EVALUATION OF GLUTAMINE SUPPLEMENTATION ON BROILER PERFORMANCE, INTESTINAL IMMUNE PARAMETERS, AND IMMUNITY ACQUISITION DURING EXPERIMENTAL ENTERIC CHALLENGE MODELS

by

JARRED HUGH OXFORD

(Under the Direction of Ramesh K. Selvaraj)

ABSTRACT

Enteric challenges play a significant role in poultry production due to their effects on nutrient digestibility and utilization. Glutamine was traditionally considered as a non-essential amino acid in humans and other animals; however, recent research has begun to provide evidence that glutamine is conditionally essential during times of enteric challenge. Three experiments were conducted to evaluate the effects of glutamine supplementation during enteric challenges and immunity acquisition. The first study demonstrated that glutamine supplementation improved intestinal health during an experimental coccidiosis infection through increased tight junction mRNA expression and improved jejunal morphology. The second study demonstrated that serum glutamine concentrations were significantly affected during an experimental necrotic enteritis infection and that glutamine supplementation helped improve serum glutamine concentrations during this time. Supplementation of glutamine at 0.5% increased IL-10 and Claudin-1 mRNA expression, which translated to reduced intestinal permeability. This study also showed that supplementation of glutamine at 0.5% may be beneficial during necrotic enteritis and that supplementation of glutamine at 1.0% negatively

affected the bird during infection. The third study demonstrated that glutamine supplementation did not affect fecal oocyst cycling in birds given a non-attenuated coccidiosis vaccine. It was also observed in this study that glutamine supplementation helped reduce the negative effects of challenge in immunized birds through decreasing IFN-γ mRNA expression and increasing IL-10 mRNA expression. This research demonstrates that glutamine supplementation may be beneficial in broilers during times of enteric challenge or immunity acquisition. There is also evidence from this work that glutamine may be a conditionally essential amino acid in broilers, during an enteric challenge.

INDEX WORDS: glutamine, broiler, coccidiosis, necrotic enteritis, nutrition, immunology

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DEDICATION

There are three people in which I would not have been able to make it through this process. I dedicate this achievement to:

The first of which is my dear mother Aleisa Oxford, without your example of hard work and persistence, no matter the circumstance, I would not be the person I am today. I will never be able to repay the sacrifices that you have made for me and thank you for all that you have done.

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CHAPTER 1

INTRODUCTION

The commercial poultry industry is undergoing changes towards antibiotic-free production due to shifts in consumer preferences. With these changes, poultry producers are losing an array of useful animal health products for the prevention of coccidiosis and/or necrotic enteritis. Coccidiosis was primarily prevented through the use of anticoccidial drugs in the past; however, many of these drugs are considered antibiotics and cannot be used anymore. These changes in the amount of usable anticoccidial drugs have led many producers to switch to the use of coccidiosis vaccines. Previously coccidiosis vaccines could be successfully used in conjunction with antibiotic growth promoters, however without the use of antibiotic growth promoters, the incidence of necrotic enteritis has become more prevalent. Due to the climate of the commercial poultry industry, there is a need for alternative products that help negate the negative effects of coccidiosis and necrotic enteritis and that can be used in birds that have been given coccidiosis vaccines. During times of infection and immunity acquisition, nutrient digestion and absorption can become limited in the intestines; therefore, we hypothesized that supplementing dietary glutamine could help reduce the negative effects of coccidiosis and necrotic enteritis and help during immunity acquisition in birds given a non-attenuated coccidiosis vaccine.

Three aims were developed to test the hypothesis mentioned above:

Aim 1: To evaluate the effects of glutamine supplementation on broiler performance and intestinal immune parameters during an experimental coccidiosis infection

Aim 2: To evaluate the effects of glutamine supplementation on broiler performance and intestinal immune parameters during an experimental necrotic enteritis infection

Aim 3: To evaluate the effects of glutamine supplementation on broiler immunity acquisition when given a non-attenuated coccidiosis vaccine

CHAPTER 2

LITERATURE REVIEW

THE IMMUNE SYSTEM

The immune system is comprised of many different cells, organs, and other tissues that aid in the protection of organisms from viruses, diseases, and other harmful substances. The two main parts of the immune system are known as the innate and adaptive immunity (Kellie and Al-Mansour, 2017). The innate immunity is considered to be the first line of defense against microbes and pathogens. This system is made up of three key components: 1) physical and chemical barriers (such as skin and villi), 2) monocytes/macrophages, neutrophils, dendritic cells, natural killer cells (NK cells), and innate lymphoid cells (ILCs), and 3) blood proteins (Yun, et al., 2000). The adaptive immune system works as a response to exposure to pathogens/microbes and from repeated exposure will "adapt" to the specific invasions. Through identification of antigens presented by pathogens and the use of T and B lymphocytes to signal immune responses, the adaptive immunity is able to target pathogen specific defenses. One of the key differences between the innate and adaptive immune system is that the innate immune system treats intrusions in a broader spectrum while the adaptive immune system is more specific to the pathogens that it is fighting and can recognize and "adapt" to multiple exposures to said pathogens. Another important difference to note is that an innate immune response is significantly more costly than that of an adaptive immune response.

