 in the duodenum of the 0.02% β-glucan birds. In the ileum, IL-18 expression declined on d14 in both β-glucan groups when compared to control. Intestinal interferon-γ expression decreased in the 0.1% β-glucan group on d7. These results suggest that β-glucans are capable of altering cytokine/chemokine levels, particularly those of the T helper

type-1cells. However, since varying levels of β-glucan result in different gene expression profiles, further research is needed to determine optimal dosage for immune modulation under various disease situations.

Introduction

During the period immediately following hatch, the immune system of the chick is immature and inefficient making the animal extremely vulnerable to infectious threats found in the environment. Due to the inadequacies in the neonatal chick immune system, immunomodulators are being sought after and studied in order to protect the chick during this immunologically liable time. Beta-glucans have been well studied in human and animal subjects and their immune-enhancing effects have been well noted. Due to their ability to enhance specific and non-specific mechanisms in the immune system, β-glucans have been termed biological response modifiers. Beta-glucans are structural components of the cell wall of many bacteria, fungi, and yeast, as well as cereal grains such as oat and barley. Beta-glucans from fungal and yeast sources have been widely studied and shown to be most effective in enhancing protective immunity against infectious agents (Soltanian et al., 2009).

Though the immune enhancing capabilities of β-glucans have been proven in mammals, limited research is available for poultry with mixed results in terms of performance and immune response. Some studies have shown that β-glucan supplementation improves body weight (Zhang et al., 2008), while other groups have found no significant effects (Chae et al., 2006). Huff and colleagues reported contradictory results where β-glucan supplementation was detrimental to BW in a non-challenge setting but was found to be beneficial when birds were challenged with *E. coli* (Huff et al., 2006). These varying results indicate that more research

needs to be carried out to determine the optimal dosage and proper usage of β-glucans in order to obtain consistent results.

Beta-glucans derived from a variety of sources have beneficial effects on both the innate and adaptive immune systems. Exposure to β-glucans results in enhanced macrophage and splenocyte proliferation as well as improved phagocytic capabilities (Chen et al., 2003; Guo et al., 2003). In terms of the adaptive immune response, β-glucans magnify plasma IgG and IgA levels indicating an up-regulation of the humoral immune response (Zhang et al., 2008). T lymphocyte subpopulations are also impacted with higher CD4+, CD8+ and CD4+/CD8+ T cells found in chickens supplemented with β-glucan (Chen et al., 2003; Chae et al., 2006).

Cytokines are powerful signaling molecules secreted by several members of the immune system. Beta-glucans have demonstrated ability to augment the secretion of several cytokines to aid in pathogen elimination. Guo et al. (2003) reported increased IL-1 production by macrophages isolated from birds fed β-glucans. Similar results were found by Zhang et al. (2008) who observed that β-glucan supplementation not only enhanced IL-1 production but also increased tumor necrosis factor (TNF)-α, IL-2 and interferon (IFN)-γ levels. These results not only demonstrate the ability of β-glucans to augment the cell-mediated immune response but also imply a pro-inflammatory function that should aid in the clearing of pathogens.

The organs responsible for regulating the production and differentiation of B and T lymphocytes are known as primary lymphoid organs (Tizard, 2009). In avian species, the bursa of Fabricius is a primary lymphoid organ responsible for B lymphocyte maturation and differentiation. The thymus, being another primary immune organ, is responsible for T lymphocyte maturation. Once mature, lymphocytes migrate to the spleen, commonly referred to as secondary lymphoid organ, where they are exposed to circulating antigens (Olah and

Vervelde, 2008). Dietary β-glucan increased the size of the primary and secondary lymphoid organs providing further evidence of their immunomodulating capabilities (Guo et al., 2003; Zhang et al., 2008).

The objective of this pilot study was to evaluate the effects of dietary administered yeast β-glucans on performance, immune organ weights, blood cell profiles, and immune-related gene expression in early post-hatch chicks in a non-infectious setting.

Materials and methods

Birds and diets

This project was approved and conducted under the guidelines of the Institutional Animal Care and Use Committee at Virginia Tech. On embryonic day 17, 100 fertilized Cobb 500 eggs were picked up from a commercial hatchery (Pilgrim's Pride, Broadway, VA), transported, incubated, and hatched at the Virginia Tech Poultry Research Farm. On day of hatch (DOH), chicks were wing banded, weighed, separated for equal weight distribution and placed into battery-brooders. There were two replicate pens per treatment group with 15 birds per pen (30 birds/treatment). Chicks had ad libitum access to water and a non-medicated corn/soy based starter diet in mash form containing either 0%, 0.02% or 0.1% YGT Auxoferm (β-glucan extracted from *S. cerevisiae*, AB Vista, UK). The complete diet formulation is presented in Table 3.1.

Performance parameters

Birds were weighed individually prior to placement on DOH and on d7 and d14. Mean BW for each treatment group was calculated from the individual bird replicates for each weigh day. Average body weight gain (BWG) was calculated based on individual bird weights. Mortality was recorded on a daily basis throughout the trial.

Immune organ weights

On d7 and d14, 10 birds/treatment were randomly selected and euthanized by cervical dislocation. The bursa of Fabricius and spleen were weighed, and relative organ weights were expressed as a percentage of live BW.

Peripheral blood cell profiling

Heterophil: lymphocyte (H:L) ratios were determined on d7 and d14 using peripheral blood collected via the jugular vein from the same 10 birds/treatment. Ratios were determined by preparing blood smears using a Morph Slide Spinner (Salem Specialties Inc., Salem, VA) and Modified Wright's stain with heterophils and lymphocytes counted to a total of 60 cells per slide.

Tissue sampling for gene expression analysis

The same ten birds per treatment were sampled on d7 and d14 post hatch. Sampled chicks were killed by cervical dislocation, and the small intestine was subsequently collected. The small intestinal segments (duodenum, jejunum, and ileum) were rinsed in cold PBS, minced, and stored at -80°C until analysis.

Total RNA extraction

Total RNA was extracted using the RNeasy kit according to the animal tissue protocol (Qiagen, Germantown, MD). Intestinal samples were removed from -80°C and placed on dry ice. A 20-30 mg aliquot of each sample was weighed and placed into a 2-mL microcentrifuge tube. The aliquots were placed back into -80°C until it was time for RNA extraction. For a short period of time, the aliquots were removed from -80°C and kept on dry ice until homogenization. Before homogenization, 600 µL of RLT Buffer (Qiagen) were added to the aliquot. The tissues were homogenized using a 7 mm tip on a PowerMax AHS200 homogenizer (VWR) for 30-45

sec. The manufacturer's protocol was followed and the RNA was eluted by rinsing the column membrane twice with 25 µL of RNase-free water. The RNA concentration was determined at OD 260 in a spectrophotometer (NanoDrop ND-1000). The RNA purity was verified by evaluating the ratio of OD 260 to OD 280.

Reverse transcription

Total RNA was diluted to 0.2 µg/µL in nuclease-free water. Reverse transcription was accomplished by using the high capacity cDNA archive kit (Applied Biosystems, Carlsbad, CA). A 2X reverse transcription master mix was prepared consisting of 2 µL 10X reverse transcription buffer, 0.8µL 25X dNTPs, 2µL 10X random primers, 1 µL MultiScribe reverse transcriptase (50U/µL), and 4.2 µL nuclease-free water per reaction. Each reverse transcription reaction contained 10 µL of 2X RT master mix and 10 µL of 0.2 µg/µL RNA. The reverse transcription reaction was performed using a Veriti 96 well thermocycler (Applied Biosystems). Hybridization occurred at 25°C for 10 min followed by extension at 27°C for 120 min and inactivation of the reverse transcriptase at 85°C for 5 sec. The cDNA was stored at -20°C.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using an ABI 7500 FAST Real-Time PCR System (Applied Biosystems). The cDNA was diluted 1:30 in nuclease-free water then 1 µL of the diluted cDNA was added to each well of a 96-well plate. Next, 9 µL of real time PCR master mix containing 5 µL FAST SYBR Green Master Mix (Applied Biosystems), 0.5 µL each of 2 µM forward and reverse primers and 3 µL of sterile nuclease-free water per reaction were added to each well for a final volume of 10 µL. During the PCR reaction, samples were subjected to an initial denaturation phase at 95°C for 20 sec followed by 40 cycles of denaturation at 95°C for 3 sec and annealing and extension at 60°C for 30 sec. Interleukin (IL)-

4, IL-8, IL-13, IL-18, inducible nitric oxide synthase (iNOS), mucin-1 (Muc-1) and Muc-2 gene expression was analyzed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. Each reaction was run in duplicate. Primers were designed using the Primer Express 3.0 software (Applied Biosystems) and synthesized by MWG Operon (Huntsville, AL). Primer sequences are listed in Table 3.2.

Quantitative real-time PCR analysis

Results from qRT-PCR were analyzed using the 7500 Real-Time PCR software (Applied Biosystems). Average gene expression relative to the GAPDH endogenous control for each sample was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The calibrator for each gene was the average ΔCt value from the duodenum of the dietary control group on d4 (first sampling).

Statistical analysis

Results were analyzed using the Fit Model platform in JMP 7.0 (SAS Institute Inc., Cary, NC). The effects of diet were compared using Tukey-HSD. Values were considered statistically different at $P < 0.05$. Results are reported as least squares means (LS Means) with standard errors (SE).

Results

Performance parameters, immune organ weights and peripheral blood cell profiles

No significant differences in BW or BWG were found between the control and YGT fed birds on d7 or d14. However, birds fed 0.1% YGT exhibited significantly higher BW than birds fed 0.02% YGT on d7 ($P = 0.041$) (Figure 3.1). There were no differences seen in mortality since no deaths occurred during the study.

The relative weights of the immune organs, bursa of Fabricius and spleen, were not significantly different between treatment groups throughout the experiment (Table A.1). Additionally, peripheral blood cell populations as measured by H:L ratios did not reveal significant differences associated with the dietary treatments (data not shown).

Intestinal gene expression

There was an effect of dietary treatment on IL-8 gene expression in the duodenum (P = 0.001) and jejunum (P = 0.005) on d7 (Figure 3.2). IL-8 gene expression was significantly reduced in the duodenum of both β-glucan supplemented groups and in the jejunum of the 0.1% YGT supplemented group as compared to controls ($P < 0.05$). No influence of dietary treatment groups was observed in the ileum on d7. On d14, dietary treatment resulted in a decrease in IL-8 gene expression in the duodenum ($P < 0.0001$) and ileum (P = 0.020) of chicks fed β-glucan. There was also an effect of diet on IL-18 expression in the jejunum on d14 (P = 0.037) with expression being down-regulated in the 0.02% YGT fed group when compared to controls ($P <$ 0.05). IL-8 expression in the 0.1% YGT fed birds was not found to be different from that of the controls or the 0.02% YGT fed group in the jejunum.

With respect to the expression of the proinflammatory cytokine IL-18, an effect of diet was observed in the jejunum on d7 (P = 0.030). Birds fed 0.02% β-glucan had significantly higher IL-18 gene expression in the jejunum when compared to the control fed birds , while the 0.1% YGT fed group was intermediate to these ($P < 0.05$) (Figure 3.3). A similar trend of IL-18 up-regulation in β-glucan supplemented birds was seen in the duodenum and ileum, but these results were not significant (P = 0.093 and P = 0.055, respectively). On d14, an effect of dietary treatment on IL-18 gene expression was seen in all intestinal segments ($P \leq 0.019$). Birds that received 0.02% YGT supplemented feed had reduced IL-18 gene expression in the duodenum

when compared to the controls ($P < 0.05$). IL-18 expression in the 0.1% YGT fed group was not found to be different from the controls or the 0.02% YGT treated birds. IL-18 gene expression was down-regulated in the jejunum of the 0.1% YGT supplemented group while not different in the 0.02% YGT fed birds when compared to the control group ($P < 0.05$). IL-18 expression was significantly down-regulated in both YGT supplemented groups in the ileum ($P < 0.05$).

On d7, IFN-γ expression was down-regulated ($P < 0.009$) in birds fed 0.1% YGT supplemented diet in all intestinal segments ($P < 0.05$) (Figure 3.4). No differences in IFN-γ gene expression among treatment groups were observed on d14 in any intestinal segment.

There was no influence of diet observed for iNOS gene expression on d7 in the small intestine (Figure 3.5). An effect of dietary treatment on iNOS expression was evident in all intestinal sections on d14 ($P < 0.003$). In the 0.1% YGT supplemented group, iNOS gene expression was significantly increased in the duodenum and ileum when compared to the controls ($P < 0.05$). In the jejunum, neither β-glucan supplemented group was found to be significantly different from the controls, but the two supplemented groups were different from each other with the 0.1% YGT fed birds having higher iNOS expression ($P < 0.05$). Gene expression in the 0.02% YGT fed birds was not found to be different from the controls in any intestinal segment. A general summary of relative gene expression results is provided in Table 3.3.

Discussion

In this study, the effects of β-glucan supplementation in broiler chick diets were investigated. Regarding performance, no significant differences were found in BW or BWG among the treatment groups. Previous studies have found varying results debating the effects of β-glucans on performance in poultry. These data confirm previous research where β-glucan

supplementation did not negatively impact performance. Zhang et al. (2008) found that β-glucans derived from the yeast *S. cerevisiae* at 50 and 75mg/kg in the diet significantly increased BW and BWG at 50 and 75 mg/kg in the diet. In a comparable study, Rathgeber et al. (2008) found higher BW in broilers during the grower phase of production. Similar results have been observed in pig studies where β-glucan supplementation improved performance (Dritz et al., 1995; Hahn et al., 2006; Li et al., 2006). Some researchers have reported a decrease in performance following β-glucan supplementation in chickens (Huff et al., 2006). These results could be due to reallocation of energy towards immune development resulting in inefficient nutrient utilization for advancing growth. Correlating with our findings, several studies have found no significant effects of β-glucans on growth performance, suggesting that β-glucans do not have a negative impact on performance in either non-challenged (Cheng et al., 2004; Chae et al., 2006; Morales-Lopez et al., 2009) or challenged settings (Chen et al., 2006; Chen et al., 2008). The inconsistent results found in these studies could be due to a variety of reasons such as differences in the source of the β-glucan or the presence and type of challenge utilized. Further research is required in order to pinpoint the optimal dosage of β-glucan in order to achieve consistently favorable results in poultry.

In this study, the relative size of the bursa and spleen were unaffected by dietary treatment. These results were consistent with the findings of Rathgeber et al. (2008) where immune organ weights of broilers were not influenced by yeast β-glucan treatment at day 14 or 38. However, in another trial presented in the same paper, bursas in the treated group were found to be smaller than in the control birds at day 38 (Rathgeber et al., 2008). These results contradict the findings of a previous study by Guo et al. (2003) in which yeast β-glucan supplementation resulted in increased relative bursa and spleen weights.

Interleukin-8 is a chemokine produced primarily by macrophages and is an important mediator of the innate immune response. Its primary function is as a chemoattractant that induces the migration of its target cells to the site of inflammation. The observed reduction in IL-8 gene expression in the intestine on both d7 and 14 suggests dietary β-glucan acts as an anti-inflammatory immunomodulator. Considering the function of IL-8, these data appear to contradict previous findings where increased migration of neutrophils was seen as a result of β-glucan treatment (Liang et al., 1998; LeBlanc et al., 2006). However, these studies were performed in challenged rats and utilized PGG, a soluble form of β-glucan, whereas we used an insoluble fraction and no challenge, which may account for the differences seen. These conflicting results may suggest that β-glucans have different immunomodulatory functions in challenged versus non-challenged situations. It is also possible that, given the fact we only looked at gene expression at two time points and the dynamic nature of cytokine production, we may have missed an initial up-regulation of IL-8.

Interleukin-18 is a pro-inflammatory cytokine that is also primarily produced by macrophages. IL-18 works in connection with IL-12 to induce a cell-mediated immune response following exposure to a pathogen. Its major targets are Th1 cells, which subsequently secrete IFN-γ that plays an essential role in activating macrophages (Gobel et al., 2003). On d7, an up-regulation of IL-18 gene expression was observed due to 0.02% β-glucan treatment in the jejunum with similar, but non-significant, trends being seen in the duodenum and ileum. This was followed by a decrease in IL-18 expression in the duodenum of the 0.02% YGT supplemented group as well as a decrease in both supplemented groups in the ileum on d14. Though differences among groups were subtle, these preliminary data indicate a possible initial proinflammatory immune response followed by an enhanced anti-inflammatory response due to

dietary β-glucan treatment. To our knowledge, the effects of β-glucan supplementation on IL-18 mRNA levels have not been reported in avian or mammalian species.

Interferon-γ is a vital cytokine that plays a central role in regulating the innate and adaptive immune responses. This cytokine is responsible for promoting Th1 cell differentiation, suppressing Th2 cell activity, and enhancing innate immune cell activation and function (Tizard, 2009). We observed a down-regulation of IFN-γ gene expression in the 0.1% YGT fed birds in all intestinal segments on d7. These data suggest a flux in the Th1/Th2 paradigm favoring more of a Th2 and anti-inflammatory response. These data oppose the findings of Zhang et al. (2008) where IFN-γ production was increased due to β-glucan supplementation in poultry. Supporting our findings, it has been reported that intraperitoneal injections of β-glucan reduced IFN-γ production induced by concanavalin A in mice (Pelizon et al., 2003). Contrary to these findings, Xiao et al. (2004) found that yeast β-glucans had no effect on IFN-γ production by peripheral blood monocytes isolated from treated pigs. However, this study utilized pigs that had been experimentally infected with porcine reproductive and respiratory syndrome virus, which may account for the differences observed (Xiao et al., 2004).

When exposed to antigens or chemotactic agents, macrophages will begin to produce iNOS. This enzyme leads to the production of nitric oxide, which will subsequently react with superoxide anions to generate toxic derivatives, allowing macrophages to proficiently kill several types of pathogens (Tizard, 2009). We observed no significant differences in iNOS gene expression on d7, though a similar trend was observed in all three intestinal sections where iNOS expression appeared to increase in the 0.02% YGT fed group but decrease in the 0.1% YGT fed group. On d14, relative iNOS gene expression was up-regulated in the 0.1% YGT supplemented birds in all intestinal segments. It has been reported in poultry that the introduction of β-glucan

induces the production of nitrite by macrophages once they have been activated. Nitrite is a product of nitric oxide synthase gene activity (Guo et al., 2003). This up-regulation in iNOS implies an enhanced capability of macrophages to kill invading pathogens allowing the host to eliminate infectious threats more efficiently.

The down-regulation of IL-8, IL-18 and IFN-γ seen in this study suggest an anti-inflammatory effect of β-glucan. Similar results have been noted in mammalian species where pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α were reduced due to β-glucan treatment (Li et al., 2005; Bedirli et al., 2007). Furthermore, Li et al. (2005) found that production of the anti-inflammatory cytokine IL-10 was amplified during β-glucan exposure. However, those experiments utilized challenges in order to determine the effects of β-glucan treatment. Contradicting studies have noted greater relative abundance of TNF-α mRNA in spleens from lipopolysaccharide challenged pigs fed β-glucan as well as heightened IL-1β expression in the intestine (Eicher et al., 2006). Pelizon et al. (2005) also found that splenocytes from mice previously treated with β-glucan produced higher IL-12p40, IL-12p70, and TNF-α following stimulation with *S. aureus* suggesting more of a pro-inflammatory response. These varying results may be due to the variety of sources, extraction and purification procedures, and administration methods of β-glucans, as well as the challenge utilized.

In conclusion, these data suggest that dietary inclusion of yeast derived β-glucan does not carry a negative impact on broiler performance in a non-infectious setting to 14d of age. When looking at the immune response, β-glucans appear to have an anti-inflammatory function by altering cytokine profiles, especially those cytokines characterized as being of Th1 cells. To our knowledge, this study is the first to evaluate the gene expression profiles of these particular cytokines in response to β-glucan supplementation in poultry in a non-challenge model. Because

of the variation in responses found due to the different levels of β-glucan included in the diet, as well as a multitude of different results found throughout the literature, more research is required to determine optimal dosage for performance and immune modulation in a non-challenge setting as well as various disease situations.

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Table 3.1. Composition of basal diet

Ingredient	**%**
Corn	57.894
Soybean Meal	32.874
Poultry by Product	3.000
Poultry Fat	2.563
Dicalcium Phosphate	1.434
Limestone	0.943
Salt	0.412
DL-Methionine	0.329
L-Lysine	0.268
Trace Mineral Premix	0.100
Vitamin Premix	0.100
L-Threonine	0.072
Selenium Premix	0.011
TOTAL	**100.000**

Table 3.2. Primers used for relative real-time PCR[1]

Target	Accession No.	Nucleotide sequence (5' → 3')
GAPDH_F	NM_204305	CCTAGGATACACAGAGGACCAGGTT
GAPDH_R		GGTGGAGGAATGGCTGTCA
IL-8_F	NM_205498	TCCTGGTTTCAGCTGCTCTGT
IL-8_R		CGCAGCTCATTCCCCATCT
IL-18_F	NM_204608	AGGTGAAATCTGGCAGTGGAAT
IL-18_R		TGAAGGCGCGGTGGTTT
IFN-γ_F	NM_205149	GCTCCCGATGAACGACTTGA
IFN-γ_R		TGTAAGATGCTGAAGAGTTCATTCG
iNOS_F	D85422	CCTGTACTGAAGGTGGCTATTGG
iNOS_R		AGGCCTGTGAGAGTGTGCAA

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

Table 3.3. Summary of results: Changes in relative gene expression due to dietary β-glucan supplementation

Gene	**Leukocyte Association**	**Gene Expression**
IL-8	Innate	↓
iNOS	Innate	↑
IL-18	Th1	↑↓
IFN-γ	Th1	↓

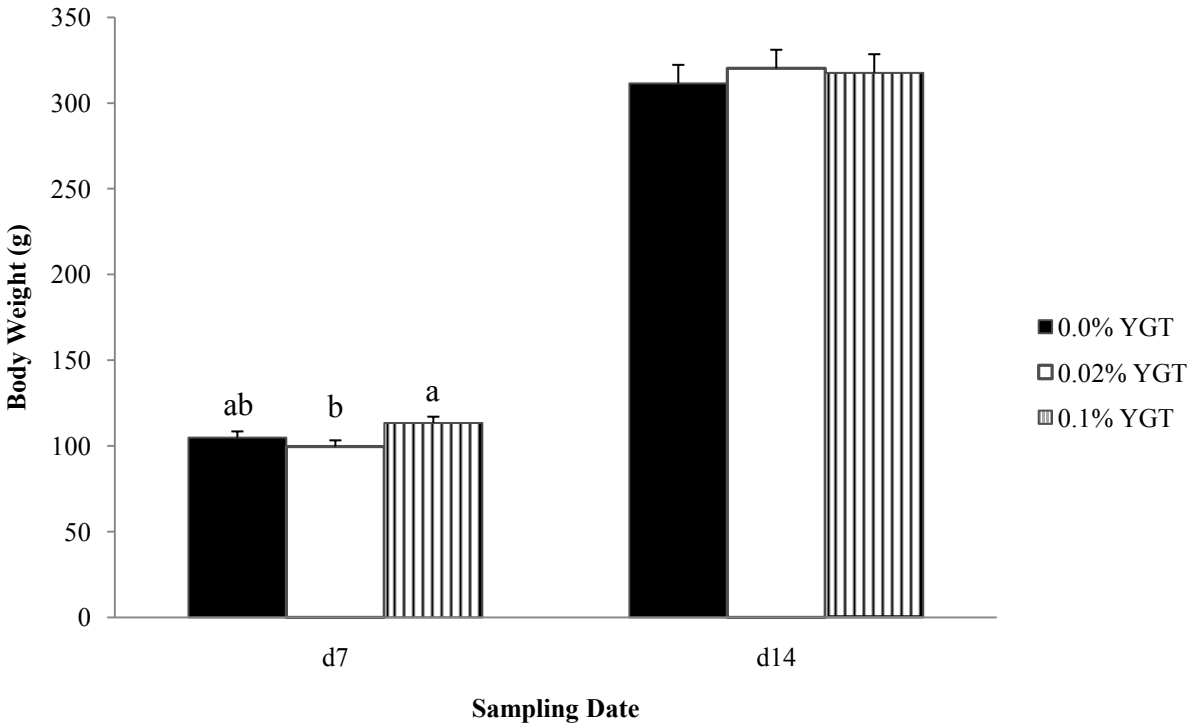

Figure 3.1. Effect of β-glucan supplementation on body weight of Cobb 500 broiler chicks on d7 and d14. Data are represented as LS Means + SEM (n=10 birds/treatment). YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant effect of dietary treatment on d7 ($P = 0.041$).