IMMUNE CELLS

Macrophages are used for both innate and adaptive immune responses. These cells are derived from monocytes and originate from bone marrow. One of the main functions of macrophages is during times of cell damage or infection they will ingest and break down microbes, damaged cells, and apoptotic cells to prevent further cellular damage (Martinez and Gordon, 2014). These cells are important for their antigen presenting (APC) properties used to stimulate other immune cells. A key characteristic of macrophages is their ability to differentiate phenotypically based on their activation stimulus. Depending on the stimulus during activation, macrophages will either differentiate into M1 or M2 macrophages. M1 activation has been shown to be induced by pathogens, components of bacteria cell walls, the cytokines interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) as well as toll-like receptor (TLR) agonist like lipopolysaccharide and follow a TH1 immune response; whereas M2 macrophages are considered to be more geared to cellular repair (TH2 response) and are activated via IL-4 (Rath, et al., 2014; Wang, et al., 2014; Jha, et al., 2015; Rőszer, 2015). One key mechanism that macrophages use against pathogens is the production of nitric oxide (Hibbs, et al., 1987). This molecule is produced from L-arginine and nitric oxide synthase when macrophages are activated and has been shown to have an effect on many different pathogens. Like macrophages, dendritic cells originate from monocytes in the bone marrow. These cells are also key APC cells that phagocytize pathogens and have high expression of surface MHC-class II. Little work has been done showing the roles of dendritic cells in coccidiosis or necrotic enteritis, but it is believed that their main function is antigen presentation. Both macrophages and dendritic cells release cytokines and chemokines to alert other immune cells of present pathogens (Gallo and Gallucci, 2013).

B cells develop in the spleen and migrate to the bursa in which they complete their differentiation (Masteller and Thompson, 1994). When activated by extracellular stimuli, these cells produce antibodies that are used to recognize and neutralize pathogens. There are three types of immunoglobulins produced by B cells; IgA, IgM, and IgY (the avian version of IgG) which is considered to be similar to the mammalian IgG (Carlander, et al., 1999). IgA antibodies are mainly localized in the mucosa, IgY are the predominant antibodies found in the chicken, whereas IgM plays a role in the innate immunity (Lebacq-Verheyden, et al., 1974).

T Cells are important adaptive immune cells that are developed in the thymus. There are two main types of T cells found in chickens, $CD4^+$ (T helper cells) and $CD8^+$ (cytotoxic T cells). $CD8^+$ T cells are the main T cells found in the gut and are responsible for recognizing intracellular pathogens and recognize major histocompatibility (MHC)-class I molecules (Sanchez-Garcia and McCormack, 1996). Whereas $CD4^+$ cells are mainly found in the spleen and recognize antigens associated with MHC-class II molecules (Lillehoj and Trout, 1996). T helper cells in the inactivated state are called Th2 cells and are skewed towards an anti-inflammatory state, once activated they begin to secrete IFN-γ and are referred to as TH1 cells and have pro-inflammatory characteristics (Atkins, et al., 2003). T cells also have the ability to develop memory to pathogens, therefore when a second exposure to the pathogen occurs there is a much strong and precise immune response.

Natural killer (NK) cells are granulocytes that produce perforin and granzymes which are used to lyse pathogens. As of now there are no validated specific marker for avian NK cells so they are usually identified as $CD8^+$ cells that lack Ig or CD3 markers (Gobel, et al., 1994). In work looking at the distribution of NK cells in different tissues of chickens, the largest percentage of NK cells were found in the IEL and consisted of around 30% of the lymphocyte

populations (Göbel, et al., 2001). These cells have been shown to have little to no role in the immune response to *Eimeria* or necrotic enteritis infections, but play and integral role in protection against viruses (Smith, et al., 1994).

GUT-ASSOCIATED LYMPHOID TISSUE (GALT)

In poultry, the GALT is considered to be one of the most important immune systems in the body. The GALT is made up of the bursa, Peyer's patches, cecal tonsils, and clusters of IEL within the epithelium and lamina propria (Lillehoj, et al., 2004). The main intestinal epithelial lymphocytes that can be found in the GALT include B cells, T cells, macrophages, dendritic cells, heterophils, M cells, and NK cells. The M cells found in the GALT play an important role in up taking luminal contents that is then provided to dendritic cells and macrophages to be evaluated for pathogens (Jeurissen, et al., 1999; Kitagawa, et al., 2000). If pathogens are identified, the dendritic cells and macrophages will then present specific antigens to the local T and B cells to orchestrate an immune response. The GALT has three main functions, tolerance of nutritional molecules, antigen presentation, and stimulation of the cellular immune system (Shivaramaiah, et al., 2014).

INTESTINAL PHYSICAL AND CHEMICAL BARRIERS

Within the intestine there are a multitude of cells that make up finger like projections called villi (Potten, 1997). These villi act as a primary barrier for protection from pathogens as well as they provide a large surface area used for the absorption of nutrients that pass through the lumen. The villi are made up of intestinal epithelial cells that are formed in a region at the base of the villi called the "villi crypt" (Willing and Van Kessel, 2007). Cellular division occurs in the

crypts to produce new cells that will mature and migrate from the crypts up towards the tips of the villi (Ensari and Marsh, 2018). During times of villus damage the crypt regions will become larger to create more epithelial cells to replace cells in the villi. It has been shown in birds infected with coccidiosis that there is villus atrophy that occurs and in response the crypts become "deeper" to produce more cells (Fernando and McCraw, 1973). Increases in villus height to crypt depth ratios show a reduction in cellular turnover and has been correlated with improved nutrient absorption due to increased surface area. It has previously been shown that glutamine supplementation can increase villus height, decrease crypt depths, and improve villus height to crypt depth ratios in both birds at normal health status and those that are under enteric challenge (Yi, et al., 2005; Bartell and Batal, 2007; Mussini, et al., 2012; Luquetti, et al., 2016; Xue, et al., 2018).