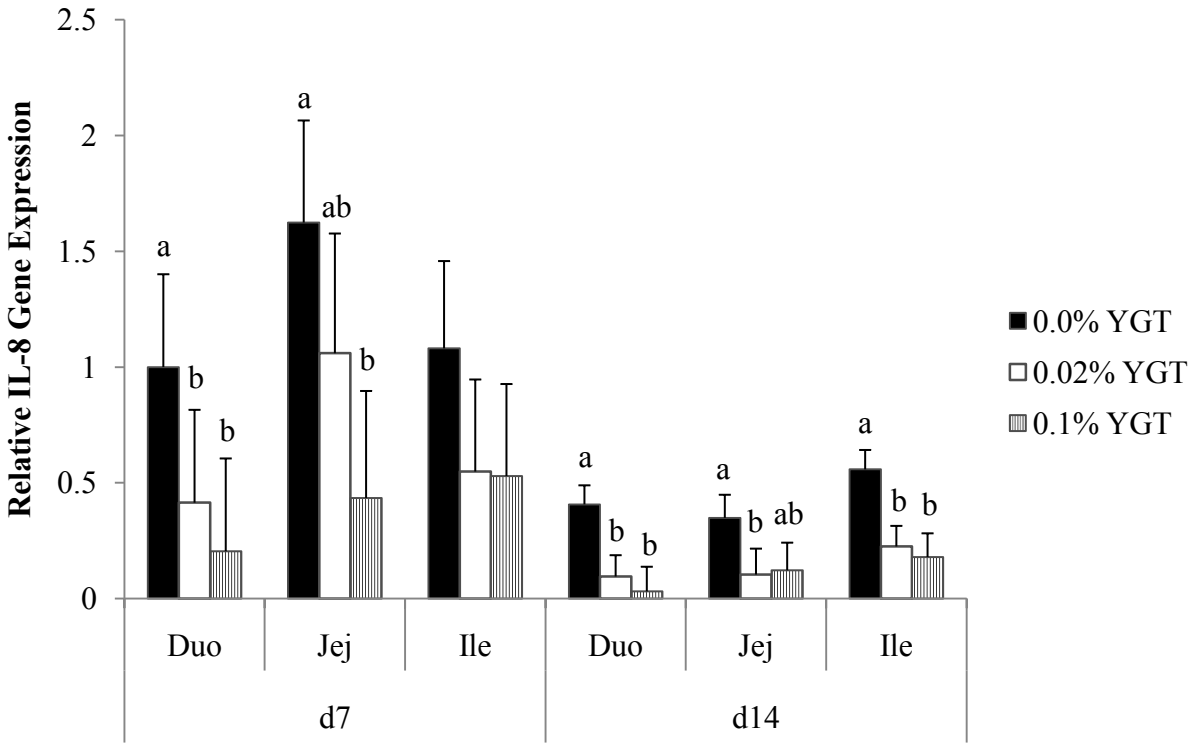

Figure 3.2. Effect of β-glucan supplementation on relative IL-8 expression in the small intestine of Cobb 500 chicks on d7 and d14. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d7 as the calibrator. Data are represented as LS Means + SEM. YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant effect of dietary treatment on d7 in the duodenum ($P = 0.001$) and jejunum ($P = 0.005$) and on d14 in the duodenum ($P < 0.0001$), jejunum ($P = 0.037$), and ileum ($P = 0.020$).

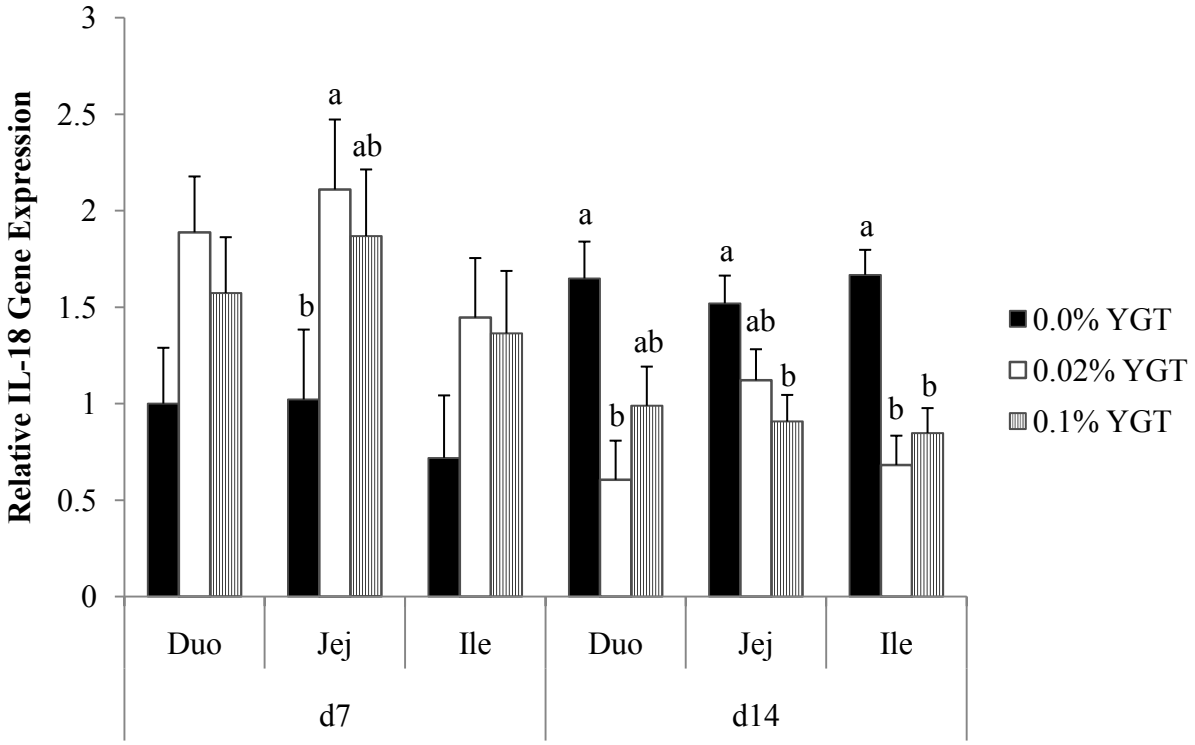

Figure 3.3. Effect of β-glucan supplementation on relative IL-18 expression in the small intestine of Cobb 500 chicks on d7 and d14. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d7 as the calibrator. Data are represented as LS Means + SEM. YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant effect of dietary treatment on d7 in the jejunum ($P = 0.030$) and on d14 in the duodenum ($P = 0.008$), jejunum ($P = 0.019$), and ileum ($P = 0.0003$).

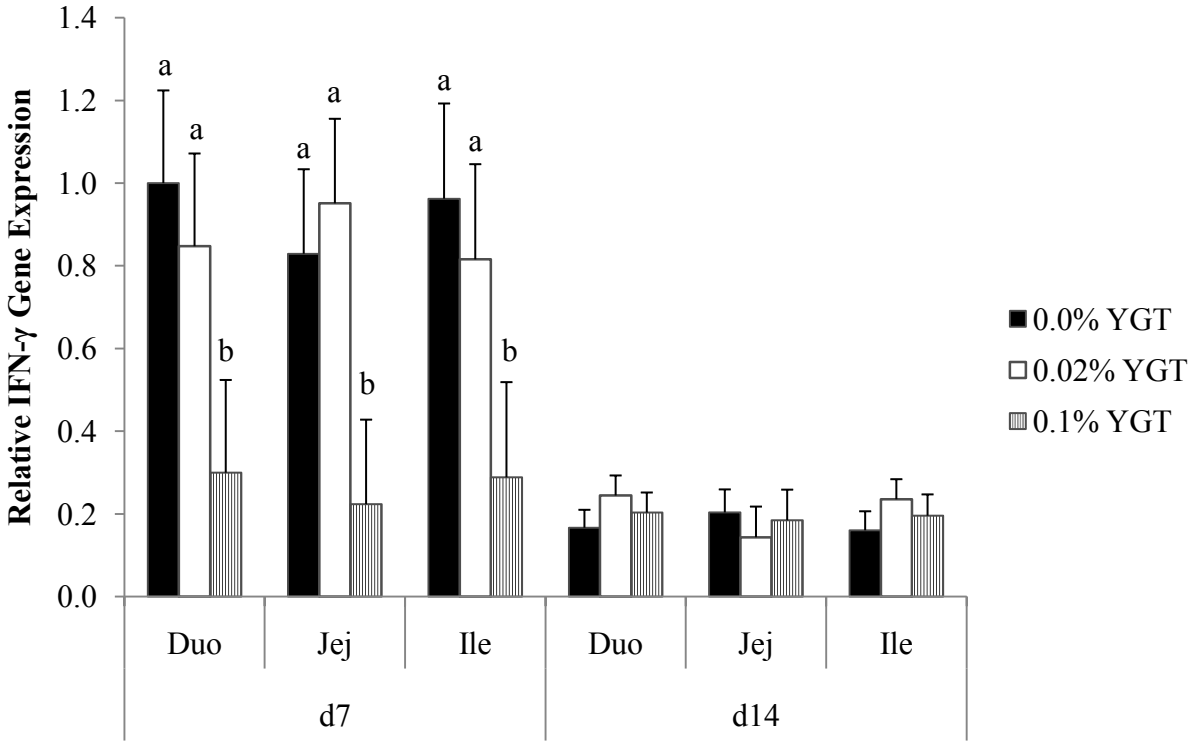

Figure 3.4. Effect of β-glucan supplementation on relative IFN-γ expression in the small intestine of Cobb 500 chicks on d7 and d14. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d7 as the calibrator. Data are represented as LS Means + SEM. YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant effect of dietary treatment on d7 in the duodenum ($P = 0.001$), jejunum ($P = 0.0002$), and ileum ($P = 0.009$).

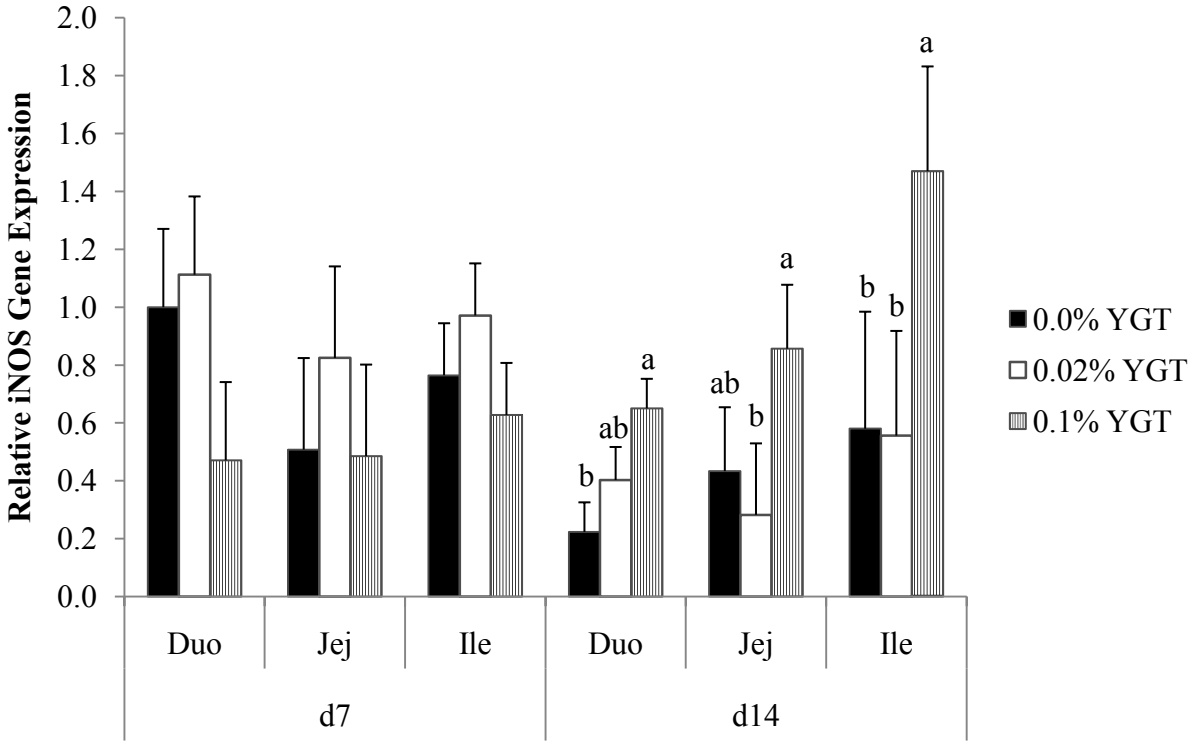

Figure 3.5. Effect of β-glucan supplementation on relative iNOS expression in the small intestine of Cobb 500 chicks on d7 and d14. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d7 as the calibrator. Data are represented as LS Means + SEM. YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant effect of dietary treatment on d14 in the duodenum ($P = 0.002$), jejunum ($P = 0.003$), and ileum ($P = 0.0002$).

CHAPTER IV

Effects of Dietary β-glucan on Performance and Immune Response of Broiler Chicks during an *Eimeria* Challenge

ABSTRACT: Escalating consumer concerns regarding the presence of chemical residues in poultry products and bacterial resistance have placed the poultry industry under mounting pressure to eliminate the use of chemotherapeutic agents as feed additives. One possible alternative receiving increased attention is the use of immunomodulators such as β-glucan. A study was conducted to investigate the effects of a yeast derived β-glucan (Auxoferm YGT) on broiler chick performance, immune organ weights, lesions scores, peripheral blood cell profiles, and immune-related gene expression during a mixed *Eimeria* infection. On day of hatch, 1440 broiler chicks were randomly assigned to one of three dietary treatments: 0%, 0.02% or 0.1% YGT. On day (d) 8 post-hatch, 8 of the 16 replicate pens/treatment were challenged with a mixed inoculum of *Eimeria acervulina* (50,000 oocysts/bird), *E. maxima* (10,000 oocysts/bird) and *E. tenella* (2,500 oocysts/bird). Measurements were taken and samples collected on days 4, 7, 10, 14 and 21 post-hatch. There were no significant differences among the dietary treatment groups for BW, BWG, feed intake, feed conversion, immune organ weights or mortality over the course of the experiment. On d14, 3 birds per pen (n=24/treatment) were scored for intestinal coccidia lesions. Gross lesion severity was significantly reduced in the duodenum and jejunum of birds supplemented with 0.1% YGT. Also, β-glucan supplementation at 0.1% significantly increased heterophil to lymphocyte ratio prior to the *Eimeria* sp. challenge on d7. A similar trend was observed on d21 where challenged birds fed 0.02% YGT had significantly higher ratios than the control fed birds. Dietary treatment had no effect on Interleukin (IL)-4, IL-8, IL-13, IL-18, inducible nitric oxide synthase (iNOS), and mucin (Muc)-1 or Muc-2 during the first

week post-hatch. After d8, IL-4 expression was down-regulated in the non-challenged birds with 0.1% YGT diet supplementation. On d10, iNOS expression was down-regulated in the ileum of challenged birds fed 0.1% YGT. Expression of iNOS was up-regulated in the ileum and jejunum of challenged birds fed 0.1% YGT on d14 when compared to the non-challenged birds fed the same diet. Muc-1 expression was significantly decreased after d8 due to 0.1% YGT dietary supplementation. On d14, Muc-2 expression was decreased due to the *Eimeria* infection in the 0.1% YGT fed birds. The addition of dietary β-glucan did not significantly affect IL-8, IL-13 or IL-18 expression after d8. These results suggest that β-glucans do not have detrimental effects on performance and are capable of altering immune-related gene expression profiles favoring a T helper type-1 (Th1) cell response. More research is needed to determine optimal dosage for immune modulation in various production settings and disease situations.

Introduction

Parasitism of the intestinal tract is a major stress factor leading to decreased nutrient utilization and reduced performance in livestock and poultry. Coccidiosis is an intestinal infection brought about by intracellular protozoan parasites belonging to the genus *Eimeria* (Dalloul and Lillehoj, 2005). There are seven *Eimeria* species that infect chickens: *E. acervulina*, *E. maxima*, *E. tenella*, *E. brunetti*, *E. necatrix*, *E. mitis* and *E. praecox*. *Eimeria* sp. invade the intestinal lining and disrupt the enterocyte layer resulting in gross lesions, nutrient malabsorption and reduced performance (Brake et al., 1997). Coccidiosis is regarded as the parasitic disease that has the greatest economic impact on the poultry industry with total losses estimated at a devastating $3 billion annually worldwide (Dalloul and Lillehoj, 2006). The monetary loss related to coccidiosis is primarily due to the costs of prophylactic measures, treatments, decontamination, restocking and lost revenues due to decreased production.

Currently, the poultry industry relies heavily on the use of anticoccidial feed additives to prevent coccidiosis outbreaks. Recently, consumers have become exceedingly concerned about chemical residues in poultry products and increased pathogen resistance. These concerns have resulted in mounting pressure to ban chemotherapeutic agents from animal feeds. The controversy and potential risk have resulted in the ban of antibiotics as feed additives by the European Union as of January 1, 2006. Unfortunately, this ban has led to a decline in animal health and higher variability in carcass size and meat characteristics (Casewell et al., 2003).

The need to move away from chemotherapeutic control of coccidiosis has prompted intense search for alternatives capable of maintaining animal health without negatively affecting performance. Beta-glucans have received rising interest due to their immunomodulating capabilities and higher acceptance by consumers. Beta-glucans belong to a group of physiologically active compounds termed biological response modifiers due to their ability to activate the immune system. Beta-glucans are glucose polymers that are structural components of the cell wall of many bacteria, fungi, algae and yeast, as well as cereal grains such as oat and barley. Large variation exists in the structure of β-glucans from these different sources that ultimately results in differences in their physiological functions (Volman et al., 2008). Those β-glucans derived from yeast and fungi are termed 1,3/1,6-β-D-glucans and consist of 1,3 β-linked glycopyranosyl backbone with variable frequency of 1,6 β-linked side chains (Harada and Ohno, 2008). Due to their highly branched structure, 1,3/1,6-β-D-glucans are considered the most effective type of β-glucans in terms of stimulating the immune system (Vetvicka and Vetvickova, 2007).

The effects of β-glucans as biologically active immunomodulators have been well noted for over 50 years. In mammals, β-glucans enhanced migration, phagocytic activity, and overall

efficiency of innate immune cells such as neutrophils (Liang et al., 1998; Mucksova et al., 2001; Pelizon et al., 2005; LeBlanc et al., 2006; Vetvicka and Vetvickova, 2007). Not only do β-glucans enhance innate immunity, but they also augment the adaptive immune system by increasing B and T lymphocyte proliferation and antibody production (Estrada et al., 1999; Krakowski et al., 1999; Li et al., 2005; Hahn et al., 2006; Wang et al., 2008).

Only recently has research been published concerning the effects of β-glucans in poultry. Exposure to β-glucan enhanced proliferation and phagocytizing efficiency of avian macrophages (Guo et al., 2003) and heterophils (Lowry et al., 2005). Similar to what has been found in mammals, birds supplemented with β-glucans had amplified humoral (Guo et al., 2003; Zhang et al., 2008) and cell-mediated immune responses (Chen et al., 2003; Chae et al., 2006). The immune enhancing capabilities of β-glucans have resulted in the clearance of several economically important pathogens such as *Salmonella enterica* and *Escherichia coli*, further asserting their potential use as an antibiotic alternative.

The objective of this study was to determine the effects of dietary supplementation of a β-glucan derived from the yeast *Saccharomyces cerevisiae* on performance, immune organ weights, peripheral blood cell profiles, and intestinal immune-related gene expression in broiler chicks with or without an *Eimeria* sp. challenge.

Materials and methods

Birds, diets, and challenge model

This project was approved and conducted under the guidelines of the Institutional Animal Care and Use Committee at Virginia Tech. On day of hatch (DOH), 1440 straight-run Cobb 500 broiler chicks were picked up from a commercial hatchery (Pilgrim's Pride, Broadway, VA) and transported to the Virginia Tech Turkey Research Farm. Chicks were weighed by pen for equal

weight distribution and placed into 48 floor pens (8 replicate pens/treatment) consisting of concrete floors and pine shavings with 30 chicks per pen (0.1 m^2/bird). Chicks had ad libitum access to water and a non-medicated corn/soy based starter diet in mash form that contained either 0%, 0.02% or 0.1% YGT Auxoferm (β-glucan extracted from *S. cerevisiae*, AB Vista, UK). The complete diet formulation and nutrient content is presented in Table 4.1. On day 8 post hatch, half of the chicks (24 pens) were orally gavaged with 1 mL of a mixed inoculum containing 50,000 *Eimeria acervulina*, 10,000 *E. maxima*, and 2,500 *E. tenella* sporulated oocysts.

Performance parameters

Birds were weighed prior to placement on DOH and on days 4, 7, 10, 14, and 21 with birds in each pen being weighed as a group. Mean body weights (BW) for each treatment group were calculated from the pen replicates for each weigh day. Average body weight gain (BWG) was calculated based on pen weights. Feed intake (FI) of each group was measured at the same time periods as BW (d4, 7, 10, 14, and 21) with cumulative averages calculated. Feed conversion ratio (FCR) was calculated using BW and FI and adjusted for mortality. Mortality was recorded on a daily basis throughout the trial.

Immune organ weights

On d4, 7, 10, 14, and 21, 8 birds/treatment (1 bird/replicate) were randomly selected and euthanized by cervical dislocation. The bursa of Fabricius and spleen were weighed and relative organ weights were expressed as a percentage of live BW.

Lesion scoring

On d14, 3 birds per pen (n=24/treatment) were randomly selected and euthanized for

scoring of lesions from intestinal *Eimeria* challenge. Lesions were scored in the duodenum, jejunum and ceca by the method of Johnson and Reid (1970) based on score range from 0 (no gross lesion) to 4 (most severe lesion).

Peripheral blood cell profiling

Heterophil: lymphocyte ratios (H:L) were determined on d4, 7, 10, 14, and 21 using peripheral blood collected via the jugular vein from 8 birds/treatment (1 bird/replicate). Ratios were determined by preparing blood smears using a Morph Slide Spinner (Salem Specialties Inc., Salem, VA) and Modified Wright's stain with heterophils and lymphocytes counted to a total of 60 cells per slide.

Tissue sampling for gene expression analysis

Eight birds per treatment (1 bird/replicate) were sampled on d4, 7, 10, 14, and 21 post-hatch. Sampled chicks were killed by cervical dislocation and the small intestine was subsequently collected. The small intestinal segments (duodenum, jejunum, and ileum) were rinsed in cold PBS and minced. Samples were placed in an 8x8 cm piece of aluminum foil, snap frozen in liquid nitrogen, and stored at -80°C until analysis.

Total RNA extraction

Total RNA was extracted using the RNeasy kit according to the animal tissue protocol (Qiagen, Germantown, MD). Intestinal samples were removed from -80°C and placed on dry ice. A 20-30 mg aliquot of each sample was weighed and placed into a 2-mL microcentrifuge tube. The aliquots were placed back into -80°C until it was time for RNA extraction. For a short period of time, the aliquots were removed from -80°C and kept on dry ice until homogenization. Before homogenization, 600 µL of RLT Buffer (Qiagen) were added to the aliquot. The tissues

were homogenized using a 7 mm tip on a PowerMax AHS200 homogenizer (VWR) for 30-45 sec. The manufacturer's protocol was followed and the RNA was eluted by rinsing the column membrane twice with 25 µL of RNase-free water. The RNA concentration was determined at OD 260 in a spectrophotometer (NanoDrop ND-1000). The RNA purity was verified by evaluating the ratio of OD 260 to OD 280.