Within the intestinal epithelium there are specialized secretory cells called goblet cells, the main function of these cells is the secretion of glycoprotein rich mucin (Lillehoj, et al., 2004). This mucin is considered to be the first barrier against pathogens in the chicken's intestines. Other secretory cells can also be found in the intestinal epithelium; their main function is to produce antimicrobial peptides (AMP) for protection of the gut (Zhao, et al., 2001). As of now there have only been two types of antimicrobial peptides identified in the intestines of poultry, cathelicidin proteins and defensins. AMPs are naturally produced antimicrobials that are capable of killing and disrupting bacteria, yeasts, fungi, and other pathogens (De Smet and Contreras, 2005). The main method in which AMPs work is by forming holes in the cellular membranes of the pathogens which ultimately leads to the death of the pathogen (Kagan, et al., 1990).

Another important barrier that is found in the gut are tight junctions. Tight junctions are made up of many different proteins (claudins, occludens, zonula occludens, and junction

adhesion molecules) with the main function of connecting the epithelial cells to each other (Awad, et al., 2017). These connections form barriers to ensure that foreign objects from the lumen are not allowed to pass through the epithelium. Tight junctions also form channels between the epithelial cells to allow for permeation of the intestines, these channels allow for ion exchange and water absorption (Van Itallie, et al., 2008). While maintained properly tight junctions provide many benefits to the birds, however during times of stress and damage, tight junctions can provide a direct path for pathogens to enter the birds (Shen, et al., 2011). When there is a loss in tight junction structure and function, increased intestinal permeability can occur which can lead to the infiltration of the epithelium by pathogens and ultimately lead to septicemia.

COCCIDIOSIS

Coccidiosis is an intestinal disease that can cause losses to performance and is a known precursor of necrotic enteritis. It is estimated that the global impact of coccidiosis to the poultry industry is around 3 billion dollars annually (McDonald and Shirley, 2009). Due to the extensive damage that occurs to the intestines during coccidiosis; there are losses to body weight, feed conversion, pigmentation, and in severe cases there is extensive mortality. This disease is self-limiting in nature meaning that through replication in the bird, immunity is developed.

Coccidiosis is caused by multiple species of apicomplexan parasites from the genus of *Eimeria* (Tyzzer, 1929). In chickens there are a total of nine *Eimeria* species that have been described, however two of the nine have not been validated using modern molecular technology (Albanese, et al., 2018). The French researchers Raillet and Lucet evaluated oocysts that were isolated from the ceca of chickens suffering from coccidiosis and described a new parasite called

Coccidium tenellum, which was later changed to *E. tenella* (Raillet and Lucet, 1891a; Raillet and Lucet, 1891b; Chapman, 2003). Harold Benjamin Fanthom was the first person to document a detailed life cycle of *Eimeria* in poultry (Wenyon, 1926; Chapman, 2003). At this time, he concluded that there was only one species of *Eimeria* that infected poultry which he named *Eimeria avium*. Approximately 32 years after the original discovery and description of *E. tenella*, Tyzzer described three new chicken *Eimeria* species: *E. acervulina*, *E. maxima*, and *E. mitis* (Tyzzer, 1929). Following Tyzzer's discovery, W.T. Johnson described two new species, *E. necatrix* and *E. praecox*, and confirmed the three species previously described by Tyzzer (Johnson, 1930). Of the seven confirmed *Eimeria* species, *E. brunetti* was described last in 1942 (Levine, 1942).

EIMERIA LIFE-CYCLE

Eimeria infections begin when a bird ingests sporulated oocysts. These oocysts then travel down to the gizzard where they are crushed by the grinding action of the gizzard and release four sporocysts per oocyst (Long, 1982; Conway and McKenzie, 2007). From here the sporocysts travel to the duodenum where trypsin and bile activates the release of two sporozoites per sporocyst (total of eight sporozoites per oocyst). The released sporozoites then enter the host's mucosal cells using organelles called micronemes and rhoptries. Micronemes release proteins that aid the sporozoites in attaching and infiltration of the mucosal cells, rhoptries release proteins that are used to form a parasitic vacuole within the host cell for the *Eimeria* to grow and develop in. After invasion the immature *Eimeria* is called a trophozoite and will feed for 12-48 hours. Once the trophozoite has retained enough nutrients it will undergo asexual reproduction to produce around 900 merozoites within a schizont in a process called schizogony.

The schizont then ruptures to release the merozoites into the intestinal lumen to re-infect the host's mucosal cells as trophozoites. A second generation of schizogony is completed however only 200-350 merozoites are produced in this cycle. The merozoites are then released from the second schizogony and they will enter the hosts mucosal cells and either conduct a third schizogony or they will differentiate to be microgametocytes (males) or macrogametocytes (females). The microgametocytes will mature and produce model microgametes that are released to fertilize the macrogametes. Once the macrogamete is fertilized, a thick wall is formed around the zygote to become an immature oocyst. From here the oocyst is shed from the intestinal cells into the lumen and excreted in the bird's fecal material. With adequate moisture, temperature, and oxygen, the oocysts will sporulate and become infective again. The prepatent period can vary based on the species of *Eimeria* that is being cycled and can range from 84 to 138 hours. Due to the nature of the life cycle of *Eimeria* these parasites are self-limiting.

IMMUNE RESPONSE TO *EIMERIA*

Most of the immune response to *Eimeria*, within the intestine, takes place in the GALT. In the GALT the primary immune function to *Eimeria* is through cell-mediated responses (Lillehoj and Lillehoj, 2000). *Eimeria* profilin has been shown to be one of the main TLR agonists during an *Eimeria* infection and has been shown to induce the increased expression of IFN-γ (Lillehoj, et al., 2000; Tang, et al., 2018). *Eimeria* infection is first recognized by the various APCs, including dendritic cells, macrophages, B cells, and epithelial cells, in the intestinal environment (Lillehoj and Trout, 1996). Upon recognition of the antigen, these immune cells release many cytokines and chemokines to induce an immune response (Table 2.1). These cytokines and chemokines notify other immune cells of the infection and induce

activation and migration of T cells, NK cells, and macrophages. In a response to *Eimeria* infection macrophages have been shown to increase production of nitric oxide, this is believed to be stimulated by the increase production of IFN-γ that occurs during the initial response (Lillehoj and Li, 2004).

Table 2.1: Cytokines involved in the immune response of birds to an infection with *Eimeria*. Adapted from Shivaramaiah, et al. (2014)

Type	Cytokine	Role
Pro-inflammatory	IFN-y	initial immune response and prevention of developmental stages of *Eimeria*
	TNF-like factor	Stimulation of immune response and increased during oocyst release
	IL-1ß	Promotes inflammatory immune response
	IL-2	Activation of B cells and NK cells
	IL-6	Promotes development of acquired immunity
	IL-8	Signals recruitment of $CD4_+$ T cells and macrophages
	IL-12	Signals recruitment of $CD4_+$ T cells
	IL-17	Promotes mucosal response and stimulates production of other cytokines
	IL-18	Signals recruitment of $CD4_+$ T cells and upregulation of immune response
Anti-inflammatory	TGF- ß	Down regulates immune response and promotes mucosal repair
	IL-4	Helps reduce mucosal tissue damage
	IL-10	Helps reduce mucosal tissue damage

There are two main types of T cells that are involved in the immune response of birds to *Eimeria*, $CD4_+$ T helper cells and $CD8_+$ cytotoxic lymphocytes (Min, et al., 2013). $CD8_+$ T cell percentages have been shown to increase in chickens infected with *E. acervulina* with most of the sporozoites being found within $CD8_+$ T cells (Lillehoj and Bacon, 1991; Trout and Lillehoj, 1995). Vervelde, et al. (1996), demonstrated that macrophages and $CD4_+$ T cells were used during the initial infection of chickens with *E. tenella* and that $CD8_+$ cells were observed at

higher concentrations in challenged immune chickens (Vervelde, et al., 1996). In more recent research, it was shown that $CD4^+$ and $CD8^+$ T cells were found at higher concentrations in the cecal tonsils of birds challenged with a mixed *Eimeria* infection compared to non-challenged birds (Walston, et al., 2016). Unfortunately, there is yet to be a complete detailed mechanism of what T cells do to protect the bird during *Eimeria* infections, however there is indication that they play a key role in the immune response (Min, et al., 2013). It has also been shown that *Eimeria* have the capability to secrete macrophage migration inhibitory factor (MIF) to help reduce the host immune response during primary infection (Hong, et al., 2006).

There have been conflicting opinions on the role of antibodies in the immune response to *Eimeria*. It was previously believed that antibodies played no role in protective immunity against *Eimeria*; however, more recently there has been evidence showing that antibodies may provide protection during an *Eimeria* infection (Wallach, 2010; Min, et al., 2013). Pierce and Long (1965), demonstrated that in bursectomized birds, the loss of antibody production did not inhibit acquired immunity to coccidiosis. In previous research, it was shown that antibodies only played a role in immunization and had no effect on initial infection (Rose and Long, 1971). In contrast, there is relatively high IgA binding capacity to *E. tenella* sporozoite membrane antigens demonstrated using immunofluorescences (Trees, et al., 1989). In the same study, the authors observed antibody concentrations to peak between 7-13 days post infection (dpi) and remain detectable to 21 dpi. Furthermore, *E. maxima* specific IgG that was subcutaneously injected into naïve chicks was shown to reduce oocyst production and reduce negative effects on performance when the birds were challenged with *E. maxima* oocysts (Rose, 1974). Researchers have also shown the protective capabilities of passive IgG antibodies to *E. acervulina*, *E. maxima*, and *E. tenella* (Lee, et al., 2009a; Lee, et al., 2009b).

Immunity development to *Eimeria* can be problematic because there is no cross protection against different *Eimeria* species, and the sites of infection differ between species (Yun, et al., 2000). Even when immunity has been developed, the host response primarily inhibits the development of the *Eimeria* once intracellular but has little effects on epithelial cell infiltration (Shirley, et al., 2005; Dalloul and Lillehoj, 2006). The most immunogenic phase of the lifecycle occurs during the asexual phases with the sexual phase not being very immunogenic (Rose, 1987). For species like *E. maxima*, there have been incidences where immunity to one *E. maxima* strain does not always correlate to immunity to other *E. maxima* strains (Martin, et al., 1997; Smith, et al., 2002). While different strains can affect immunity there are also other parameters that can have direct effects on immunity including inoculate dose, age of the bird, genetics of the bird and many other factors (Lillehoj, 1988; Blake, et al., 2005). When using coccidiosis vaccines, the bird can usually acquire immunity within 2-4 cycles, this can depend on which vaccine and species immunogenicity. *E. maxima* and *E. praecox* have been shown to be the most immunogenic followed by *E. acervulina*, *E. brunetti*, and *E. mitis*; with *E. tenella* and *E. necatrix* being the least immunogenic of the species (Long, et al., 1976; Long and Johnson, 1988). It is important to note that pathogenicity does not always correlate with immunogenicity; *E. tenella* and *E. necatrix* are very pathogenic however they are the least immunogenic, in contrast, *E. praecox* is very immunogenic with relatively low pathogenicity. Fortunately, once immunity is developed to an *Eimeria* species, the bird will retain that immunity throughout the rest of its life.