Reverse transcription

Total RNA was diluted to 0.2 µg/µL in nuclease-free water. Reverse transcription was accomplished by using the high capacity cDNA archive kit (Applied Biosystems, Carlsbad, CA). A 2X reverse transcription master mix was prepared consisting of 2 µL 10X reverse transcription buffer, 0.8µL 25X dNTPs, 2µL 10X random primers, 1 µL MultiScribe reverse transcriptase (50U/µL), and 4.2 µL nuclease-free water per reaction. Each reverse transcription reaction contained 10 µL of 2X RT master mix and 10 µL of 0.2 µg/µL RNA. The reverse transcription reaction was performed using a Veriti 96 well thermocycler (Applied Biosystems). Hybridization occurred at 25°C for 10 min followed by extension at 27°C for 120 min and inactivation of the reverse transcriptase at 85°C for 5 sec. The cDNA was stored at -20°C.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using an ABI 7500 FAST Real-Time PCR System (Applied Biosystems). The cDNA was diluted 1:30 in nuclease-free water then 1 µL of the diluted cDNA was added to each well of a 96-well plate. Next, 9 µL of real time PCR master mix containing 5 µL FAST SYBR Green Master Mix (Applied Biosystems), 0.5 µL each of 2 µM forward and reverse primers and 3 µL of sterile nuclease-free water per reaction were added to each well for a final volume of 10 µL. During the PCR reaction, samples were subjected to an initial denaturation phase at 95°C for 20 sec followed by 40 cycles of

denaturation at 95°C for 3 sec and annealing and extension at 60°C for 30 sec. Interleukin (IL)-4, IL-8, IL-13, IL-18, inducible nitric oxide synthase (iNOS), mucin-1 (Muc-1) and Muc-2 gene expression was analyzed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. Each reaction was run in duplicate. Primers were designed using the Primer Express 3.0 software (Applied Biosystems) and synthesized by MWG Operon (Huntsville, AL). Primer sequences are listed in Table 4.2.

Quantitative real-time PCR analysis

Results from qRT-PCR were analyzed using the 7500 Real-Time PCR software (Applied Biosystems). Average gene expression relative to the GAPDH endogenous control for each sample was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The calibrator for each gene was the average ΔCt value from the duodenum of the dietary control group on d4 (first sampling).

Statistical analysis

Data were analyzed using the Fit Model platform in JMP 7.0 (SAS Institute Inc., Cary, NC). For performance parameters, on days 4 and 7, diet, time and their interaction were placed in the model. On d10-d21, diet, challenge and time and all appropriate two- and three-way interactions were placed in the model. For intestinal gene expression, intestinal segment was also placed in the model. Differences among groups were compared with Tukey-HSD following analysis. Values were considered statistically different at $P < 0.05$. Results are reported as least squares means (LS Means) with standard errors (SE).

Results

Performance parameters and immune organ weights

During the first week post-hatch, there was a trend for birds fed 0.02% YGT to have higher BWG than both the control and the 0.1% YGT fed birds ($P = 0.052$) (Figure 4.1). Following the *Eimeria* infection, time and challenge presented a two-way interaction ($P < 0.0001$) with the challenged birds having an expected decrease in BW compared to the non-challenged birds on both d14 and d21 ($P < 0.05$) (Figure 4.2). The *Eimeria* challenge also resulted in decreased BWG regardless of dietary treatment from d8 to d21 ($P < 0.0001$) (Figure 4.3). Dietary treatment had no effect on BW (Table A.2) or BWG (Table A.3) following the *Eimeria* infection.

Beta-glucan supplementation did not have an effect on FI (Table A.2) or FCR (Table A.3) at any time point during the study. Following *Eimeria* inoculation, challenged birds had a decrease in FI ($P = 0.0001$) (Figure 4.4) and increase in FCR ($P = 0.004$) (Figure 4.5). When compared to the non-challenged controls, mortality was increased by 42% due to the *Eimeria* challenge ($P < 0.0001$) (Figure 4.6). The addition of β-glucan had no effect on mortality (data not shown).

During the first week post-hatch, β-glucan supplementation had no effect on bursa or spleen weights (Table A.2). Neither diet nor challenge had an effect on bursa weight after d8 (Table A.2). Time, dietary treatment and the *Eimeria* challenge presented a three-way interaction for spleen weight ($P = 0.01$) though no differences were seen among groups (Figure 4.7).

Lesion scoring

Dietary treatment had an effect on lesion scores in the duodenum ($P = 0.044$) and

jejunum ($P = 0.021$) on d14. Gross lesion severity was significantly reduced in the duodenum and jejunum of birds supplemented with 0.1% YGT when compared to controls ($P < 0.05$) (Figure 4.8). Though a similar trend was observed, there were no significant differences among dietary treatment groups in the ceca ($P = 0.180$).

Peripheral blood cell profiling

There was a main effect of diet ($P < 0.01$) resulting in an increase in H:L in the 0.1% YGT fed group over the control and 0.02% YGT fed groups ($P < 0.05$) (Figure 4.9). Following *Eimeria* inoculation, time, diet and challenge presented a 3-way interaction ($P =0.03$). On d21, challenged birds fed 0.02% YGT had higher H:L than the challenged control fed birds ($P \leq 0.05$) (Figure 4.10). The H:L was also elevated in the challenged birds fed 0.1% YGT though this was not found to be significantly different from the controls. There were no differences observed among groups on d10 or d14.

Intestinal gene expression

Intestinal IL-4 expression was higher on d7 than on d4 ($P < 0.01$) (Figure 4.11). There was no effect of β-glucan supplementation seen during the first week post-hatch. Following administration of the *Eimeria* oocysts, dietary treatment and challenge presented a two-way interaction in the intestine ($P = 0.01$) where non-challenged birds fed 0.1% β-glucan had the lowest level of IL-4 expression when compared to the other treatment groups ($P < 0.05$) (Figure 4.12). After d8, there was a time by intestinal section interaction ($P < 0.01$). Interleukin-4 expression on d10 was higher in the ileum than in the duodenum; however, on d14, IL-4 expression was found to be higher in the duodenum than in the ileum. On d21, IL-4 expression was higher in the duodenum than in the other intestinal segments ($P < 0.05$) (Figure 4.13).

Overall, IL-8 expression on d7 was significantly up-regulated compared to d4 ($P < 0.01$)

(Figure 4.14). There was no effect of dietary treatment seen in the intestine on d4 or d7. After d8, a two way interaction among time and dietary treatment was observed ($P = 0.04$). In the 0.1% YGT fed birds, IL-8 expression was higher on d14 and d21 when compared to d10 ($P < 0.05$). There were no differences in IL-8 expression observed among the control fed groups over time (Figure 4.15). Intestinal segment and time presented a two-way interaction after d8 ($P = 0.03$). Interleukin-8 expression was higher in the ileum than in the duodenum on d10 and higher in both the jejunum and ileum on d14 ($P < 0.05$) (Figure 4.16). On d14, IL-8 expression was higher in the ileum than in the jejunum ($P < 0.05$). There were no differences among intestinal sections observed on d21 (Figure 4.16). An interaction of intestinal segment and challenge ($P = 0.02$) resulted in the ileum having the higher IL-8 expression than the duodenum regardless of the *Eimeria* challenge ($P < 0.05$) (Figure 4.17). Time and challenge also demonstrated a two-way interaction ($P = 0.01$). Challenged birds had the highest IL-8 expression on d14 when compared to d10 or d21 (Figure 4.18).

Interleukin-13 expression was not affected by time, intestinal section, or dietary treatment during the first week post-hatch. After d8, time, intestinal section and challenge displayed a three-way interaction ($P = 0.04$). On d10, there were no differences among groups observed (Figure 4.19). Interleukin-13 expression was higher on d10 than on d14 in the ileum of non-challenged birds ($P < 0.05$) (Figure 4.19). There were no differences detected on d14 or d21 based on challenge or intestinal section. Though not significant, after d8, there was a tendency for birds fed 0.1% YGT to have lower intestinal IL-13 expression than the control fed birds ($P = 0.063$) (Figure 4.20).

On d7, IL-18 expression was up-regulated in the intestine when compared to d4 ($P < 0.01$) (Figure 4.21). There was no effect of dietary treatment on d4 or d7 in any intestinal

segment. Following inoculation on d8, intestinal segment, time, and *Eimeria* challenge demonstrated a three-way interaction for IL-18 gene expression ($P = 0.04$) (Figure 4.22). When compared to d10, IL-18 expression was higher on d14 and d21 in all three intestinal sections ($P < 0.05$). There were no differences observed among groups based on intestinal section or challenge (Figure 4.22). Intestinal section and dietary treatment also produced a two-way interaction ($P = 0.02$) (Figure 4.23). In the 0.1% YGT fed birds, IL-18 expression was highest in the jejunum, intermediate in the duodenum and lowest in the ileum ($P < 0.05$) (Figure 4.23). Despite the fact that no significant differences were seen among treatment groups, there was a strong trend for IL-18 expression to be up-regulated in the challenged birds due to β-glucan exposure ($P = 0.07$) (Figure 4.24).

Intestinal expression of iNOS increased on d7 above what was seen on d4 ($P < 0.01$) (Figure 4.25). During the first week, an effect of intestinal section was detected ($P = 0.01$) (Figure 4.26). The ileum had significantly higher iNOS expression than the duodenum ($P < 0.05$) while the jejunum was intermediate and statistically similar to other sections (Figure 4.26). Dietary supplementation of β-glucan had no effect on iNOS gene expression on d4 or d7. After d8, time, intestinal segment, diet, and challenge presented a four-way interaction for iNOS gene expression ($P < 0.01$) (Figure 4.27a,b,c). On d10, no differences were seen among groups in the duodenum or jejunum (Figure 4.27a and 4.27b respectively). Challenged birds fed 0.1% YGT showed a down-regulation of iNOS gene expression in the ileum compared to non-challenged birds fed the same diet ($P < 0.05$) (Figure 4.27c). There were no differences observed among the control fed groups on d10 in the ileum (Figure 4.27c). On d14, there were no differences among groups observed in the duodenum (Figure 4.27a). Expression of iNOS was up-regulated in the jejunum and ileum of challenged birds fed 0.1% YGT on d14 when compared to the non-

challenged birds fed the same diet ($P < 0.05$)(Figure 4.27b and 4.27c respectively). The relative expression of iNOS on d14 was not affected by challenge in the control fed birds. There were no differences in iNOS gene expression seen among groups on d21 (Figure 4.27a,b,c).

Time and intestinal section exhibited a two-way interaction for relative Muc-1 gene expression during the first week post-hatch ($P = 0.02$) (Figure 4.28). However, differences among intestinal sections were not distinguished on d4 or d7. There was no effect of dietary treatment present in the intestine on d4 or d7. Following the administration of the *Eimeria* challenge, time and challenge produced a two-way interaction ($P < 0.01$) (Figure 4.29). The effects of the challenge were obvious on d14 where challenged birds had significantly higher Muc-1 expression than the non-challenged birds ($P < 0.05$). There were no differences based on challenge observed on d10 or d21 (Figure 4.29). Time and intestinal section also exhibited a two-way interaction after d8 ($P = 0.02$) (Figure 4.30). On d14, Muc-1 expression was higher in the duodenum than in the ileum ($P < 0.05$). There were no differences among intestinal sections seen on d10 or d21 (Figure 4.30). There was a main effect of treatment observed over time where supplementation of 0.1% YGT resulted in decreased Muc-1 expression ($P = 0.01$) (Figure 4.31).

Muc-2 expression was significantly decreased on d7 compared to d4 ($P = 0.01$) (Figure 4.32). During the first week, there was a significant effect of intestinal section ($P < 0.01$) (Figure 4.33). The ileum had the highest Muc-2 expression followed by the jejunum then the duodenum ($P < 0.05$) (Figure 4.33). There was no effect of dietary treatment on Muc-2 gene expression in the intestine from d4 to d7. Time, dietary treatment, and challenge presented a three-way interaction after d8 ($P < 0.01$) (Figure 4.34). Differences among groups were not discerned on d10 or d21. On d14, however, Muc-2 expression was decreased due to the *Eimeria* challenge in

the 0.1% YGT fed birds ($P < 0.05$). A similar trend was seen in the control fed birds on d14 though these differences were not found to be significant (Figure 4.34). A general summary of relative gene expression results is provided in Table 4.3.

Discussion

Although 1,3/1,6-β-D-glucans are well known to activate the innate and adaptive immune systems of mammals, their impact on the avian immune system still remains elusive. In this study, the influence of dietary β-glucan supplementation on broiler chicks with and without an *Eimeria* challenge was investigated. Regarding performance, there was an effect of treatment on BW observed prior to the *Eimeria* infection. Though no significant differences were found among treatment groups, there was a trend for birds fed 0.02% YGT to have higher body weight gains than the control fed birds during the first week post-hatch. These data confirm previous studies where β-glucan supplementation did not negatively or positively impact performance. Zhang et al. (2008) found that β-glucans derived from the yeast *S. cerevisiae* significantly increased BW and BWG at 50 and 75mg/kg in the diet. In a comparable study, Rathgeber et al. (2008) found enhanced BW in broiler chicks during the grower phase of production. Similar results were observed in pig studies where β-glucan supplementation improved performance (Dritz et al., 1995; Hahn et al., 2006; Li et al., 2006). Contrary to these findings, there have been reports of a decrease in performance following β-glucan supplementation in chickens (Huff et al., 2006). These results could be due to reallocation of energy towards immune development resulting in inefficient nutrient utilization for growth. Correlating with our findings, several studies have noted no significant effects of β-glucans on growth performance suggesting that β-glucans do not have a negative impact on performance in either non-challenge (Cheng et al., 2004; Chae et al., 2006; Morales-Lopez et al., 2009) or challenge settings (Chen et al., 2006;

Chen et al., 2008). The variable results of those studies could be due to a variety of reasons such as differences in the source and preparation of the β-glucan or the presence and type of challenge utilized. Further research is required in order to define the optimal source and dosage of β-glucans in order to achieve consistently favorable results in poultry.

The decreases in BW, BWG and FI, and increase in FCR and mortality due to the *Eimeria* inoculation were not surprising since coccidial infections are known to cause disruption to the intestinal mucosa resulting in nutrient malabsorption (Dalloul and Lillehoj, 2005). Furthermore, parasitic infections elicit nutrient demanding immune responses, which may have also contributed to the impaired growth.

In this study, the relative size of the bursa was unaffected by dietary treatment. Time, dietary treatment and the *Eimeria* challenge did present a three-way interaction for relative spleen weight; however, no differences among groups were observed. These findings support our previous work where β-glucan supplementation did not affect immune organ weights (unpublished data). Rathgeber et al. (2008) reported similar findings where immune organ weights of broiler chicks were not influenced by yeast β-glucan treatment at days 14 or 38. However, in another trial presented in the same paper, bursas in the treated birds were found to be smaller than in the control birds on day 38 (Rathgeber et al., 2008). These results contradict the findings of Guo et al. (2003), who reported increased relative bursa and spleen weights with yeast β-glucan supplementation.

Coccidia parasites are known to cause significant intestinal damage as they destroy enterocytes during the progression of their lifecycle (Allen and Fetterer, 2002). Even though the *Eimeria* challenge only resulted in a mild infection (based on lesion scores), dietary β-glucan supplementation at 0.1% resulted in significant decrease in lesion severity in the duodenum and

jejunum. Though not significant, a similar trend was observed in the ceca. These results indicate that dietary β-glucan acted as an immunoprotective agent and enhanced host defenses against *E. acervulina* and *E. maxima* in the duodenum and jejunum respectively.

Beta-glucans are known for their potent ability to induce nonspecific inflammatory reactions. An increase in H:L was observed prior to the *Eimeria* challenge due to β-glucan supplementation at 0.1%, as well as after the challenge on d21 in the 0.02% supplemented group. A similar trend was seen in the challenged birds on d10 but those results were not significant. This higher H:L ratio was due to an increase in the numbers of heterophils counted. The avian heterophil is considered to be equivalent to the mammalian neutrophil. These innate immune cells are highly phagocytic and make up the first line of cellular defense against a broad spectrum of microbial pathogens. Heterophils are the principal granulated leukocytes responsible for the acute inflammatory response in gallinaceous birds such as chickens (Harmon, 1998). In previous studies, the inclusion of β-glucan as a feed additive enhanced innate immunity through improved phagocytosis of live bacteria by heterophils isolated from chickens challenged with *Salmonella enterica*. Those heterophils were more efficient and capable of engulfing a significantly higher number of bacteria per cell than heterophils isolated from birds not supplemented with β-glucan (Lowry et al., 2005). In mammalian studies, yeast derived β-glucans have also demonstrated their ability to activate and enhance the function of phagocytic innate immune cells. Le Blanc et al. (2006) found that systemic treatment of poly-[1-6]-D-glucopyranosyl-[1-3]-D-glucopyranose glucan (PGG), a soluble form of yeast β-glucan, increased the migration of neutrophils to areas of inflammation in rats with experimentally induced acute bacterial pneumonia. These results were confirmed by Liang and colleagues (1998) where neutrophil numbers increased in β-glucan treated rats when exposed to

Staphylococcus aureus. Furthermore, neutrophils obtained from β-glucan treated rats and mice show heightened levels of respiratory burst resulting in enhanced clearance of pathogens (Liang et al., 1998; Mucksova et al., 2001; LeBlanc et al., 2006). Beta-glucan treatment also resulted in increased phagocytic activity and destructive capability of neutrophils isolated from mice (Vetvicka and Vetvickova, 2007). Our results indicate that dietary β-glucan enhances innate immunity by increasing heterophil numbers and inducing an inflammatory immune response during a challenge. In the absence of a challenge, there was no effect of β-glucan supplementation on H:L suggesting a priming of the innate immune system.

Numerous cytokines are involved in directing both innate and adaptive immunity. Interleukin-8 is a chemokine produced primarily by macrophages and is an important mediator of the innate immune response. Its primary function is as a chemoattractant that induces the migration of its target cells to the site of inflammation. While intestinal IL-8 expression in the control fed birds stayed relatively constant following the *Eimeria* challenge, the addition β-glucan at 0.1% was capable of modulating IL-8 expression resulting in enhanced expression on d14 and d21 over that of d10. This up-regulation of IL-8 is most likely responsible for the increased H:L observed on d21. Similar results have been reported in human based studies where β-glucan supplementation resulted in increased IL-8 production (Engstad et al., 2002; Vetvicka et al., 2008).

Interleukin-18 is a pro-inflammatory cytokine that is also primarily produced by macrophages. Interleukin-18 works in connection with IL-12 in order to induce a cell-mediated immune response following exposure to a pathogen. Its major targets are Th1 cells that subsequently secrete interferon (IFN)-γ, which plays an essential role in activating macrophages (Gobel et al., 2003). Though no significant differences among groups were detected, there was a

trend of IL-18 up-regulation due to β-glucan supplementation in the challenged birds primarily seen on d14 and d21. Supporting our findings, IFN-γ has also been reported to be produced in response to β-glucan exposure (Zhang et al., 2008). This cytokine is secreted by several cells including Th1 and cytotoxic T lymphocytes and is responsible for activating and enhancing antigen presentation by macrophages (Tizard, 2009). All together, the enhanced expression of IL-18 in challenged birds with β-glucan supplementation, modulation of IL-8 expression, and reports of increased IFN-γ production, provide strong evidence suggesting a favoring of the Th1 immune response.

When exposed to antigens or chemotactic agents, macrophages will begin to produce iNOS. This enzyme leads to the production of nitric oxide, which will subsequently react with superoxide anions to generate toxic derivatives allowing macrophages to proficiently kill several types of pathogens (Tizard, 2009). On d14, intestinal iNOS expression was enhanced in 0.1% YGT-fed birds due to the *Eimeria* challenge, while expression was not altered due to challenge in the control-fed birds. These results are supported by mammalian studies where β-glucan exposure augmented iNOS expression and nitric oxide production (Ljungman et al., 1998; Mucksova et al., 2001). The data presented by this study demonstrate the ability of β-glucans to induce the expression of iNOS mRNA, thus implying subsequent nitric oxide production. Because nitric oxide is a major mediator of the non-specific immune response against a wide scope of invading microbes, the ability to stimulate nitric oxide production is important (Ljungman et al., 1998). Not only does nitric oxide serve as an effector molecule leading to pathogen destruction, but it also acts as an initiator of acute inflammation through the up-regulation of pro-inflammatory cytokine gene expression including that of IL-8 (Remick and Villarete, 1996).

Interleukin-4, a representative of T_H2 cytokines, plays a key role in the stimulation of B lymphocytes, T lymphocyte proliferation and the differentiation of $CD4^+$ T cells into T_H2 cells (Fietta and Delsante, 2009). During this study, IL-4 expression was down-regulated in non-challenged birds supplemented with 0.1% β-glucan. The functions of IL-13, also characterized as a T_H2 cytokine, overlap considerably with those of IL-4. Though no significant differences were observed, there was trend for IL-13 expression to also decrease due to β-glucan supplementation. Taken together, the down-regulation of these two cytokines implies that β-glucan supplementation does not support a T_H2 cell mediated response.

Muc-1 and Muc-2 are the genes responsible for encoding for mucin production. Mucin is made up of glycoproteins and serves a protective function by binding to pathogens preventing their adhesion to the intestinal surface. T lymphocytes and T_H2 cytokines are mediators shown to regulate mucin production (Beum et al., 2005). Following the *Eimeria* challenge, β-glucan supplementation at 0.1% YGT resulted in a decrease in Muc-1 and Muc-2 expression in the intestine. These results further suggest a bias of β-glucan modulation towards a Th1 mediated response.

It is important to note that during the progression of this experiment, it became evident that our chicks had become infected with *E. coli* presumed to have been contracted from the hatchery. It is assumed that the increases in intestinal gene expression of IL-4, IL-8, IL-18 and iNOS, as well as the decrease in MUC-2 seen on d7, were a result of this "natural" *E. coli* infection. The effects of the infection appeared to diminish shortly after d7.

The small intestine is not only part of the digestive system, but it is one of the most important organs of the immune system. In this study, we demonstrate the ability of yeast derived β-glucan to modulate the Th1/Th2 balance within the small intestine. The addition of β-

glucan to the diet of broiler chicks subjected to an *Eimeria* infection skewed the host immune response toward aTh1 mediated response and consequently down-regulated the T_H2 mediated response. This response to β-glucan exposure has also been reported in human and mice studies (Kirmaz et al., 2005; Baran et al., 2007). The augmented pro-inflammatory response resulted in increased protection from the challenge as seen by the decreased lesion scores. The overall peak in immune response seen on d14 and decline by d21 further suggests that β-glucan supplementation does not result in a chronic inflammatory response, but instead primes the immune system in order to enhance its effectiveness during immunological challenges. Furthermore, the addition of β-glucan showed no signs of having a negative impact on performance parameters further suggesting that the immune system was not continuously being stimulated.

In conclusion, our data suggest that dietary inclusion of yeast derived β-glucan does not carry a negative or a positive impact on performance. When looking at the immune response, β-glucans modulate cytokine profiles, resulting in an enhanced Th1 mediated immune response during an *Eimeria* infection. Beta glucan acts as an immunoprotective agent by up-regulating the inflammatory response leading to increased protection from intracellular pathogens. To our knowledge, this is the first study to evaluate the effects of dietary β-glucan on immune-related gene expression during an *Eimeria* challenge in poultry, thus providing new evidence for the potential use of β-glucans for the prevention or amelioration of coccidial infections.

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Table 4.1. Composition of basal diet

Ingredient	**%**
Corn	57.894
Soybean Meal	32.874
Poultry by Product	3.000
Poultry Fat	2.563
Dicalcium Phosphate	1.434
Limestone	0.943
Salt	0.412
DL-Methionine	0.329
L-Lysine	0.268
Trace Mineral Premix	0.100
Vitamin Premix	0.100
L-Threonine	0.072
Selenium Premix	0.011
TOTAL	**100.000**

Table 4.2. Primers used for relative real-time PCR[1]

Target	Accession No.	Nucleotide sequence (5' → 3')
GAPDH_F	NM_204305	CCTAGGATACACAGAGGACCAGGTT
GAPDH_R		GGTGGAGGAATGGCTGTCA
IL-4_F	NM_001007079	GCTCTCAGTGCCGCTGATG
IL-4_R		GAAACCTCTCCCTGGATGTCAT
IL-8_F	NM_205498	TCCTGGTTTCAGCTGCTCTGT
IL-8_R		CGCAGCTCATTCCCCATCT
IL-13_F	NM_001007085	CATGACCGACTGCAAGAAGGA
IL-13_R		CCGTGCAGGCTCTTCAGACT
IL-18_F	NM_204608	AGGTGAAATCTGGCAGTGGAAT
IL-18_R		TGAAGGCGCGGTGGTTT
iNOS_F	D85422	CCTGTACTGAAGGTGGCTATTGG
iNOS_R		AGGCCTGTGAGAGTGTGCAA
Muc-1_F	XM_430395	CAGAGATGTGGTGGCAAAAGC
Muc-1_R		CCCTTATCACCACTTGCAGGAA
Muc-2_F	XM_421035	TTCATGATGCCTGCTCTTGTG
Muc-2_F		CCTGAGCCTTGGTACATTCTTGT

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

Table 4.3. Summary of results: Changes in relative gene expression due to dietary β-glucan supplementation.

Gene	**Leukocyte Association**	**Gene Expression**
IL-8	Innate	NS
iNOS	Innate	↑
IL-18	Th1	NS, ↑
IL-4	Th2	↓
IL-13	Th2	NS, ↓
Muc-1	Th2	↓
Muc-2	Th2	↓

NS = Not Significant.

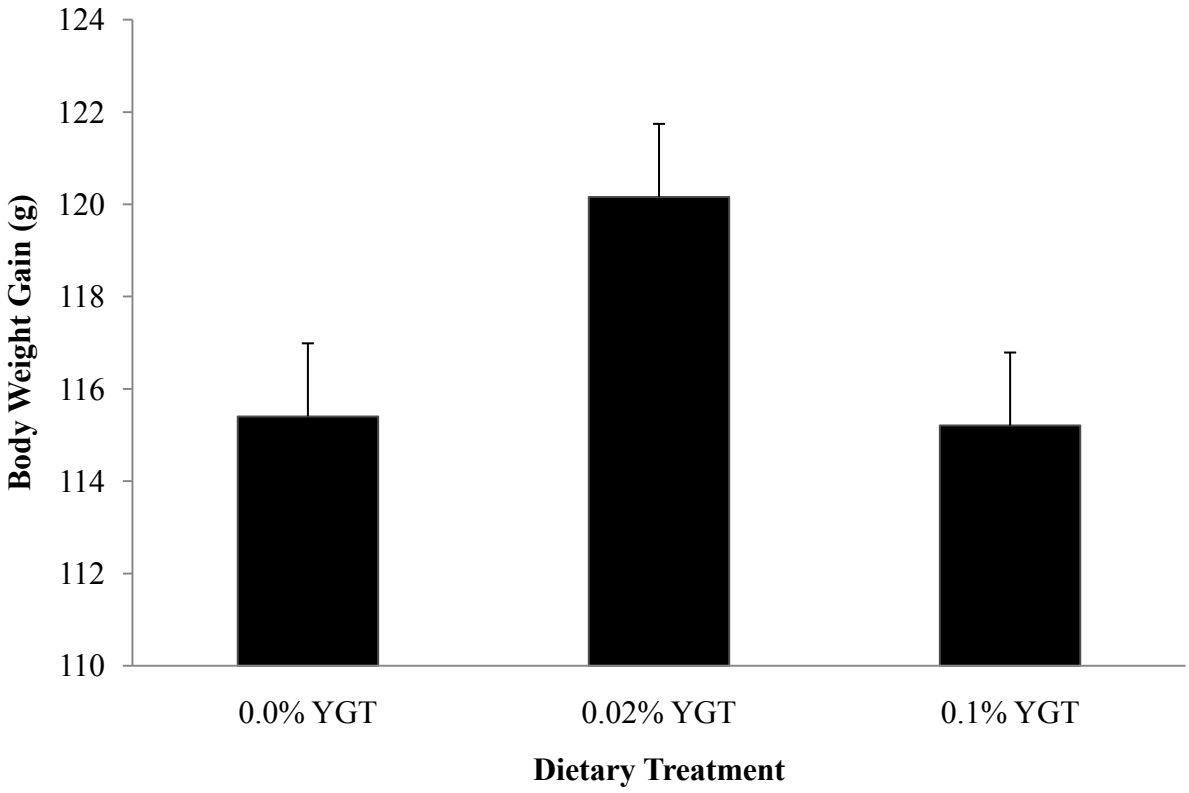

Figure 4.1 Effect of β-glucan supplementation on body weight gain of Cobb 500 broiler chicks from d1 to d7. Data are represented as LS Means + SEM (n=16 pens/treatment). YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. ($P = 0.052$).

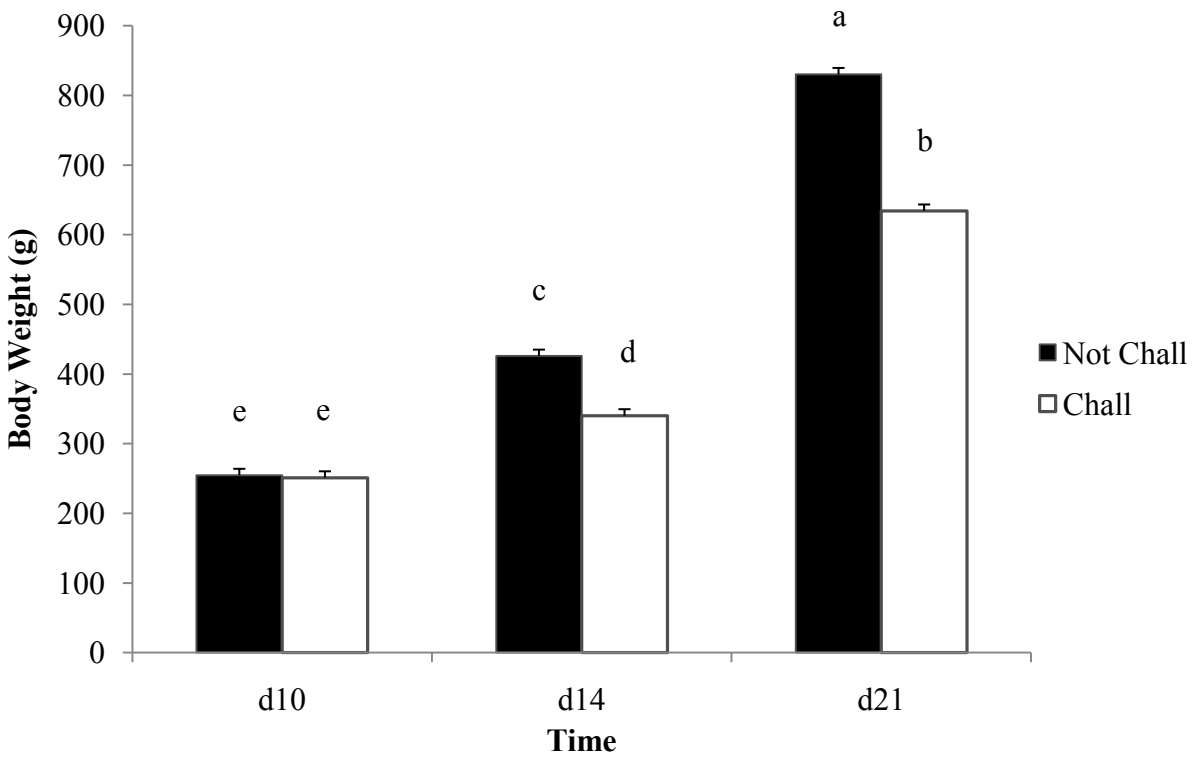

Figure 4.2. Effect of time and *Eimeria* challenge on body weight of Cobb 500 broiler chicks from d10 to d21. Data are represented as LS Means + SEM (n=8 pens/treatment). Not Chall = not challenged; Chall = challenge with Eimeria. There was a significant two-way interaction ($P < 0.0001$) of time and challenge.

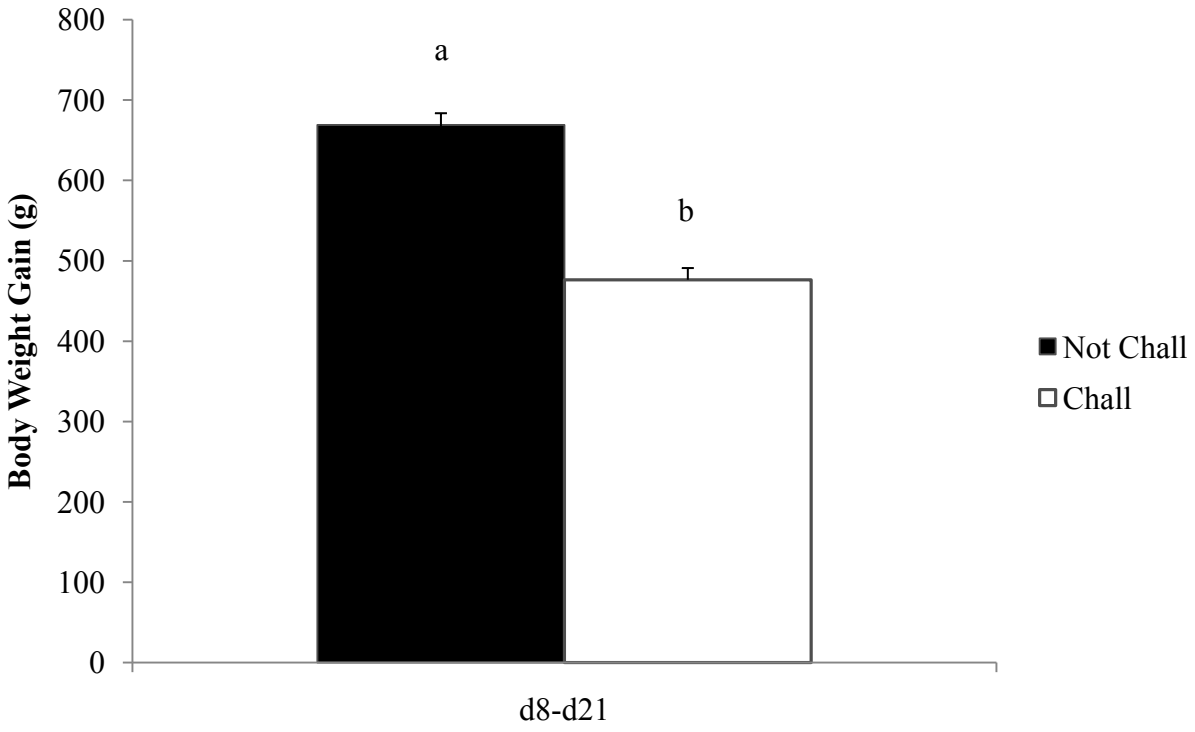

Figure 4.3. Effect of *Eimeria* challenge on body weight gain of Cobb 500 chicks from d8 to d21. Data are represented as LS Means + SEM (n=8 pens/treatment). Not Chall = not challenged; Chall = challenged with *Eimeria*. There was a significant main effect of challenge ($P < 0.0001$).

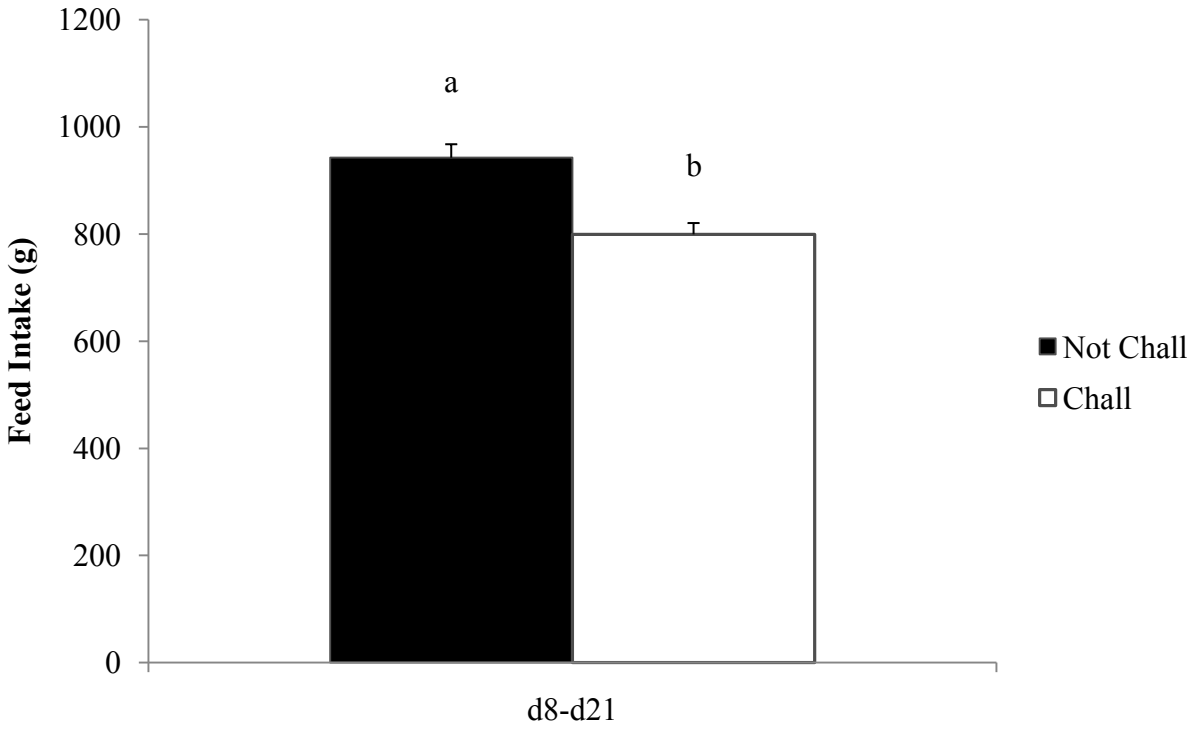

Figure 4.4. Effect of *Eimeria* challenge on feed intake of Cobb 500 broiler chicks from d8 to d21. Data are represented as LS Means + SEM. Not Chall = not challenged; Chall = challenged with *Eimeria*. There was a significant main effect of challenge ($P = 0.0001$).

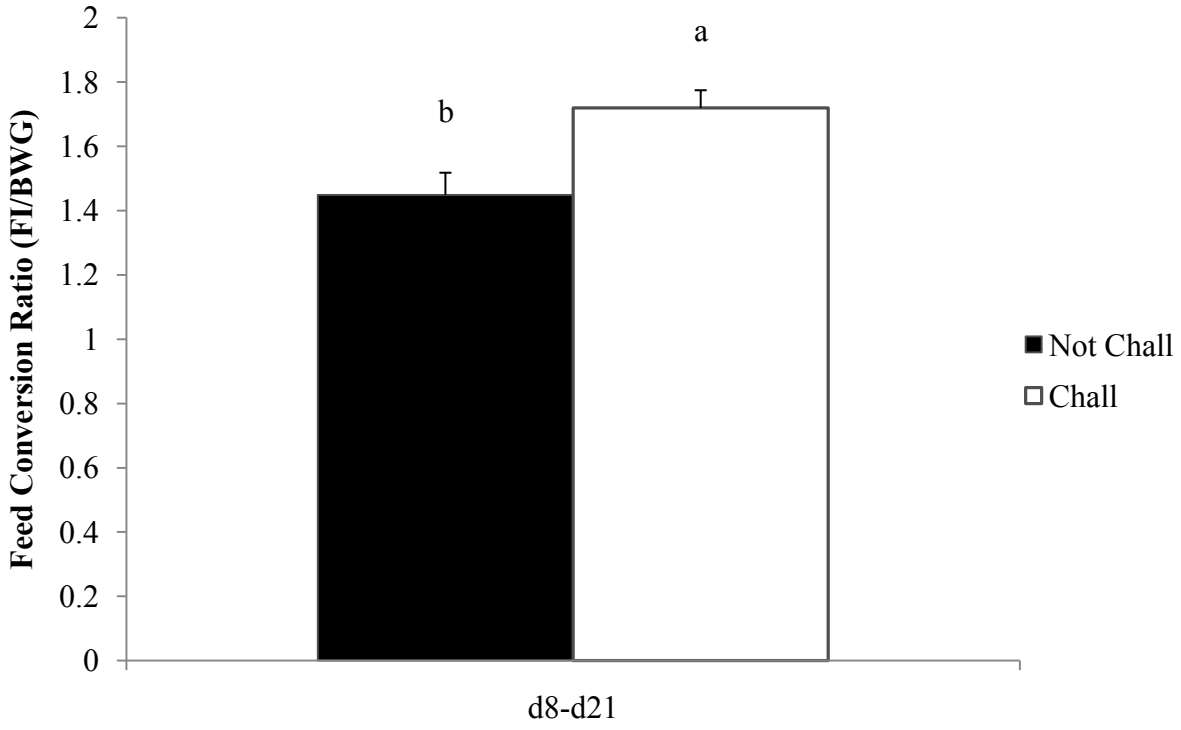

Figure 4.5. Effect of *Eimeria* challenge on feed conversion ratio of Cobb 500 broiler chicks from d8 to d21. Data are represented as LS Means + SEM. Not Chall = not challenged; Chall = challenged with *Eimeria*. There was a significant main effect of challenge ($P = 0.004$).

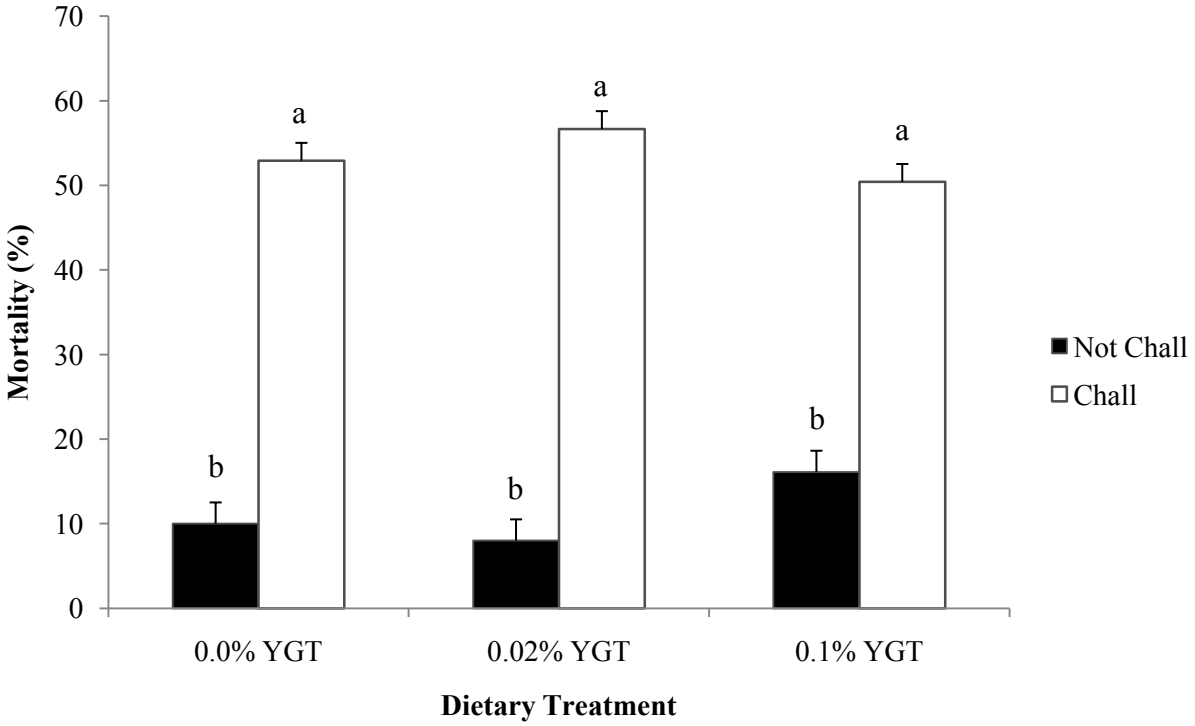

Figure 4.6. Effect of *Eimeria* challenge on percent mortality in Cobb 500 broiler chicks from d8 to d21. Data are represented as LS Means + SEM. YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan; Not Chall = not challenged; Chall = challenged with *Eimeria*. There was significant main effect of challenge ($P < 0.0001$).

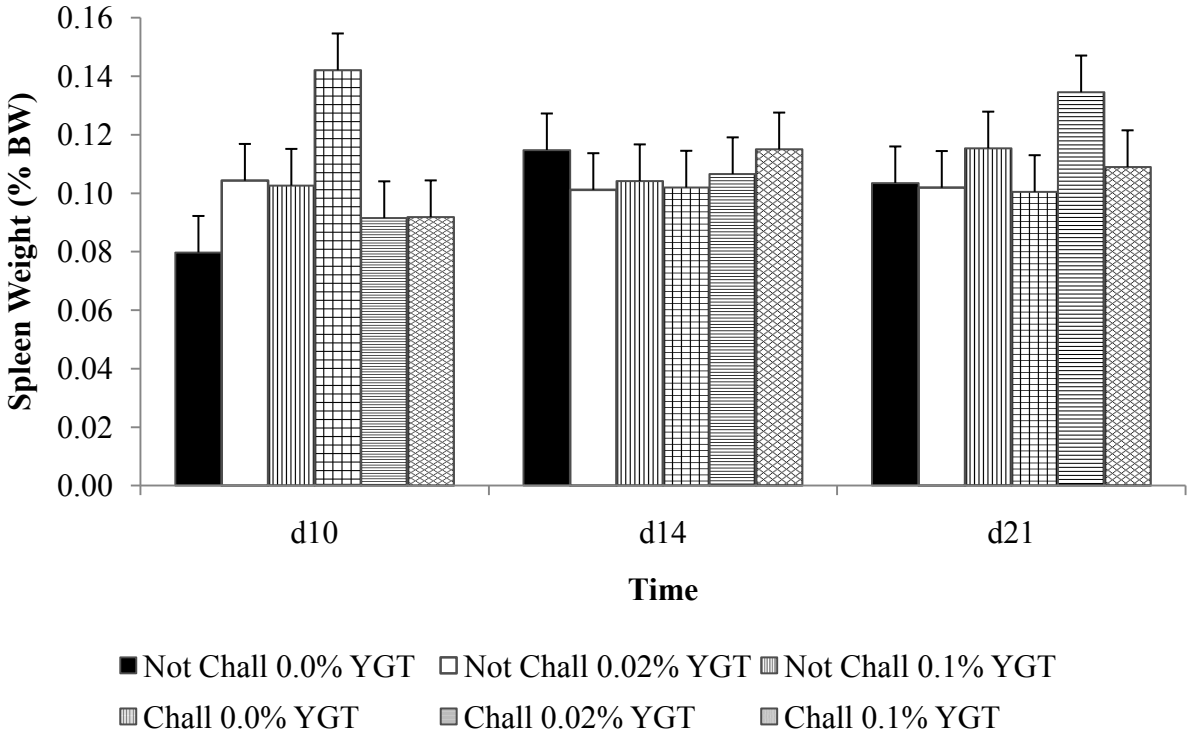

Figure 4.7. The effect of time, β-glucan supplementation and *Eimeria* challenge on spleen weights of Cobb 500 broiler chicks from d10 to d21. Data are represented as LS Means + SEM. YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan; Not Chall = not challenged; Chall = challenged with *Eimeria*. There was a significant three-way interaction (P = 0.010) of time, diet and challenge.