NECROTIC ENTERITIS

In previous years necrotic enteritis was primarily controlled using antimicrobial growth promoters (AGPs), specifically Bacitracin and Virginiamycin. With the withdrawal of Roxarsone from the market in 2011 and current consumer preferences for antibiotic free poultry production the incidence of necrotic enteritis has increased substantially, up to one percent daily (Williams, 2005; Van Immerseel, et al., 2009; Cervantes, 2017). The overall global estimated cost of necrotic enteritis to the poultry industry is around 6 billion dollars, and is considered to be the third most problematic disease behind coccidiosis and *E. maxima* (Cervantes, 2017; Fasina and Lillehoj, 2018). The causative agent of necrotic enteritis is the anaerobic gram-positive bacteria *Clostridium perfringens*, which is commonly found in nature as well as the ceca of chickens (Fasina and Lillehoj, 2018). It is estimated that there is around 10^4 CFU of *C. perfringens* per gram of luminal contents in the intestinal tract of a healthy broiler (McDevitt, et al., 2006). During times of normal health this load of *C. perfringens* has no negative effects on the bird, however when there are times of intestinal damage, excess mucin production provides a more anaerobic environment and excess nutrients for *C. perfringens* to proliferate to reach levels of around 10^7 to 10^9 CFU/g of luminal contents (Williams, 2005). As mentioned above, intestinal damage is a precursor of necrotic enteritis and coccidiosis is considered to be one of the primary predisposing factors to necrotic enteritis (Williams, 2005). Excess damage that is caused by coccidiosis can cause plasma leakage into the intestinal lumen which provides an excellent substrate for growth and proliferation of *C. perfringens* (Van Immerseel, et al., 2009).

Once *C. perfringens* reaches high levels in the intestines, α-toxin and necrotic enteritis toxin B (NetB) are produced and cause extensive damage to the intestinal mucosa which leads to losses in performance and mortality in birds (Van Immerseel, et al., 2009). Enterotoxins that are

produced by the *C. perfringens* have been shown to use tight junction proteins as cell receptor sites (Awad, et al., 2017). When the enterotoxins bind to the tight junction proteins they cause a disruption in the cellular membranes and lead to increased intestinal permeability (Saitoh, et al., 2015). This increase in permeability allows for the *C. perfringens* to go systemic as well as leads to more plasma leakage into the intestines that provides addition nutrients for the pathogen to thrive. Once intestinal barrier function is broken down *C. perfringens* will travel to the kidneys, liver, and heart via the bloodstream where it will cause hepatic congestion (Al-Sheikhly and Truscott, 1977; Shane, et al., 1985).

IMMUNE RESPONSE TO NECROTIC ENTERITIS

Within the gastrointestinal tract, *C. perfringens* is first recognized by APCs (dendritic cells, macrophages, and B cells) via pathogen-associated molecular patterns (PAMPS). The main recognition receptor for *C. perfringens* in the intestines is the Toll-like receptor 2 (TLR2), this TLR has the ability to recognize peptidoglycans located in the cell wall of *C. perfringens* (Guo, et al., 2015; Du, et al., 2016; Fasina and Lillehoj, 2018). Once activated TLR2 induces the myeloid differentiation primary response 88 (MyD88) pathway leading to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) and the induction of an immune response (Lu, et al., 2009; Guo, et al., 2015; Oh and Lillehoj, 2016). From here a large array of cytokines and chemokines are released to help direct the immune response against the pathogen. In previous work it was shown that IFN-γ, IL-4, IL-10, and IL-1β were significantly increased in birds infected with necrotic enteritis (Collier, et al., 2008; Park, et al., 2008; Oh and Lillehoj, 2016). However, in other work it was shown that *C. perfringens* reduced the expression of anti-inflammatory cytokines while increasing the expression pro-inflammatory cytokines (Hong, et

al., 2012). In the study previously mentioned, the authors also saw a significant increase in β-defensins in birds challenged with necrotic enteritis, showing that β-defensins may play an important role in the bird's immune response against necrotic enteritis.

It has been shown that when birds are challenged with *C. perfringens* alone, there is no increase in nitric oxide production and when birds are challenged with *Eimeria* alone there is a significant increase in nitric oxide production (Park, et al., 2008). However, in this study when birds were challenged with both *C. perfringens* and *Eimeria*, there was a reduction in nitric oxide production compared to the *Eimeria* only challenged birds. These results may show indication that *C. perfringens* reduces or inhibits NO production as a protection mechanism.

There is some evidence that toxin specific antibodies may play a protective role against necrotic enteritis. In work evaluating flocks infected with necrotic enteritis, it was shown that the birds that did not succumb to the infection had consistently higher α-toxin and NetB toxin antibody levels compared to their counterparts (Lee, et al., 2011). Elevated levels of *C. perfringens* antibodies have been recorded to last from 2-4 weeks post infection (Lu, et al., 2009). It was shown that the progeny of breeder hens vaccinated against *C. perfringens* had increased maternal antibodies against the pathogen and reduced lesion scores when challenged with *C. perfringens* (Thompson, et al., 2006).