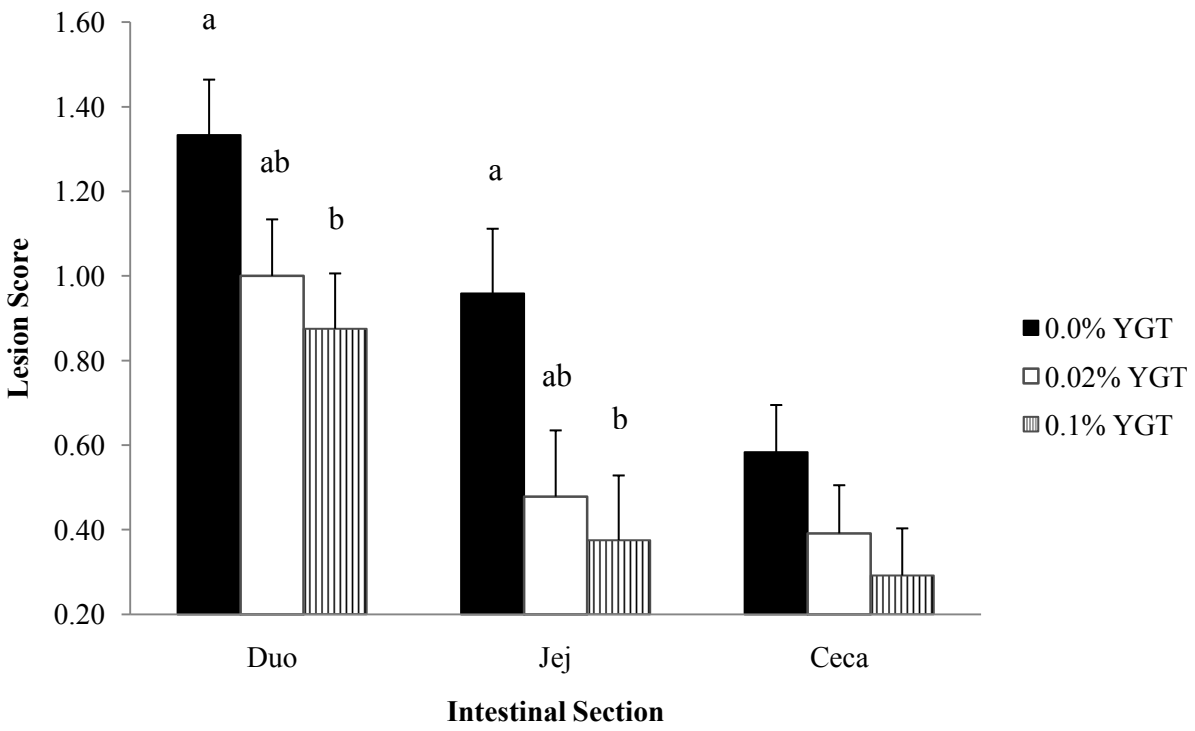

Figure 4.8. Effect of β-glucan supplementation on gross intestinal lesions scores of Cobb 500 broiler chicks. Data are represented as LS Means + SEM. Duo = duodenum; Jej = jejunum; Ile = ileum; YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant effect of dietary treatment in the duodenum ($P = 0.044$) and jejunum ($P = 0.021$).

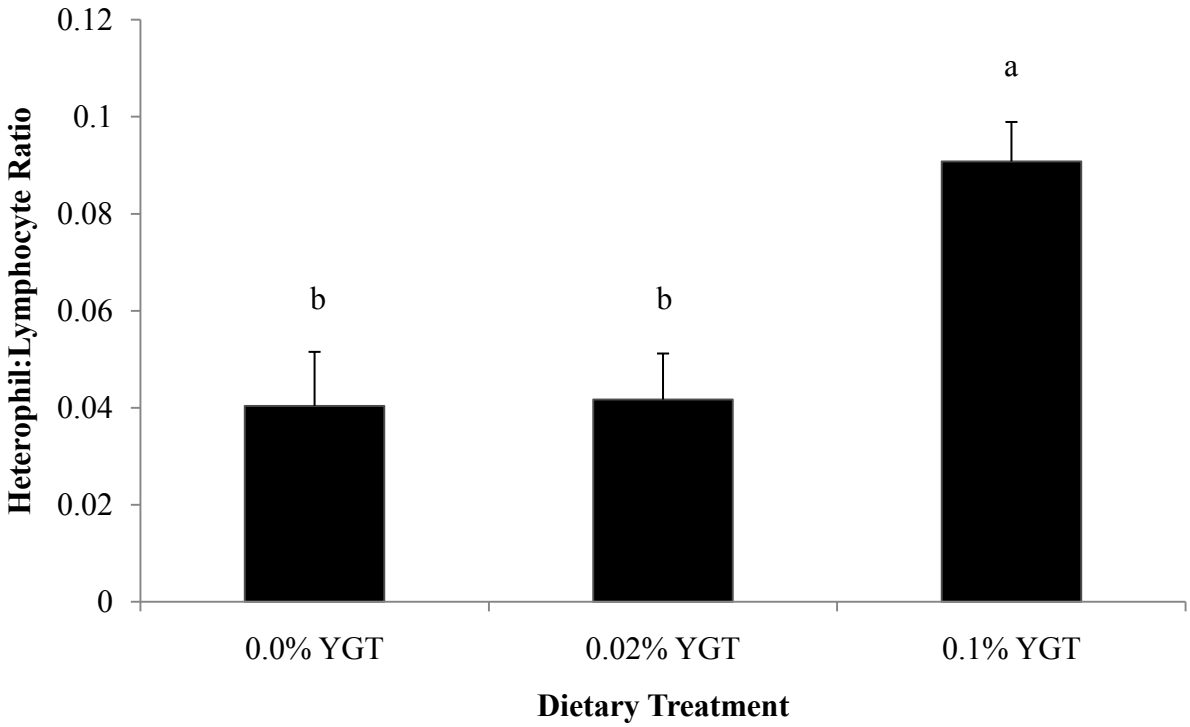

Figure 4.9. Effect of β-glucan supplementation on heterophil: lymphocyte ratio in Cobb 500 broiler chicks from d4 to d7. Data are represented as LS Means + SEM. YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant main effect of dietary treatment ($P = 0.001$).

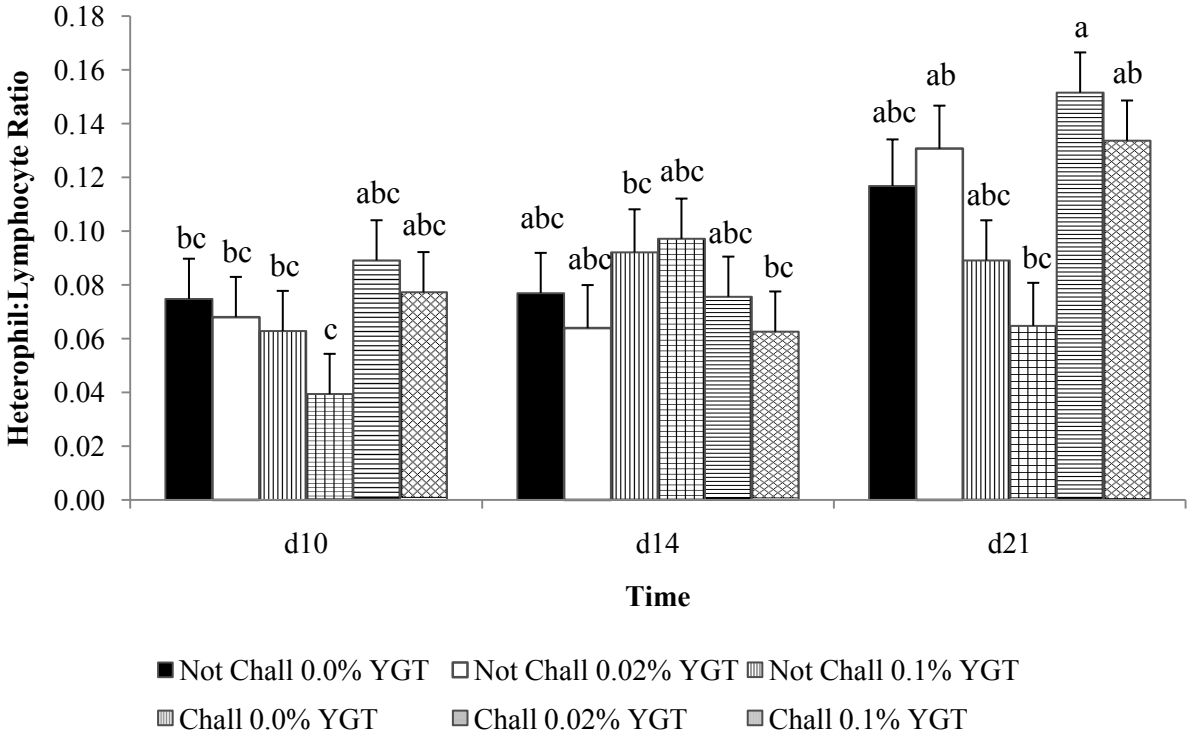

Figure 4.10. Effect of time, β-glucan supplementation, and *Eimeria* challenge on heterophil: lymphocyte ratios of Cobb 500 broiler chicks from d10 to d21. Data are represented as LS Means + SEM. YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan; Not Chall = not challenged; Chall = challenged with *Eimeria*. There was a significant three-way interaction ($P = 0.028$) of time, dietary treatment and challenge.

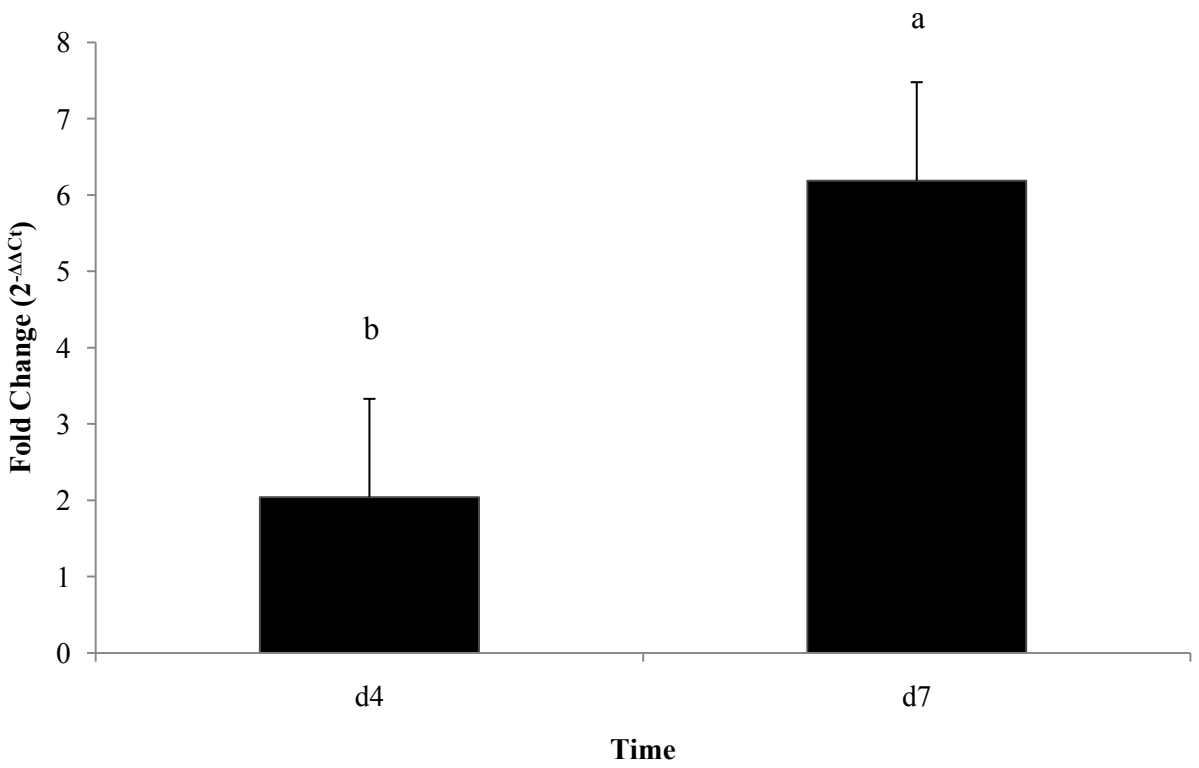

Figure 4.11. Effect of time on relative IL-4 expression in the small intestine of Cobb 500 chicks on d4 and d7. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. There was a significant main effect of time ($P = 0.004$).

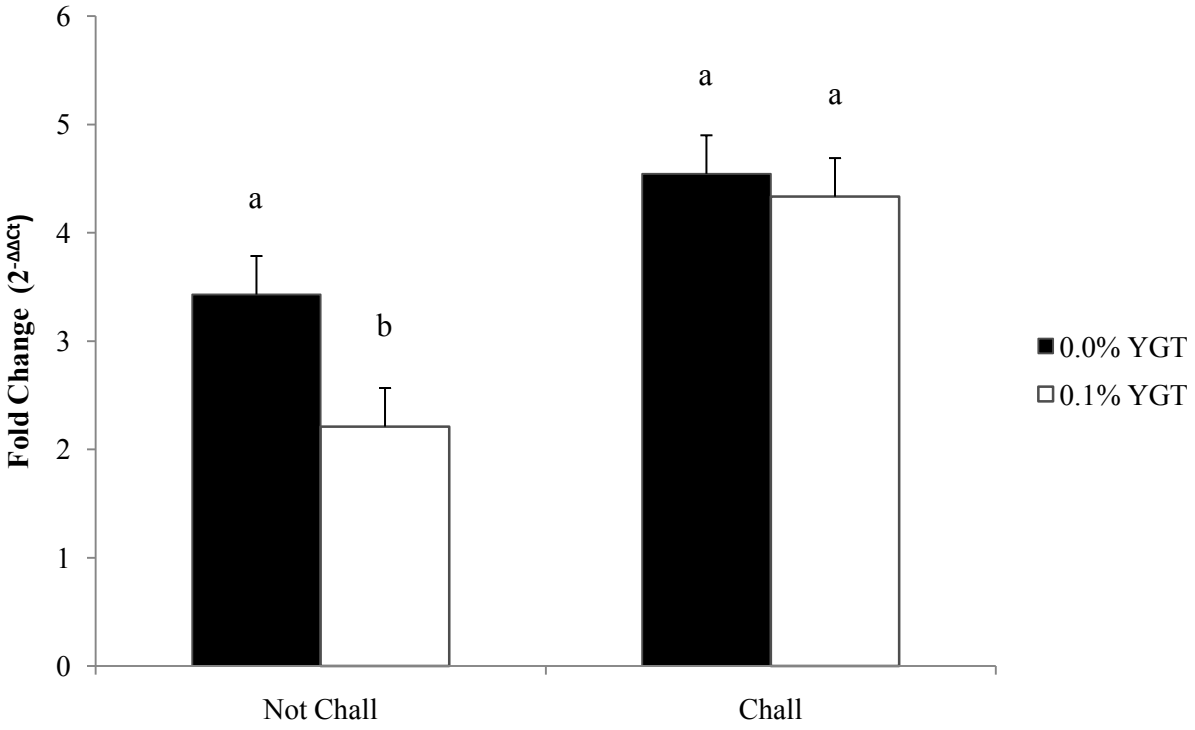

Figure 4.12. Effect of β-glucan supplementation and *Eimeria* challenge on relative IL-4 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan; Not Chall = not challenged; Chall = challenged with *Eimeria*. There was a significant two-way interaction ($P = 0.014$) of dietary treatment and challenge.

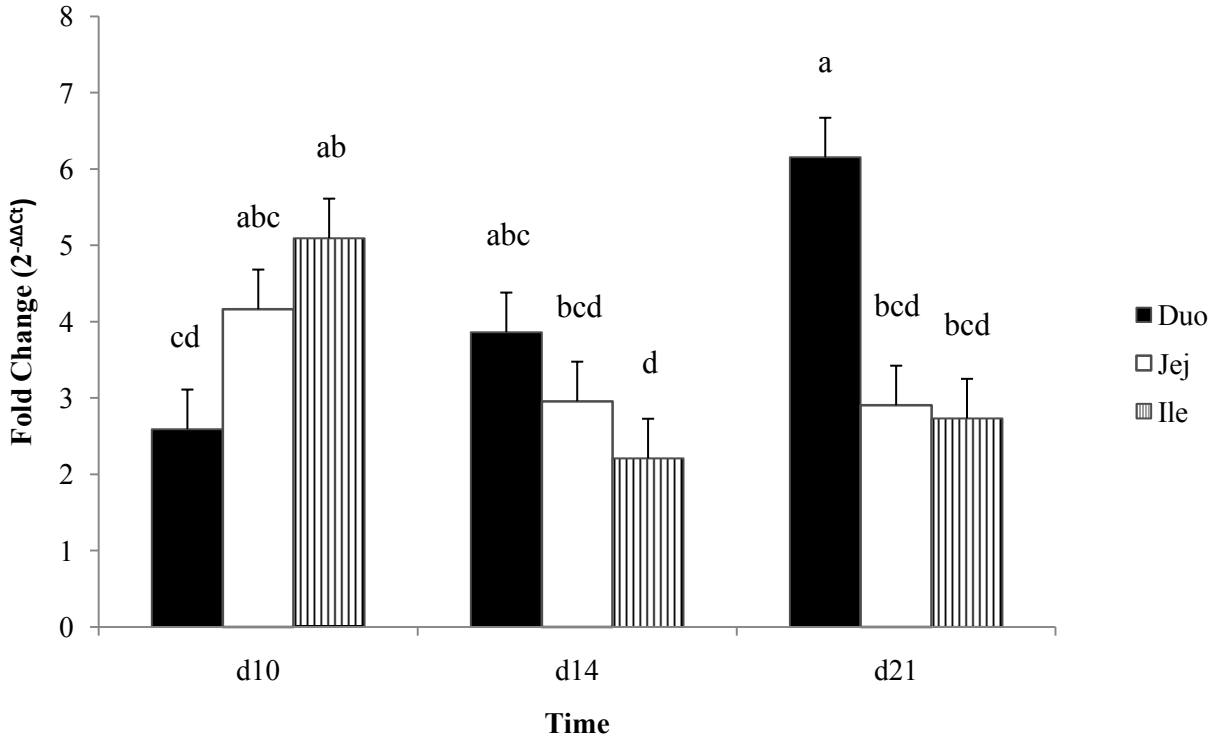

Figure 4.13. Effect of time and intestinal section on relative IL-4 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Duo = duodenum; Jej = jejunum; Ile = ileum. There was a significant two-way interaction ($P < 0.0001$) of time and intestinal section.

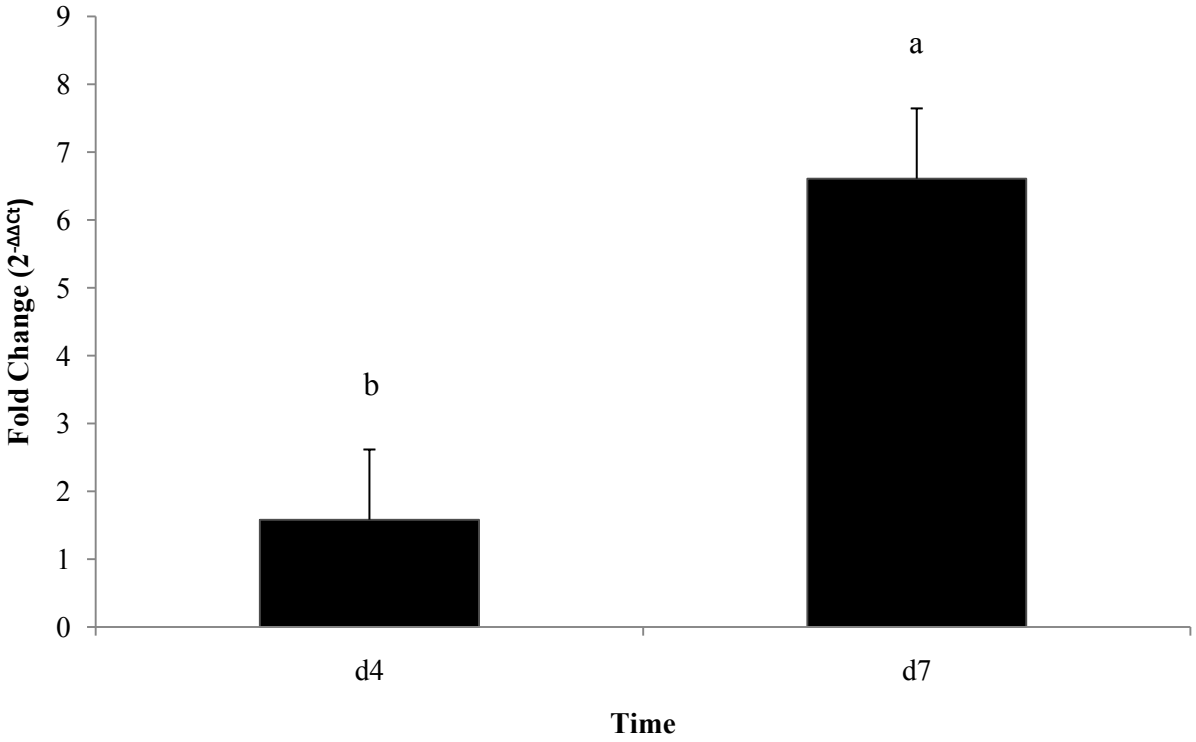

Figure 4.14. Effect of time on relative IL-8 expression in the small intestine of Cobb 500 broiler chicks on d4 and d7. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. There was a significant main effect of time ($P < 0.0001$)..

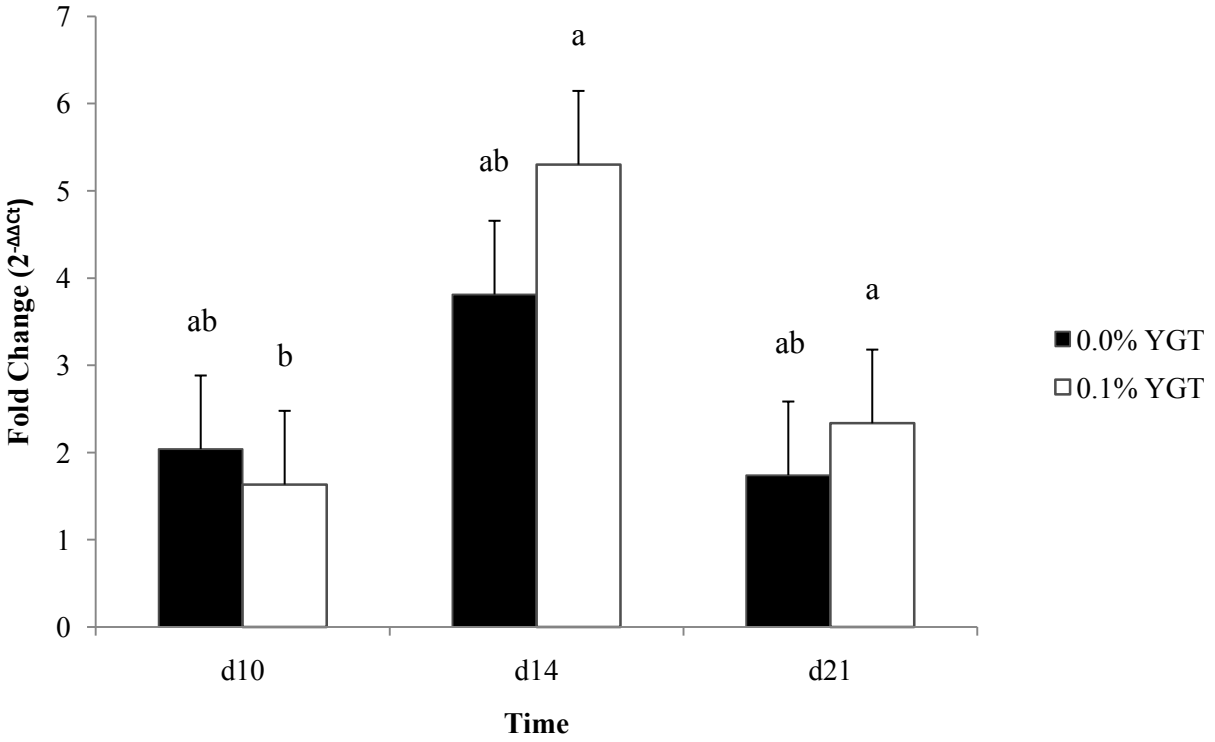

Figure 4.15. Effect of time and β-glucan supplementation on relative IL-8 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant two-way interaction ($P = 0.0358$) of time and dietary treatment.

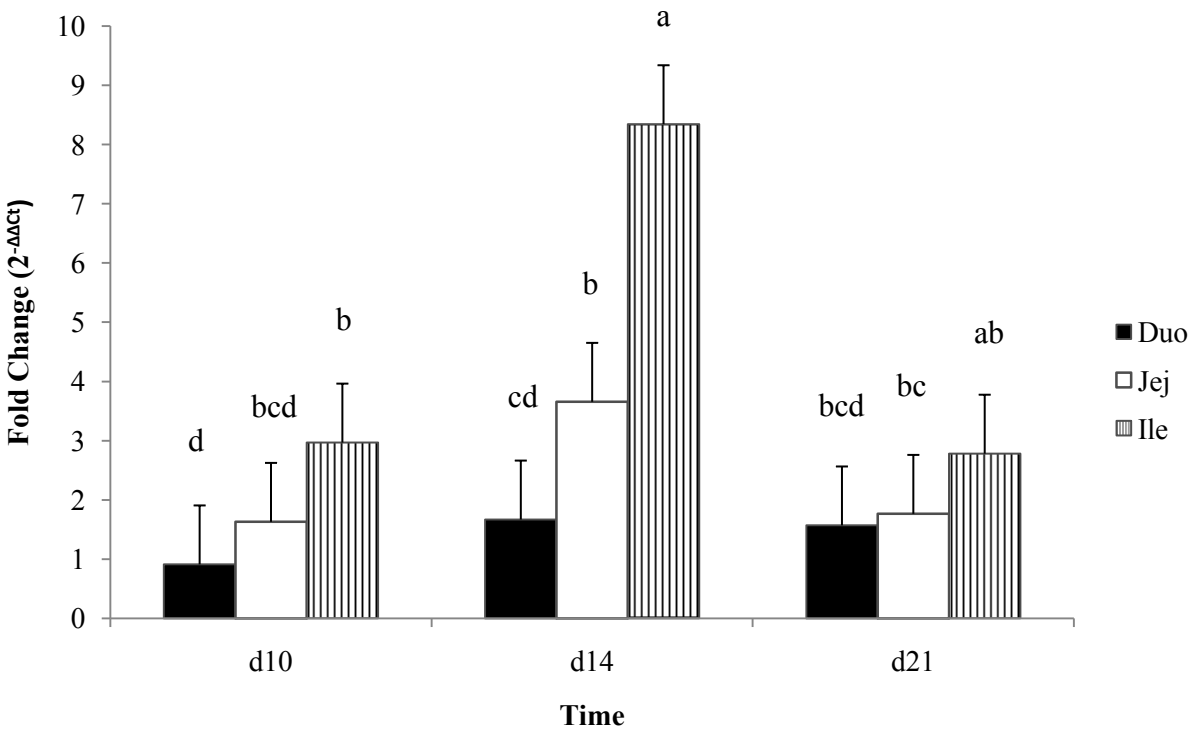

Figure 4.16. Effect of time and intestinal section on relative IL-8 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Duo = duodenum; Jej = jejunum; Ile = ileum. There was a significant two way interaction ($P = 0.033$) of time and intestinal section.

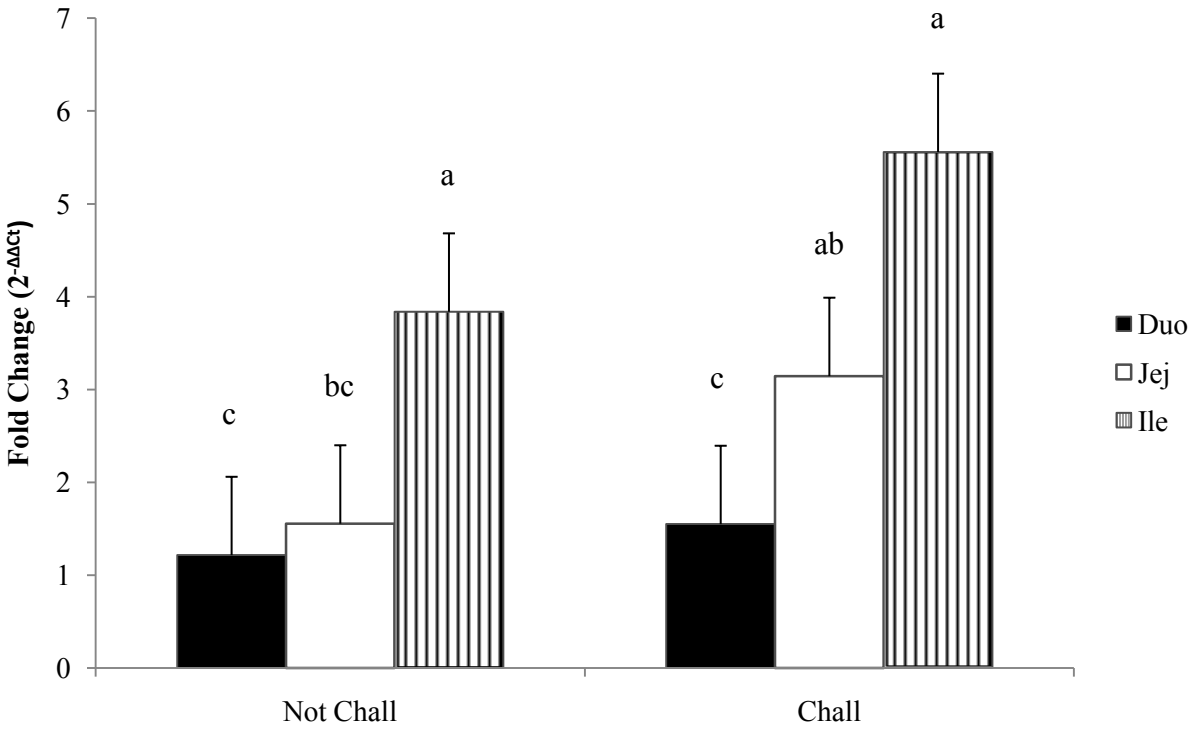

Figure 4.17. Effect of *Eimeria* challenge and intestinal section on relative IL-8 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means ± SEM. Not Chall = not challenged; Chall = challenged with *Eimeria*; Duo = duodenum; Jej = jejunum; Ile = ileum. There was a significant two-way interaction (*P* = 0.024) of challenge and intestinal section.

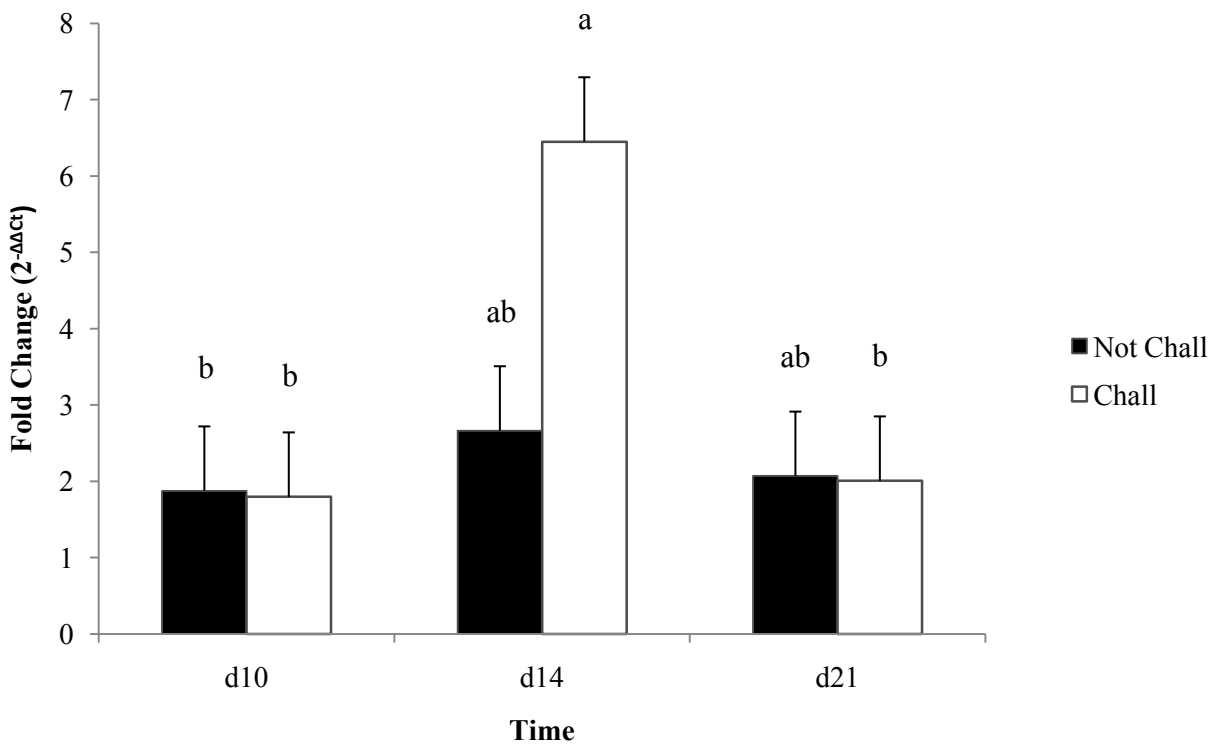

Figure 4.18. Effect of time and *Eimeria* challenge on relative IL-8 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Not Chall = not challenged; Chall = challenged with *Eimeria*. There was a significant two-way interaction ($P = 0.008$) of time and challenge.

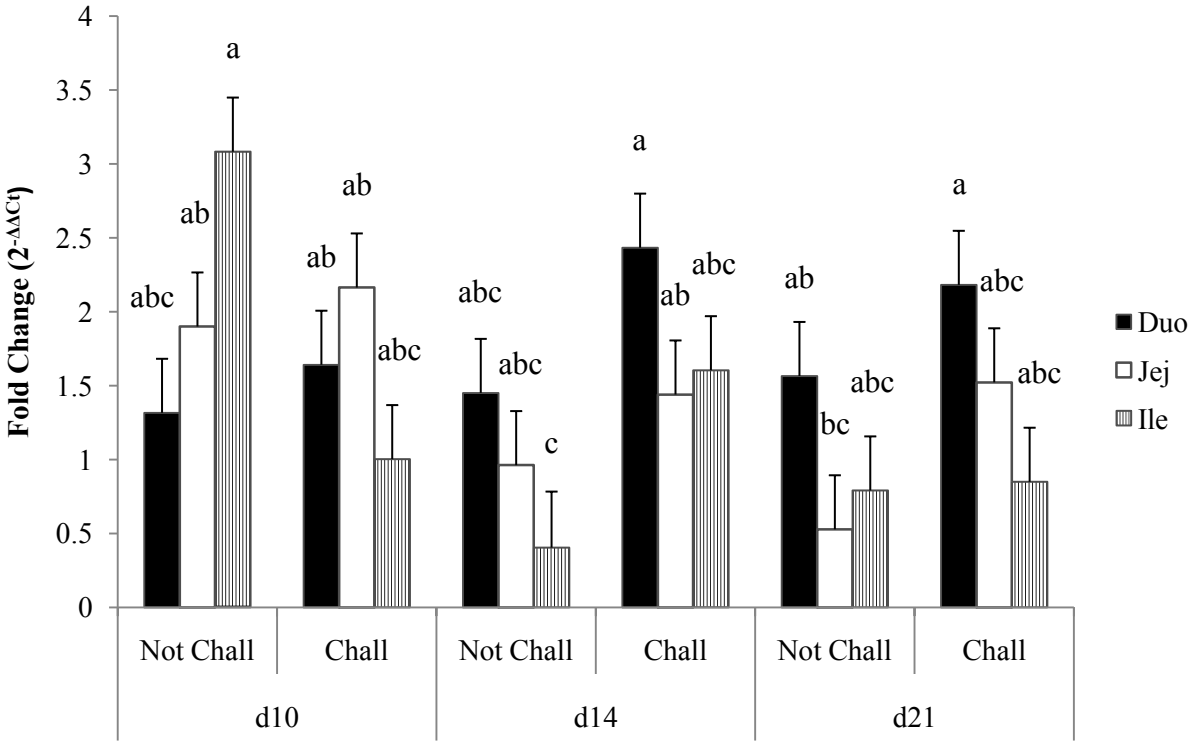

Figure 4.19. Effect of time and *Eimeria* challenge on relative IL-13 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Not Chall = not challenged; Chall = challenged with *Eimeria*; Duo = duodenum; Jej = jejunum; Ile = ileum. There was a significant two-way interaction ($P = 0.038$) of time and challenge.

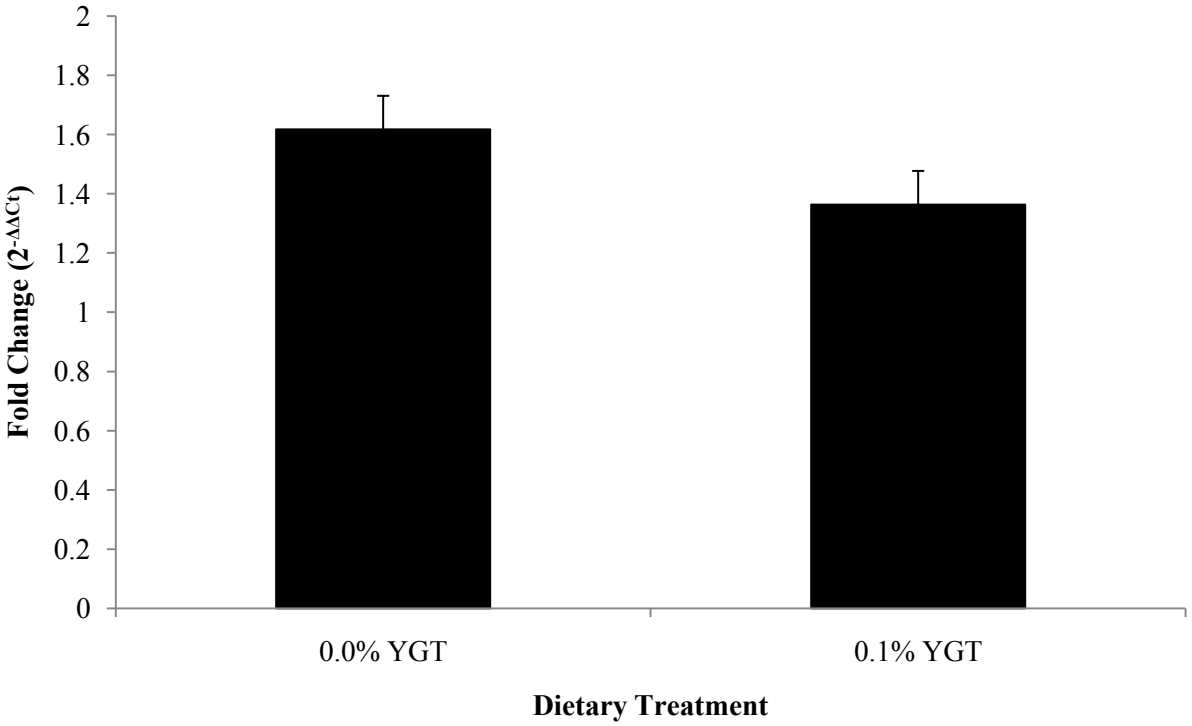

Figure 4.20. Effect of β-glucan supplementation on relative IL-13 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. (P = 0.063).

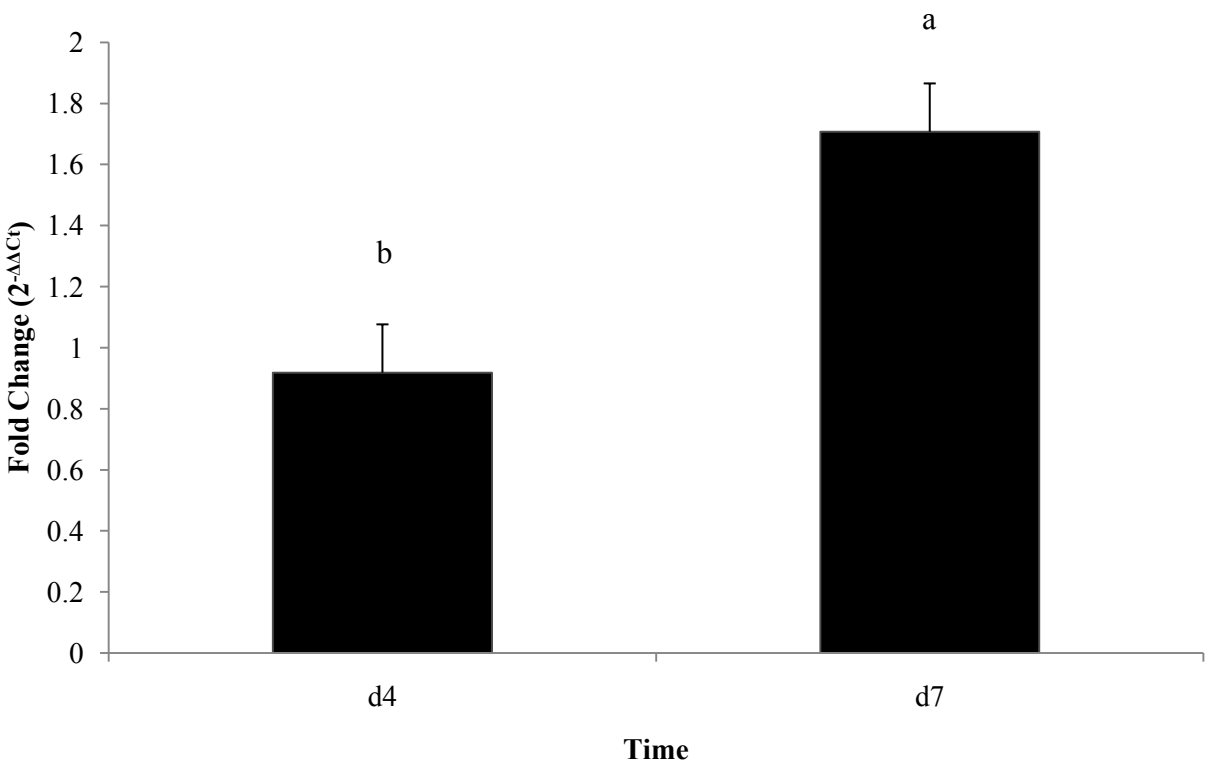

Figure 4.21. Effect of time on relative IL-18 expression in the small intestine of Cobb 500 broiler chicks on d4 and d7. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. There was a significant main effect of time ($P < 0.0001$).

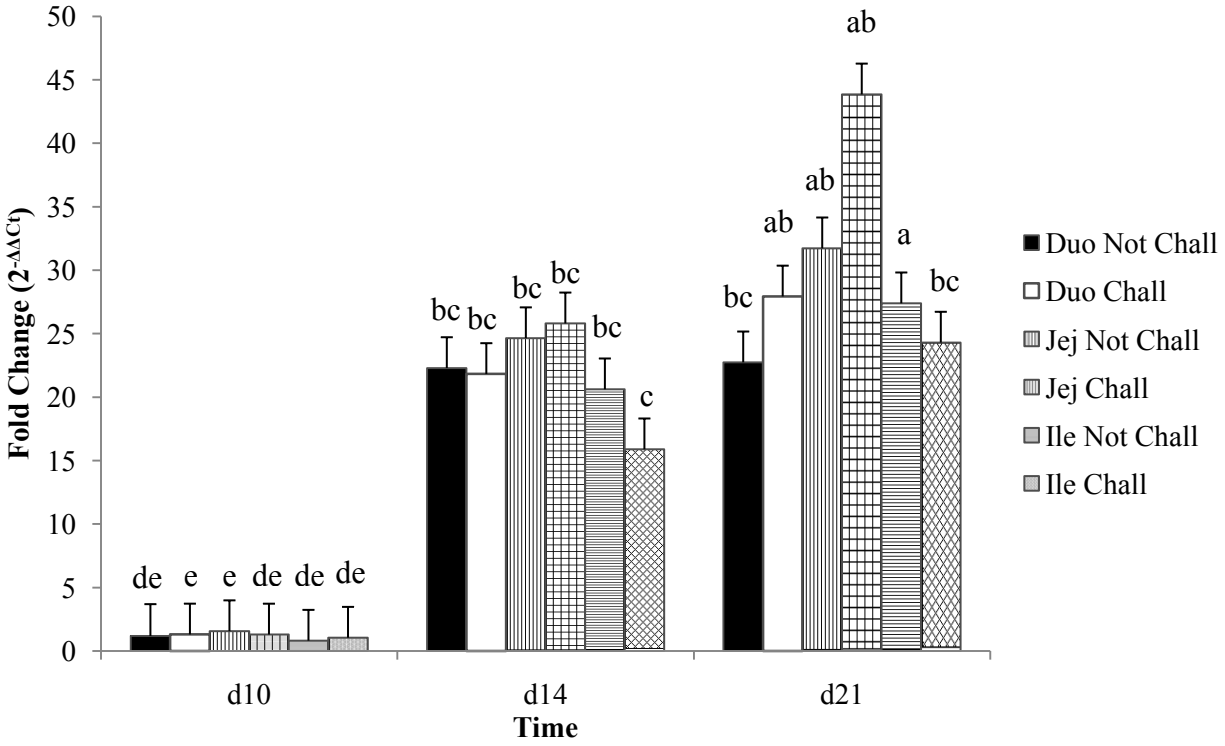

Figure 4.22. Effect of time, intestinal section and *Eimeria* challenge in the on relative IL-18 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Duo = duodenum; Jej = jejunum; Ile = ileum; Not Chall = not challenged; Chall = challenged with *Eimeria*. There was significant three-way interaction ($P = 0.045$) of time, intestinal section and challenge.

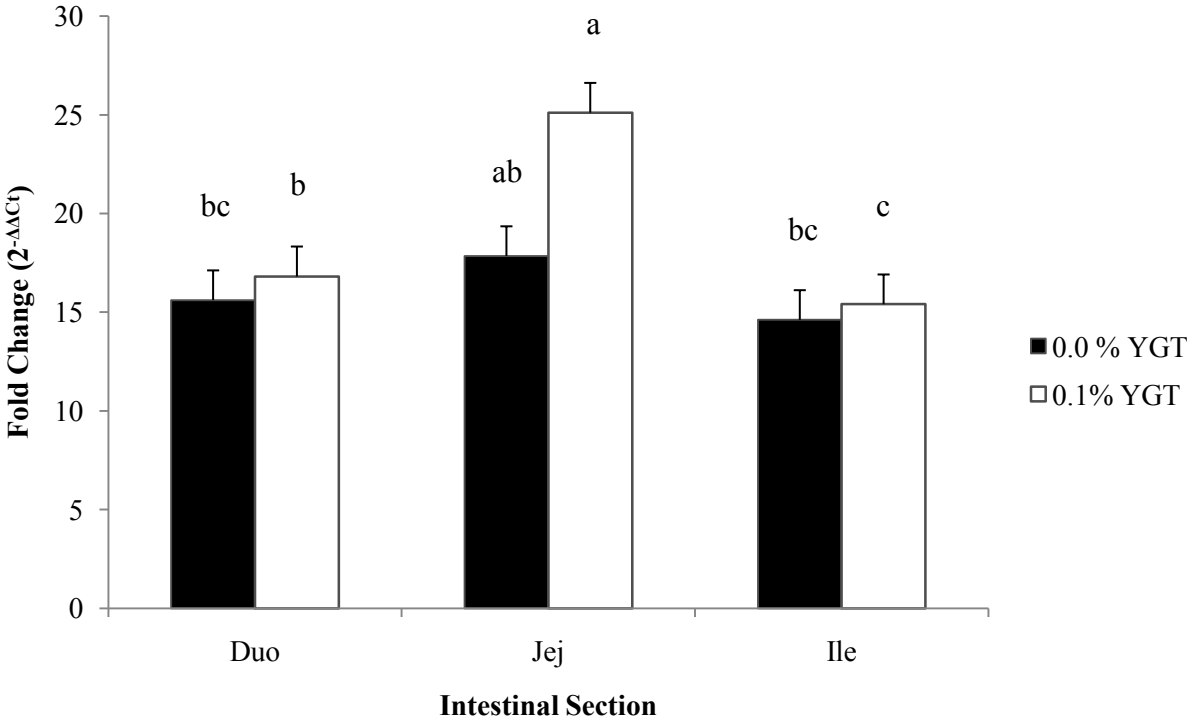

Figure 4.23. Effect of β-glucan supplementation and intestinal section on relative IL-18 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Duo = duodenum; Jej = jejunum; Ile = ileum; YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant two-way interaction ($P = 0.021$) of diet and intestinal section.