EFFECTS OF COCCIDIOSIS AND NECROTIC ENTERITIS ON NUTRITION

Due to the intrusive nature of coccidiosis, there are significant effects on the bird's nutrient absorption and utilization. In fact, 70% of weight loss that occurs during coccidiosis is from reduced feed intake, while 30% of weight loss is due to reduced absorption and utilization (Preston-Mafham and Sykes, 1970). These negative effects on absorption and utilization have

been documented on many different nutrients. For instance, as lesions increase in birds challenged with coccidiosis, average daily gain, energy consumption, retained energy, and feed efficiency all decrease while maintenance cost and excreted energy increases (Teeter, et al., 2008). Previous work showing the effects of varying inoculate levels of either *E. acervulina*, *E. maxima*, or *E. tenella* reduced lipid, carotenoid, and fat soluble vitamin absorption, with *E. maxima* and *E. acervulina* having the greatest effects on absorption (Conway, et al., 1993). There has also been a good amount of work that has demonstrated the negative effects that coccidiosis has on amino acid absorption and metabolism (Williams, 2005; Rochell, et al., 2016a; Rochell, et al., 2016b). In the work conducted by Rochell, plasma glutamine concentrations were significantly decreased during *E. acervulina* infection. Due to the lack of experimental models there is little work conducted evaluating the effects of subclinical coccidiosis on nutrition. It is estimated that 80.5% of the economic losses to subclinical coccidiosis are due to reduced body weight gain and feed conversion which shows that there is an overall effect on the birds nutritional status (Williams, 1999).

Unfortunately, there is little to no research on the effects of nutrient absorption and utilization during subclinical necrotic enteritis infections, but it is believed that the damage has significant effects based on losses to body weight and poor feed efficiency during infection (Kaldhusdal and Hofshagen, 1992). The effects of necrotic enteritis may not only last during the infection but may effect nutrient absorption and utilization in birds that are able to clear the infection (Paiva and McElroy, 2014). Inoculation of *E. maxima* in necrotic enteritis models can also make differentiating the effects of necrotic enteritis and coccidiosis on nutrient absorption and utilization difficult. It may not be imperative to know the differences though, since the

majority of incidences of necrotic enteritis in the commercial industry are in conjunction with coccidiosis infections.

GLUTAMINE

Glutamine is commonly referred to as a non-essential amino acid and is one of the most abundantly found amino acids in the body and blood of humans and animals (Newsholme, et al., 1985; Andrews and Griffiths, 2002). This amino acid can be found in both D and L isomeric forms but is predominantly found in nature in its L form. In a study conducted by Nassiri Moghaddam and Alizadeh-Ghamsari (2013), glutamine concentrations were analyzed in broiler diets formulated based on the nutrient requirements set by the National Research Council (1994), using Near Infrared Spectroscopy (NIRS); the calculated glutamine concentrations in the starter diet was estimated to be 3.21% and the grower diet was estimated to be 2.91%. The glutamine concentrations in this study were substantially higher than the concentrations of other amino acids that were estimated using NIRS.

In 1935, Hans Krebs was the first researcher to hypothesize that glutamine was an important nutrient needed for cellular function (Krebs, 1935; Brosnan, 2001). At that time Krebs believed that the main role of glutamine was to provide energy for respiratory process as well as providing nitrogen for key cellular reactions (Curi, et al., 2007). Since Krebs discoveries, there has been an extensive amount of research showing that glutamine is important for a vast amount of cellular processes and functions including cell metabolism, cell proliferation, protein degradation, cell defense and repair, and immune function. It has been well documented that glutamine is important for prolific cells like intestinal epithelial cells, lymphocytes, monocytes, macrophages, and neutrophils (Newsholme, 2001; Newsholme, et al., 2002; Curi, et al., 2007).

Within these cells glutamine can be used for many different biosynthetic processes but the main use for glutamine is as a key source of energy through gluconeogenesis (Curthoys and Watford, 1995).

As mentioned above, glutamine is an important provider of energy to intestinal epithelial cells (IEC). Epithelial cells have been shown to catabolize most of the glutamine that is received in the diet as well as catabolizing arterial glutamine showing that the requirement for glutamine in these cells is high (Wu, 1998; Reeds and Burrin, 2001). One explanation for the high glutamine need in intestinal cells is that previous research has shown that these cells have very low levels of glutamine synthetase, therefore they cannot convert glutamate to glutamine in adequate amounts (Meynial-Denis, 2017). Within IEC glutamine is converted to glutamate using glutaminase and glutamate dehydrogenase by removing a NH_3 molecule. The NH_3 molecule that is released from this reaction can then be transported through plasma to provide nitrogen for urea synthesis in the liver (Curthoys and Watford, 1995). In the liver, glutamate that is made from glutamine can then be used with acetyl-CoA to activate carbamoyl phosphate synthetase (CPS). CPS is an important enzyme for the regulation of the urea cycle by aiding the conversion of ammonia, bicarbonate, and phosphate to carbamoyl-phosphate, which then reacts with ornithine and enters the urea cycle. Watford (2000), concluded that the liver processes glutamine in the periportal region for use in the urea cycle and in the presence of excess NH_3, glutamate is converted to glutamine in the perivenous region to protect the body from NH_3 toxicity or to provide other regions of the body with glutamine. This was determined by evaluating the levels of glutaminase and glutamine synthetase in the liver and where they are localized. By conducting these two reactions in separate parts of the liver, glutamine can be closely regulated so that the needs of the organism are met. Watford (2000), concluded from previous research that net

synthesis of glutamine does not change in the liver when organisms are fed low protein diets, but the rate of urea synthesis is actually decreased. Decreasing the rate of urea synthesis allows the organism to use the glutamine for other metabolic purposes in the body.