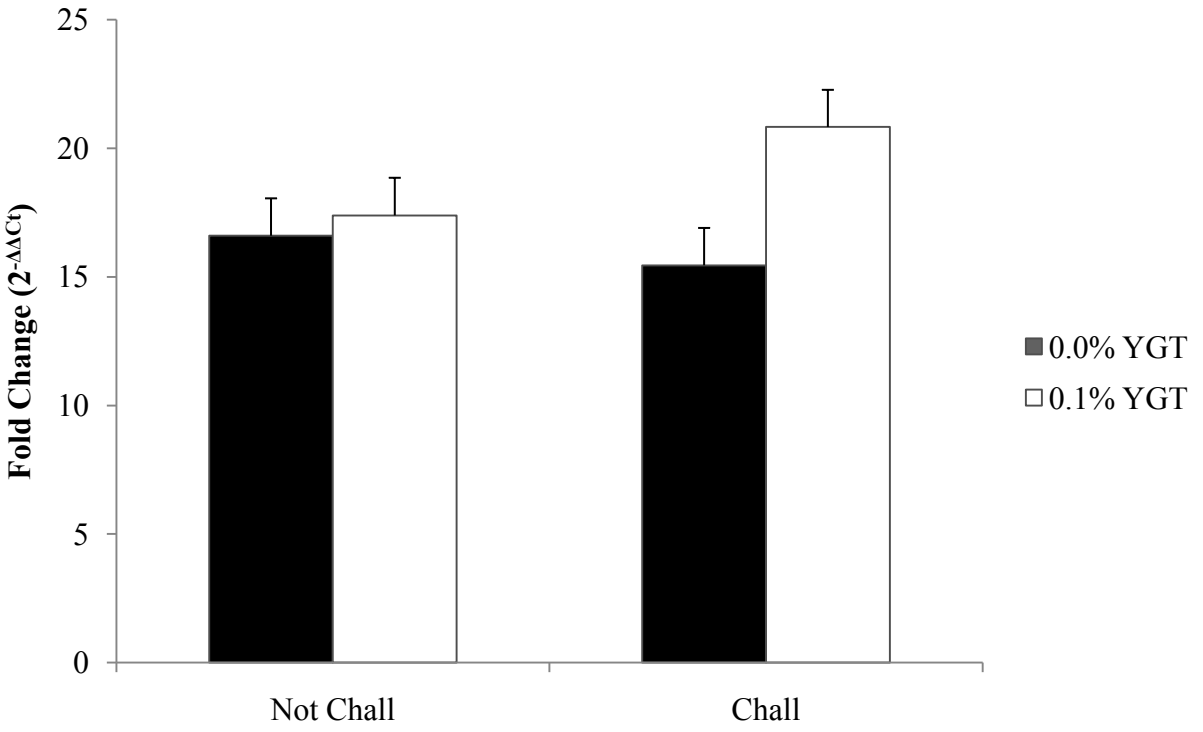

Figure 4.24. Effect of β-glucan supplementation on relative IL-18 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Not Chall = not challenged; Chall = challenged with *Eimeria*; YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. ($P = 0.071$).

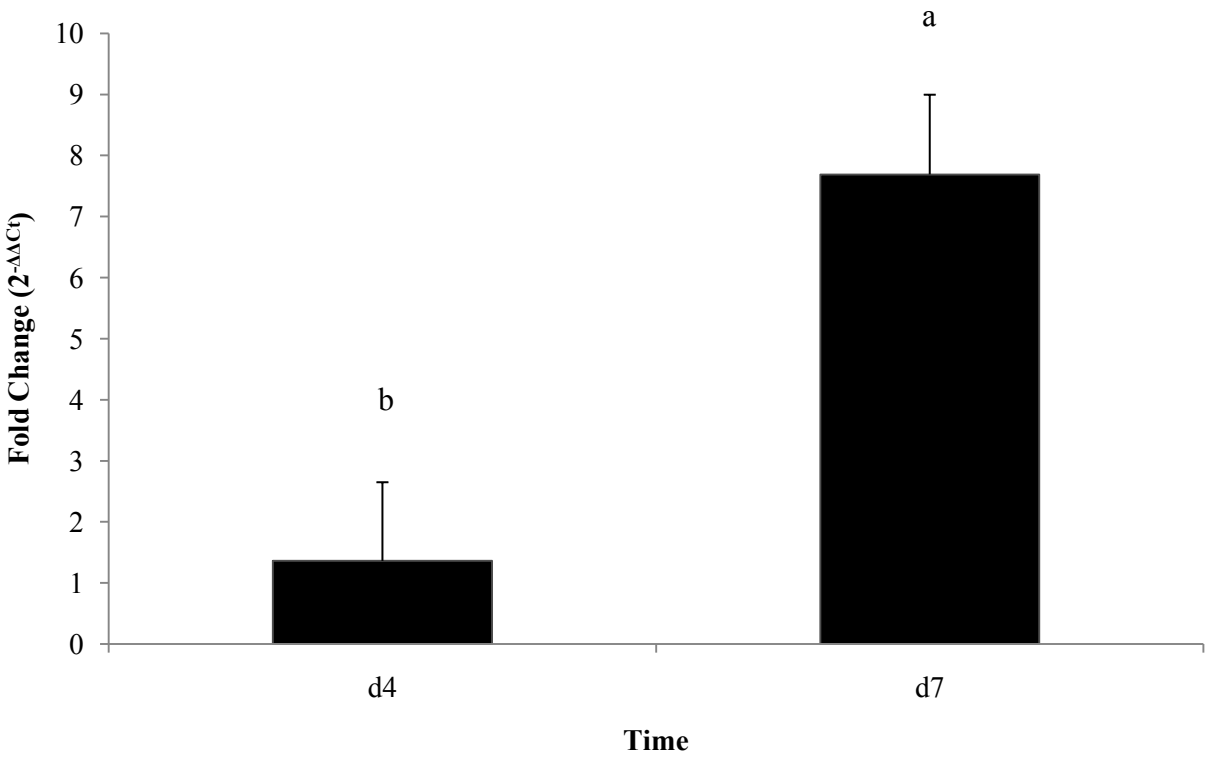

Figure 4.25. Effect of time on relative iNOS expression in the small intestine of Cobb 500 broiler chicks on d4 and d7. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. There was a significant main effect of time ($P < 0.0001$).

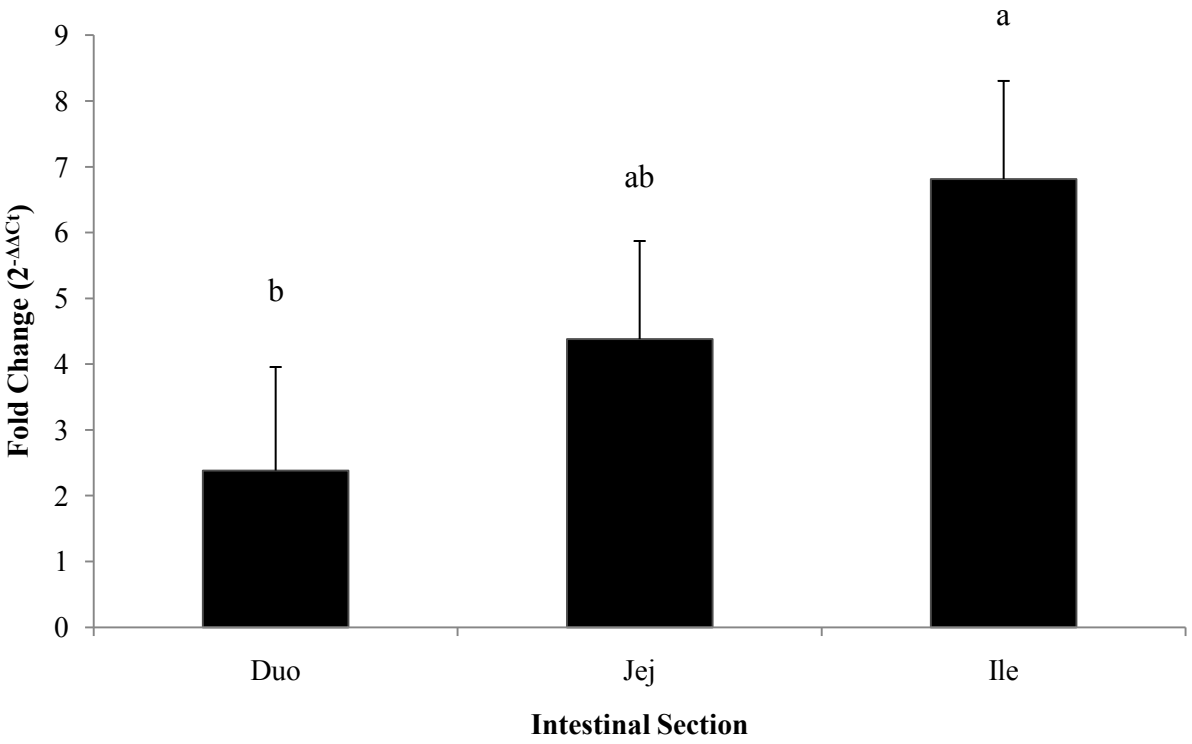

Figure 4.26. Effect of intestinal section on relative iNOS expression in the small intestine of Cobb 500 broiler chicks from d4 to d7. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Duo = duodenum; Jej = jejunum; Ile = ileum. There was a significant main effect of intestinal section ($P = 0.012$).

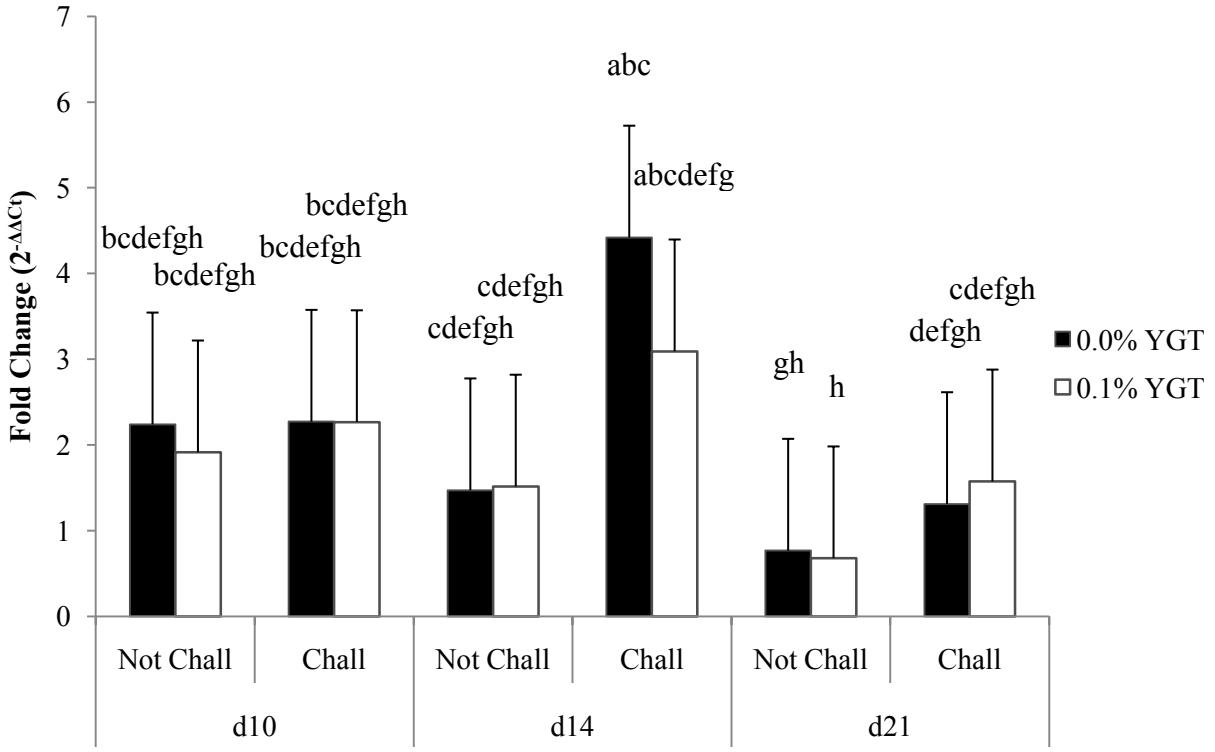

Figure 4.27a. Effect of time, β-glucan supplementation and *Eimeria* challenge on relative iNOS expression in the duodenum of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Not Chall = not challenged; Chall = challenged with *Eimeria*; YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant four-way interaction ($P = 0.039$) of time, dietary treatment, challenge and intestinal section.

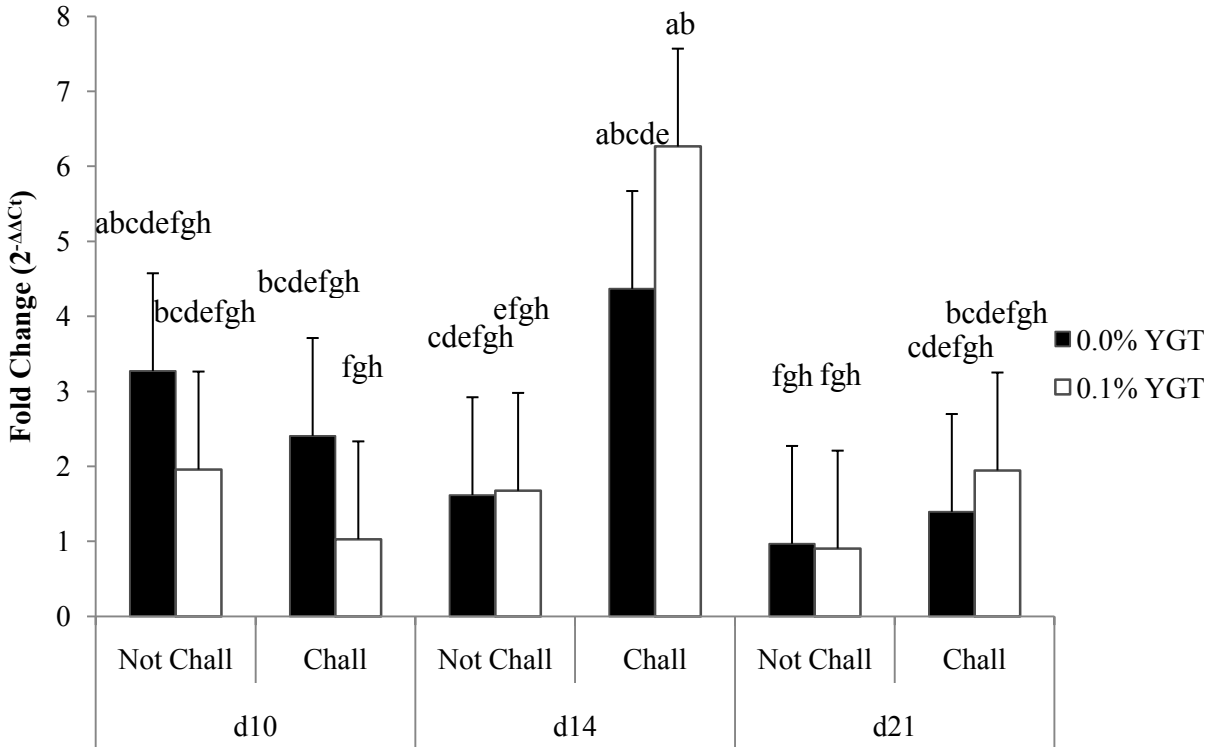

Figure 4.27b. Effect of time, β-glucan supplementation and *Eimeria* challenge on relative iNOS expression in the jejunum of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Not Chall = not challenged; Chall = challenged with *Eimeria*; YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant four-way interaction ($P = 0.039$) of time, dietary treatment, challenge and intestinal section.

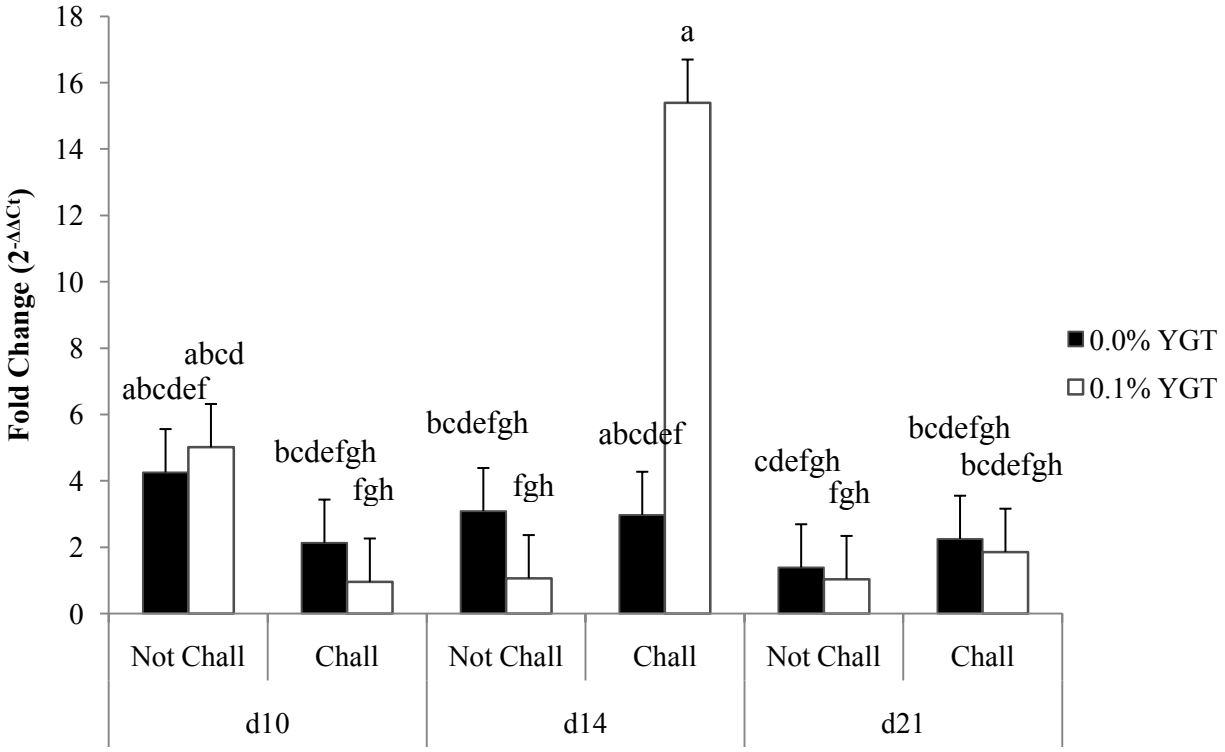

Figure 4.27c. Effect of time, β-glucan supplementation and *Eimeria* challenge on relative iNOS expression in the ileum of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Not Chall = not challenged; Chall = challenged with *Eimeria*; YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant four-way interaction ($P = 0.039$) of time, dietary treatment, challenge and intestinal section.

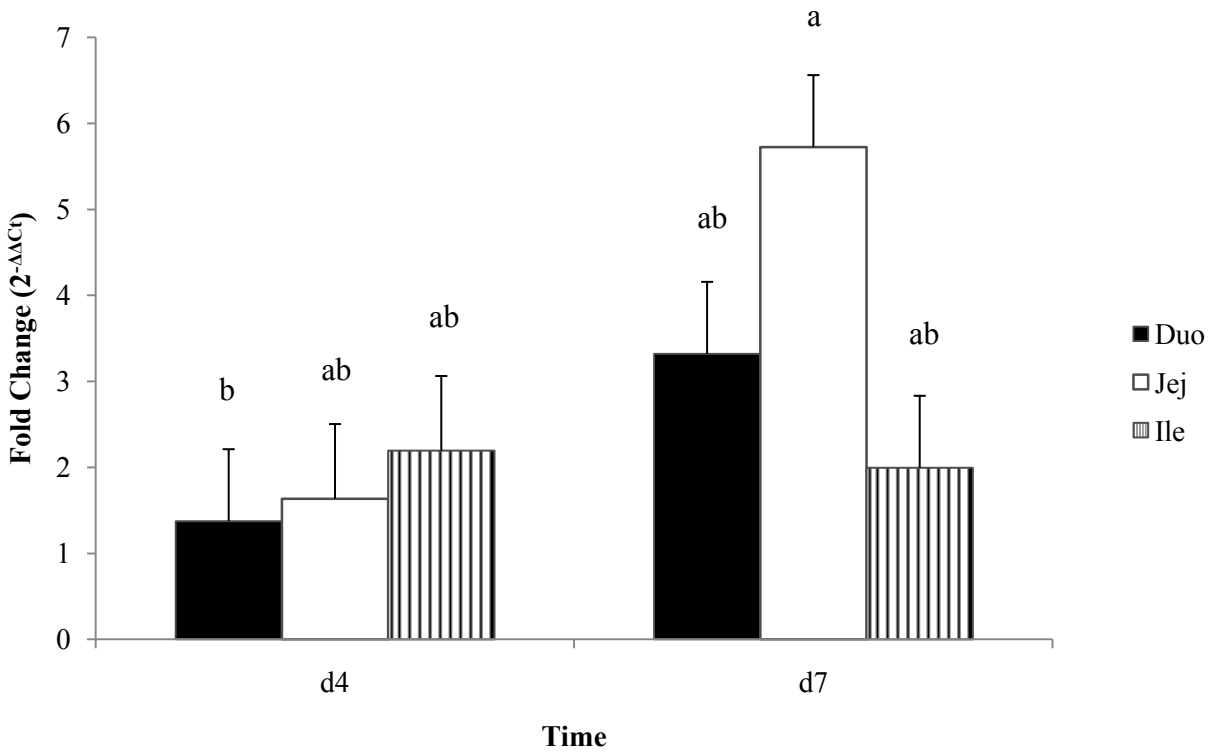

Figure 4.28. Effect of time and intestinal section on relative Muc-1 expression in the small intestine of Cobb 500 broiler chicks on d4 and d7. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Duo = duodenum; Jej = jejunum; Ile = ileum. There was a significant two-way interaction ($P = 0.024$) of time and intestinal section.

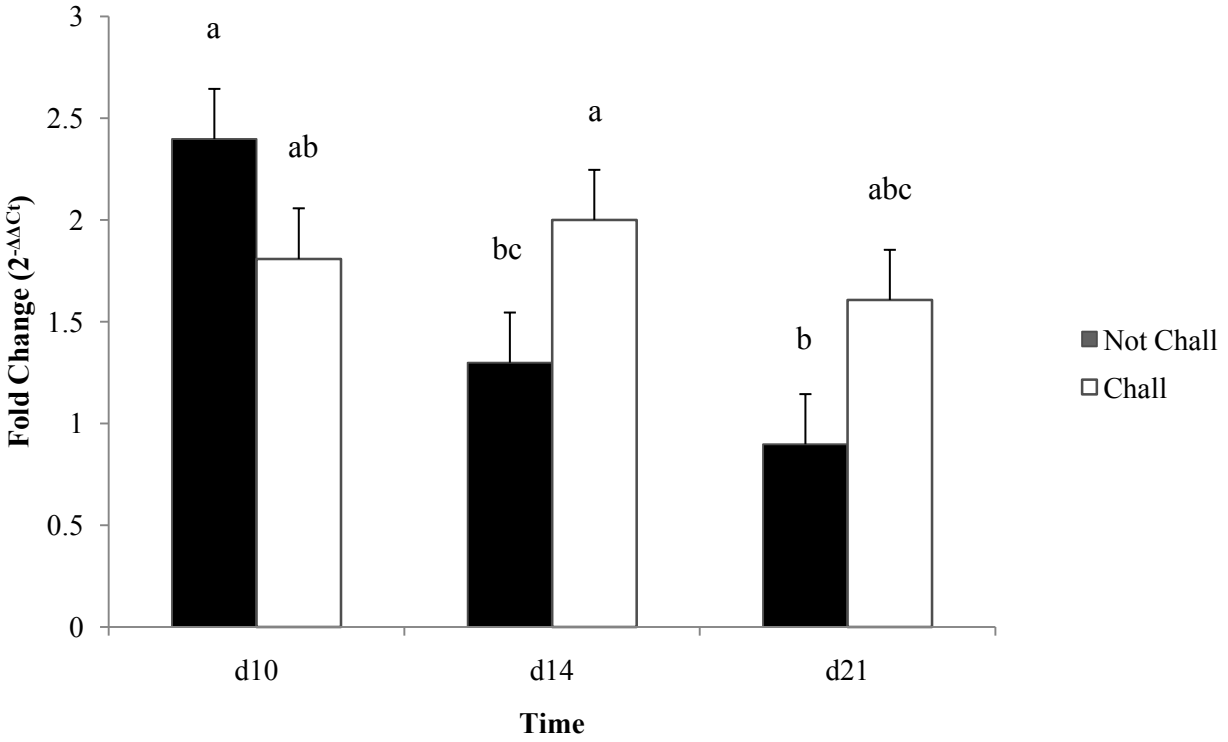

Figure 4.29. Effect of time and *Eimeria* challenge on relative Muc-1 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Not Chall = not challenged; Chall = challenged with *Eimeria*. There was a significant two-way interaction ($P = 0.001$) of time and challenge.

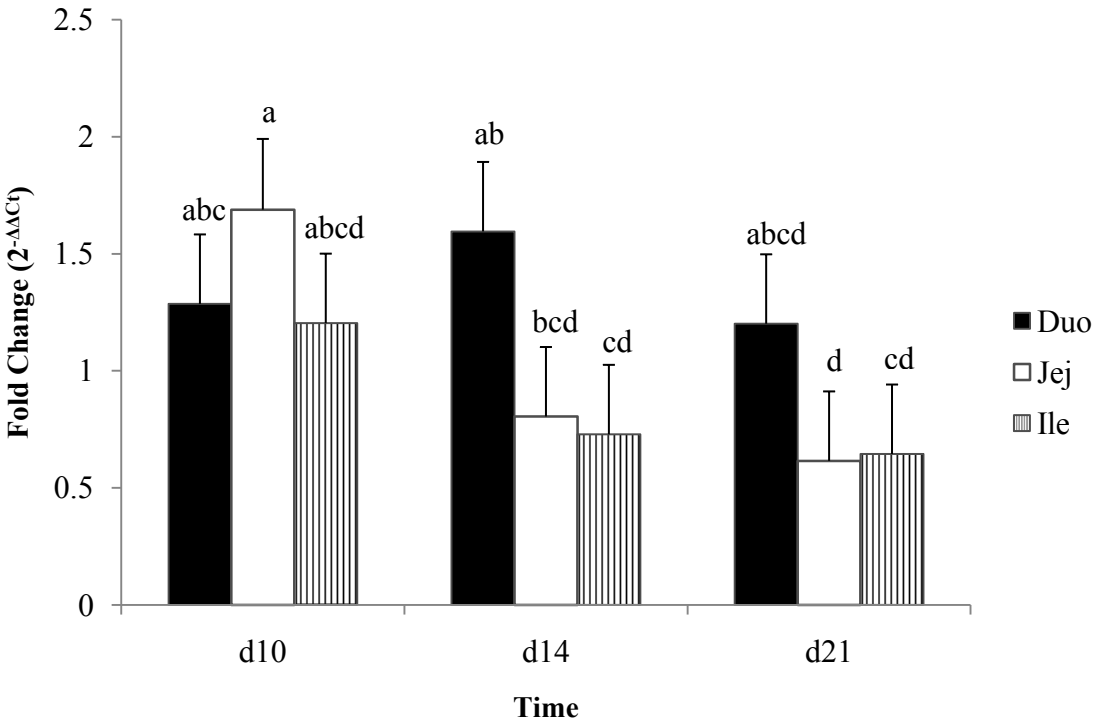

Figure 4.30. Effect of time and intestinal section on relative Muc-1 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Duo = duodenum; Jej = jejunum; Ile = ileum. There was a significant two-way interaction ($P = 0.003$) of time and intestinal section.

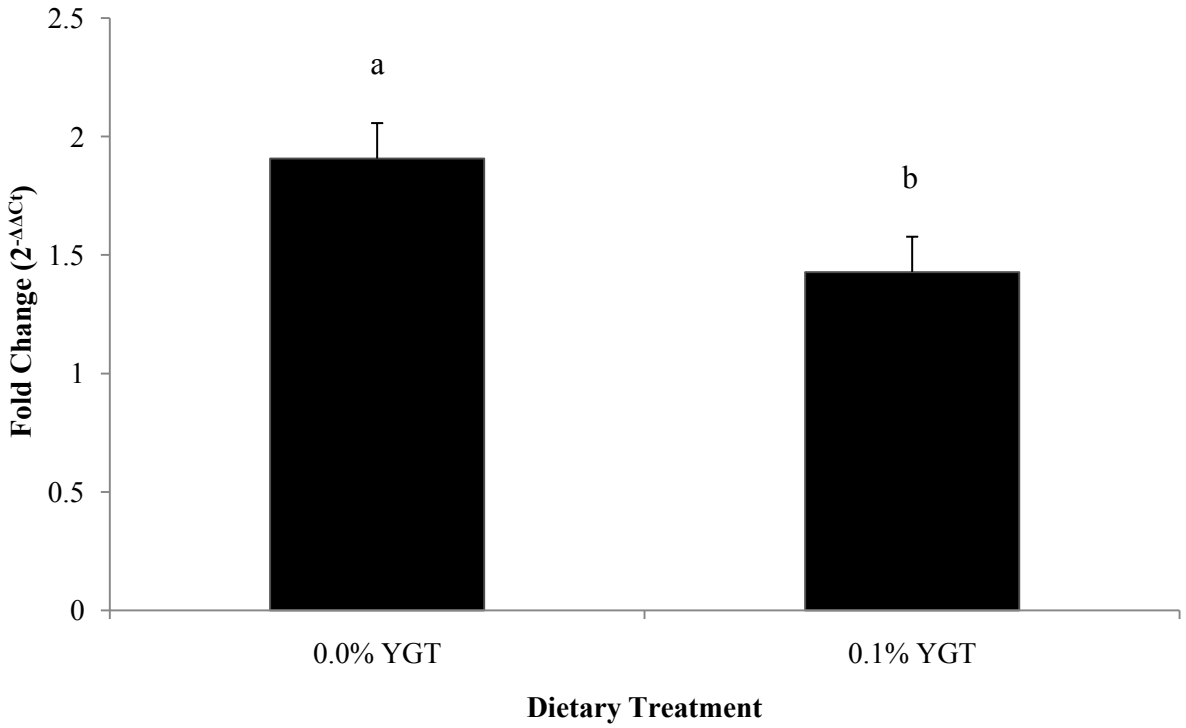

Figure 4.31. Effect of β-glucan supplementation on relative Muc-1 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant effect of dietary treatment ($P = 0.013$).

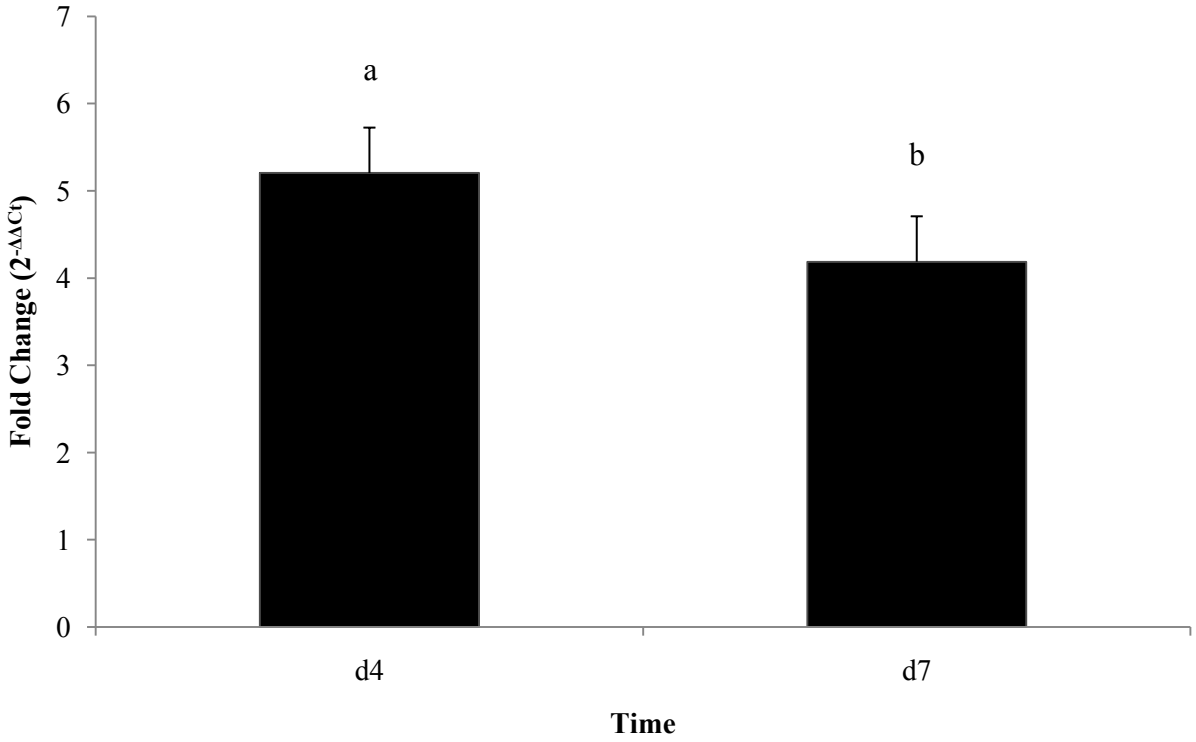

Figure 4.32. Effect of time on relative Muc-2 gene expression in the small intestine of Cobb 500 broiler chicks on d4 and d7. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. There was a significant effect of time ($P = 0.007$).

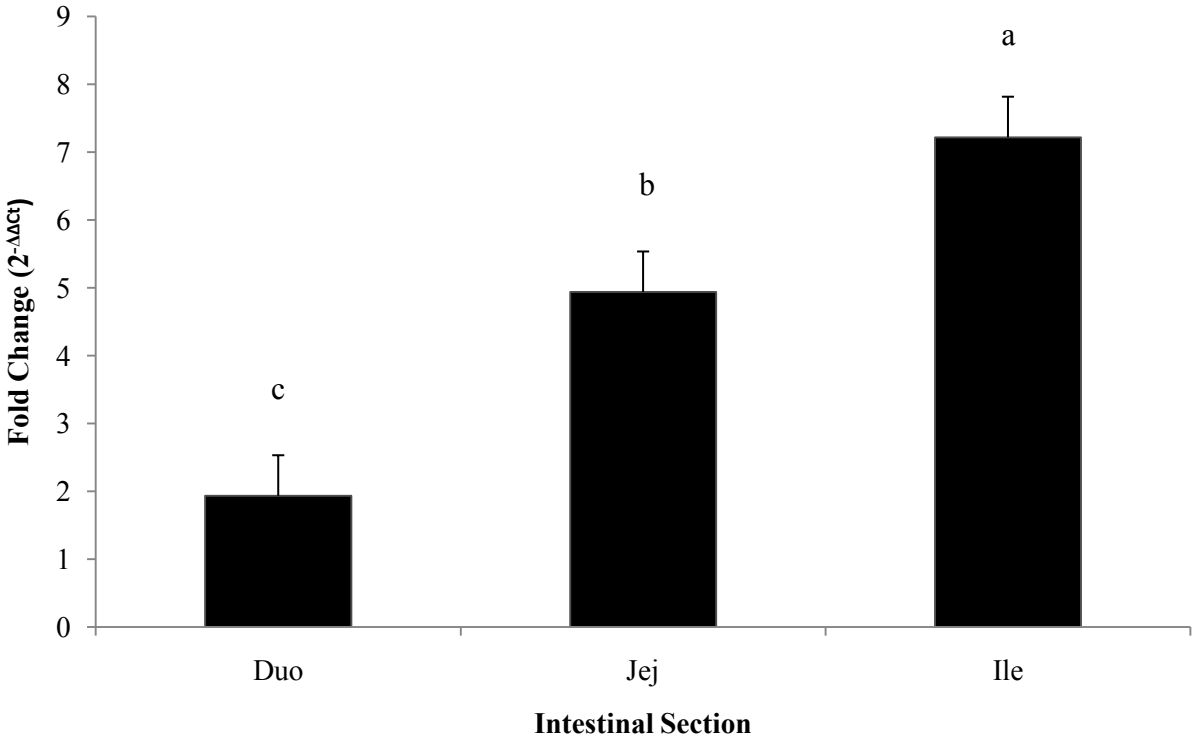

Figure 4.33. Effect of intestinal section on relative Muc-2 expression in the small intestine of Cobb 500 broiler chicks from d4 to d7. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Duo = duodenum; Jej = jejunum; Ile = ileum. There was a significant effect of intestinal section ($P < 0.001$).

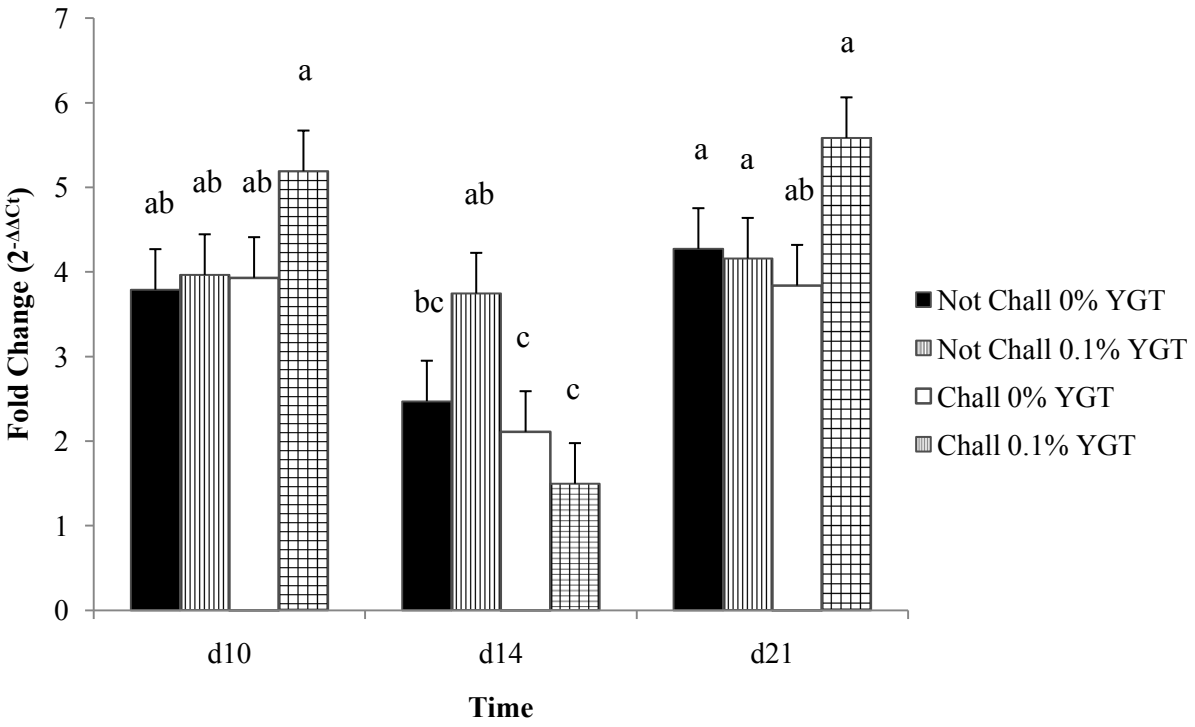

Figure 4.34. Effect of time, β-glucan supplementation and *Eimeria* challenge on relative Muc-2 expression in the small intestine of Cobb 500 broiler chicks from d10 to d21. Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average ΔCt value for the duodenum of the 0.0% YGT fed birds on d4 as the calibrator. Data are represented as LS Means + SEM. Not Chall = not challenged; Chall = challenged with *Eimeria*; YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan. There was a significant three-way interaction ($P = 0.003$) of time, diet and challenge.

Chapter V

Epilogue

The small intestine is not only a vital component of the digestive system but also the largest lymphoid organ, albeit not as defined as the other well known immune organs such as the bursa of Fabricius, thymus, and spleen. The integrity of the digestive tract is essential for protecting the host against enteric diseases such as coccidiosis. With the ban of antibiotics as feed additives by the European Union and the looming ban in the U.S., a more consumer friendly and natural alternative needs to be procured in the immediate future. The results presented in this thesis indicate that dietary β-glucan supplementation modulates the immune system, specifically gut immune responses. The evidence reported herein suggests that β-glucans exhibit different effects depending on the presence of a challenge. Though the differences were subtle, β-glucan significantly down-regulated Th1 cytokines during the pilot study where no challenge was present. The opposite was true during the subsequent study where β-glucan supplementation led to an up-regulation of Th1 cytokines and a down-regulation of Th2 mediators during an *Eimeria* challenge. This indicates that β-glucans are capable of enhancing the pro-inflammatory immune response during times of challenge and down-regulate potentially deleterious hyperactivity of the immune system after the clearing of the infection. It would have been advantageous to look at cytokine profiles in the blood in order to determine if the effects on gene expression were translated into differences in protein levels, but unfortunately those capabilities are not yet available for poultry researchers. In addition, mRNA expression levels of the Th2 cytokines should have also been evaluated during the pilot study in order to determine if β-glucan supplementation had an effect in the absence of a challenge.

Because β-glucans are not expressed on mammalian cells, they are recognized as PAMPs by PRRs on innate immune cells. Much work has been completed characterizing the many receptors responsible for recognizing β-glucans and subsequently activating the immune system in mammals. The most widely acknowledged of these receptors is Dectin-1. To date, very little information has been reported regarding the receptors responsible for recognizing β-glucans in avian species. This lack of knowledge makes it difficult to determine if the avian immune system is in fact recognizing β-glucans as foreign antigens. Once we shed some light on the molecular mode of action of β-glucans in poultry, we may better employ β-glucans to our advantage.

Though the addition of β-glucan did not prevent mortality in the *Eimeria*-challenged birds, it significantly reduced the severity of gross intestinal lesions, suggesting that β-glucans can potentially protect the host from more severe symptoms. It is important to note that during the first week of this trial, there was an unintentional *E. coli* infection presumed to have been contracted at the hatchery. This infection resulted in early mortality of several chicks. The residual of this infection, confounded with the experimental *Eimeria* infection, was obviously too much for the immune system of the chick to overcome and may have been too severe to allow the true effects of the β-glucan on mortality to be manifested. This suggests that β-glucans may not be potent enough to be efficacious against a more arduous infection or concurrent infections.

Both studies concluded that β-glucan supplementation did not carry detrimental effects on performance, an issue of concern in some previous research. In the case of the larger study, β-glucan supplementation at 0.02% YGT exerted a positive impact on body weight during the first week post hatch, even in the presence of a natural *E. coli* infection. Though differences in performance were not seen after the first week, these results further indicate that β-glucan as a

feed additive does not "over activate" the immune system leading to adverse consequences on performance.

From these two studies, there is great potential for future research. The first endeavor should be to repeat the second trial using chicks known to be free from *E. coli* in order to determine the true effects of β-glucan supplementation on an *Eimeria* infection. Although the natural *E. coli* infection did carry negative consequences in terms of our research, it provided useful information on what could potentially happen in a realistic production setting. Producers take great strides in preventing disease in their flocks, but from time to time incidences such as this do occur and it is useful to know and understand the outcomes of these situations. The unexpected *E. coli* infection has also sprouted other ideas for future work. It would be interesting to learn how β-glucan may modulate the immune system in different challenge scenarios. For example, how does the immune system react to intracellular pathogens such as *Eimeria* versus an extracellular pathogen such as *E. coli*? Since extracellular pathogens induce primarily a Th2-mediated reaction and our results indicate a favoring of the Th1 response by β-glucan, it will be interesting to see if β-glucan will utilize the same or different pathway(s) in terms of the host immune response. If a soluble fraction of β-glucan from the yeast *S. cerevisiae* could be acquired, it would be beneficial to see if in ovo administration of β-glucan could enhance the immune system of the chick even before hatch and potentially provide protection during the time immediately post-hatch where the chick is immunologically most vulnerable. Such scenario would have proven highly advantageous in the second trial with the early *E. coli* infection.

There has been much talk about how β-glucans could potentially serve as an antibiotic alternative. However, little work exists directly comparing the effects of β-glucan and antibiotics

in poultry. Such research needs to be performed in order to evaluate the credibility of β-glucan as an alternative to the sub-therapeutic use of antibiotics. It is my opinion, with our limited current knowledge of the effects of dietary β-glucan in poultry, that β-glucans will not prove to be as capable as antibiotic treatments, but they may alleviate the damaging impact of disease and thus reduce monetary losses for producers. Furthermore, this work should be extended to other avian subjects including turkeys and laying hens. The more we know about the mode of action of β-glucans as feed additives in different production settings and avian species, the more we will understand about β-glucans as immunomodulators and employ them to our advantage.

Appendix: Non-Significant Results

Table A.1. Effect of dietary β-glucan supplementation on immune organ weights

	d7	SEM	*P*-value[1]	d14	SEM	*P*-value[2]
Bursa Weight (% BW)						
0.0% YGT	0.142	± 0.01	0.59	0.201	± 0.01	0.14
0.02% YGT	0.150			0.158		
0.1% YGT	0.161			0.187		
Spleen Wight (% BW)						
0.0% YGT	0.090	± 0.01	0.48	0.078	± 0.01	0.56
0.02% YGT	0.102			0.081		
0.1% YGT	0.083			0.071		

[1] *P*-value derived from effect dietary treatment on d7
[2] *P*-value derived from effect of dietary treatment on d14
YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan

Table A.2. Effect of dietary β-glucan treatment and Eimeria challenge on body and bursa weights

	d4	d7	SEM	*P*-value[1]	d10	d14	d21	SEM	*P*-value[2]
Body Weight									
0.0% YGT, Not Chall	91.0	157.6	± 1.25	0.24	250.2	411.7	825.1	± 16.03	0.94
0.02% YGT, Not Chall	93.1	162.6			258.3	439.2	838.4		
0.1% YGT, Not Chall	90.9	157.8			255.5	426.4	826.9		
0.0% YGT, Chall	-	-			244.2	336.7	642.6		
0.02% YGT, Chall	-	-			254.1	337.0	621.4		
0.1% YGT, Chall	-	-			254.9	347.0	638.4		
Bursa Weight (%BW)									
0.0% YGT, NC	0.131	0.200	± 0.01	0.15	0.195	0.209	0.201	± 0.02	0.77
0.02% YGT, NC	0.147	0.171			0.164	0.210	0.212		
0.1% YGT, NC	0.140	0.139			0.170	0.210	0.221		
0.0% YGT, Chall	-	-			0.182	0.207	0.216		
0.02% YGT, Chall	-	-			0.179	0.236	0.212		
0.1% YGT, Chall	-	-			0.181	0.208	0.205		

[1] *P*-value derived from the interaction of time and dietary treatment from d4 to d7
[2] *P*-value derived from the interaction of time, dietary treatment, and *Eimeria* challenge from d10 to d21
Not Chall = not challenged; Chall = challenged with *Eimeria*
YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan

Table A.3. Effect of dietary β-glucan treatment and *Eimeria* challenge on body weight gain, feed intake and feed conversion ratios

	d1-d7	SEM	*P*-value[1]	d10-d21	SEM	*P*-value[2]
Body Weight Gain						
0.0% YGT, Not Chall	115.4	± 1.58	0.05	665.9	± 25.1	0.76
0.02% YGT, Not Chall	120.2			674.3		
0.1% YGT, Not Chall	115.2			667.2		
0.0% YGT, Chall	-			486.6		
0.02% YGT, Chall	-			460.3		
0.1% YGT, Chall	-			482.5		
Feed Intake						
0.0% YGT, Not Chall	149.8	± 2.45	0.32	982.6	± 36.6	0.73
0.02% YGT, Not Chall	154.6			881.3		
0.1% YGT, Not Chall	150.4			963.4		
0.0% YGT, Chall	-			803		
0.02% YGT, Chall	-			760.4		
0.1% YGT, Chall	-			834.8		
Feed Conversion Ratio						
0.0% YGT, Not Chall	1.30	± 0.02	0.81	1.48	± 0.10	0.92
0.02% YGT, Not Chall	1.29			1.43		
0.1% YGT, Not Chall	1.31			1.44		
0.0% YGT, Chall	-			1.72		
0.02% YGT, Chall	-			1.68		
0.1% YGT, Chall	-			1.76		

[1] *P*-value derived from the effect of dietary treatment from d1 to d7

[2] *P*-value derived from the interaction of dietary treatment and *Eimeria* challenge from d10 to d21

Not Chall = not challenged; Chall = challenged with *Eimeria*

YGT = Auxoferm YGT, *Saccharomyces cerevisiae* derived β-glucan