Due to the amount of cell turnover that occurs in the intestines there is a large amount of synthesis of proteins, lipids, and nucleic acids that is required to maintain the needed cell proliferation (Kim and Kim, 2017). Glutamine is regarded to be one of the main providers of nitrogen and carbon for the synthesis of nutrients previously mentioned (Meynial-Denis, 2017). There has also been research showing that glutamine can play an intrinsic role in cell regulation and proliferation by activation of mitogen-activated protein kinases (MAPKs), which are important for regulation of cell proliferation (Zhang and Liu, 2002). Rhoads, et al. (1997), showed that glutamine activates extracellular signal-regulated kinases (ERKs) and jun nuclear kinases (JNKs) which caused a four-fold increase in activator protein 1 (AP-1) dependent gene transcription. AP-1 has been shown to play a key role in regulating cellular growth and division as well as inflammation process. Glutamine is also a precursor to the antioxidant glutathione (Wang, et al., 2015). Cao, et al. (1998), demonstrated that when rats were supplemented with glutamine, there was a significant increase in intestinal glutathione concentrations.

Glutamine can be synthesized from glutamate using glutamine synthetase to add a nitrogen and hydrogen, from ammonia, to the glutamate molecule. This process primarily occurs in the skeletal muscles, lungs, adipose tissue, and the liver (Watford, 2015). When high levels of ammonia are present in the body the conversion of glutamate to glutamine can be used to help protect against ammonia toxicity. However, glutamine can be synthesized, James, et al. (1998), displayed in rats that glutamine synthesis levels were relatively low in the gastro-intestinal tract (2.5 μmol/min) when compared to that of the levels found in the muscle and liver (15.9

μmol/min and 55.0 μmol/min, respectively). Therefore, during times of reduced nutrient digestion or absorption or times of increased needs (stress and illness) the animal may use amino acids from its muscles to fulfill its glutamine requirements. In production animals, catabolism of muscles for amino acids can be very costly to body weight gain and carcass yields. A recent study on pectoralis minor muscle gaping myopathy reported that the pectoralis minor muscles of birds suffering from this myopathy had significantly lower glutamine contents (Soglia, et al., 2019).

GLUTAMINE AND CHALLENGE

Since Kreb's hypothesis on glutamine, there has been much research showing the role of glutamine in the intestinal tract. The intestinal tract is considered to have the largest requirement of glutamine of any organ in the body and depends on absorption from both the intestinal lumen and blood stream to meet its needs (Miller, 1999). As mentioned above, the intestinal enterocytes have low levels of glutamine synthetase therefore they rely heavily on dietary glutamine. A large amount of the intestinal requirement for glutamine can be attributed to its role as the main source of energy in enterocytes and highly prolific cells (Andrews and Griffiths, 2002; Bortoluzzi, et al., 2017). During times of stress or challenge the requirements of the intestinal tract increase and glutamine is taken from other organs in the body, this can be problematic in agriculture animals because an estimated 60% of the body's glutamine is stored in skeletal muscles (Miller, 1999; Coster, et al., 2004). It is believed that gluconeogenesis can be increased by 150-200% during times of infection or stress, with this increase leading to the loss of skeletal muscle tissue (Xue, et al., 2018). Rochell, et al. (2016b), demonstrated that when birds are challenged with *E. acervulina,* their plasma concentrations of glutamine are significantly reduced. This observation

may show that either glutamine absorption is decreased during infection or the intestinal requirement for glutamine is higher during infection.

Glutamine was first observed to have beneficial effects on the intestinal tract in humans and has been used during gastric diseases and in neonatal nutrition (Andrews and Griffiths, 2002; Newsholme, et al., 2002). It is believed that glutamine helps improve intestinal health by increasing enterocyte proliferation, tight junction regulation, regulation of inflammatory pathways, and preventing cellular apoptosis (Kim and Kim, 2017). In animal agriculture, 1% glutamine supplementation improved villus lengths while reducing crypt depths in swine (Kitt, et al., 2002). Furthermore, in work looking at castrated barrows challenged with LPS, The group that received supplemental glutamine had increased concentrations of IgG along with reduced concentrations of plasma cortisol (Hsu, et al., 2012). The same study looked at castrated barrows post weening and saw improved daily body weight gains and better feed efficiency in pigs supplemented with glutamine. Glutamine supplemented at 1% has also been shown to improve ATTD of dietary nutrients, increase serum superoxide dismutase, increase serum IgG, and regulate the levels of peroxisome proliferator-activated receptor y (PPARy), mammalian target of rapamycin (mTOR), and pyruvate kinase (PK) in 28 day-old pigs (He, et al., 2016).

Little work has been done on the effects of glutamine in poultry with most of the work being focused on performance results of nonchallenged birds. Early work in glutamine supplementation was conducted by Yi and colleagues and evaluated the effects of glutamine supplementation on broilers and turkey poults (Yi, et al., 2001a; Yi, et al., 2001b). In Yi's work they demonstrated the effects of glutamine supplementation on improving intestinal villus heights and decreasing crypt depths. Similar results were shown in work looking at broilers supplemented glutamine at 1%, however in this study the authors demonstrated that glutamine

supplementation increased the amount of bile, intestinal, and serum IgA and IgG levels (Bartell and Batal, 2007). Khempaka, et al. (2011), showed that in non-challenged 21-day old broilers, that 1% glutamine supplementation improved villus height to crypt depth ratio in the duodenum and jejunum, however in this study there were no differences observed in performance characteristics. In a similar study conducted by Nassiri Moghaddam and Alizadeh-Ghamsari (2013), supplementation of glutamine at 1% improved intestinal histomorphology observed at 21 and 42 days of age along with improved average daily weight gains.

The supplementation of 0.5% glutamine in heat stressed broilers helped improve final body weights in 42 day-old birds (Ayazi, 2014). Sifa, et al. (2018), demonstrated that acute heat stress caused high levels of intramuscular malondialdehyde while having reduce concentration of glutamine, glutamate, and glutaminase. When the birds in this study were supplemented glutamine at 2% the amount of malondialdehyde in the muscle was significantly decreased while glutamine, glutamate, glutaminase, glutathione, glutathione peroxidase, and total antioxidant capability was increased.

Glutamine supplemented at 1% was shown to improve intestinal morphology and feed conversion of birds vaccinated with coccidiosis vaccine but had no significant effects on body weight (Luquetti, et al., 2016). Mussini, et al. (2012), demonstrated that supplementing glutamine to birds given coccidiosis vaccine helped reduce the negative side effects of the vaccine on body weight. Furthermore, it was demonstrated in nonchallenged birds subjected to a normal commercial vaccination program, 1% glutamine supplementation improved performance, red and white blood cell counts, phagocytic activity, lymphocyte counts, antibody titers to Newcastle disease, total blood proteins, and intestinal histomorphology (Soltan, 2009). Glutamine supplementation in birds challenged with necrotic enteritis improved body weight

gain, feed conversion ratio, and intestinal histomorphology while reducing necrotic enteritis lesion scores (Xue, et al., 2018). These results show that the current commercial diets being fed to poultry may be deficient in glutamine during times of stress or enteric challenge. Glutamine supplementation has shown promising results for use during enteric challenges; however, much work needs to be conducted to evaluate the effects of glutamine supplementation in poultry.

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CHAPTER 3

EFFECTS OF GLUTAMINE SUPPLEMENTATION ON BROILER PERFORMANCE AND INTESTINAL IMMUNE PARAMETERS DURING AN EXPERIMENTAL COCCIDIOSIS INFECTION

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ABSTRACT

The objective of this study was to evaluate the effects of glutamine supplementation on performance and intestinal immune parameters of broilers during an experimental coccidiosis infection. There were three glutamine supplementation concentrations in this study: 0, 0.5, and 1.0%. At 14 d of age, each bird was given an oral gavage of 20X dose of Coccivac B52® suspended in distilled water. IFN-γ mRNA levels were downregulated 11.8- and 2.0-fold in the jejunum and 8.1- and 7.9-fold in the cecal tonsils of birds supplemented with 0.5 and 1.0% glutamine at 21 d, respectively, compared to that in the birds fed 0% supplemental glutamine ($P = 0.06$ and $P = 0.01$, respectively). At 21 d of age, IL-10 mRNA levels were down-regulated in the jejunum of the birds supplemented with 0.5 and 1.0% having a 25.3- and a 1.5-fold lower level, respectively ($P < 0.05$). Glutamine supplementation significantly increased Claudin-1, Claudin-2, and Zonula occluden-1 mRNA expression ($P < 0.01$, $P < 0.01$, and $P < 0.01$; respectively). The birds fed glutamine at 0.5 and 1.0% had reduced ($P < 0.01$) crypt depth (84 and 117%; respectively) and increased ($P < 0.01$) villus height to crypt depth ratios (97 and 161%; respectively) compared to the control group. No significant effects ($P > 0.05$) were observed on any performance parameter or T cell population percentages. This study shows that glutamine supplementation up-regulated tight junction protein gene expression, improved intestinal morphology, and down-regulated pro- and anti-inflammatory cytokine gene expression. In conclusion, these results indicate that dietary glutamine supplementation can improve intestinal health in birds challenged with an experimental coccidiosis infection.

DESCRIPTION OF PROBLEM

Recent consumer pressure for antibiotic-free production has led most commercial poultry companies to use antibiotic restrictions in their broiler production. The use of alternative dietary supplements have become increasingly popular in commercial poultry diets [1]. Antibiotics have been used in commercial poultry production to maintain intestinal health and to help reduce the incidence of enteric diseases.

Of the enteric diseases that affect poultry, coccidiosis has one of the most significant impacts on the poultry industry with an estimated cost of over 3 billion dollars annually [2]. This disease is caused by *Eimeria* which are Apicomplexa parasites that infiltrate host intestinal cells. Coccidiosis causes a reduction in performance parameters, severe intestinal damage, and increases host susceptibility to other enteric diseases such as necrotic enteritis [3]. Villus atrophy has been documented in birds with coccidial infections [4]. Villi are the site of nutrient absorption from the lumen; therefore, any damage that occurs can cause a loss in nutrient absorption and utilization in broilers.

The role of T cells during an *Eimeria* infection has been previously described [5]. Coccidiosis increases $CD4^+$ and $CD8^+$ T cell populations in birds at 6 d post-challenge [6]. Cytokines produced by T cells play a major role in the immune responses to *Eimeria* infections. IFN-γ inhibits *Eimeria* development *in vitro* and is up-regulated in birds that are infected with coccidiosis [7]. IL-10, an important anti-inflammatory cytokine, has been shown to be downregulated in birds that are resistant to coccidiosis and up-regulated in birds susceptible to coccidiosis [8].

Glutamine is considered to be a non-essential amino acid. In birds, glutamine can be synthesized in the liver, brain, kidneys, and other important organs to meet normal physiological

requirements [9]. Research in humans and other mammalian species have shown that glutamine is conditionally essential during times of stress and enteric challenge [10]. Glutamine is an important energy source for highly prolific immune cells and intestinal epithelial cells and plays a role in protein degradation, cell defense, cell repair, and is a precursor of glutathione [11]. Supplementation of glutamine improved production performance, increased villus heights in the duodenum and jejunum, and serum IgA and IgG concentrations in 21 d-old broilers [12]. Improved body weights at 21 d of age have been observed in coccidiosis vaccinated birds provided with supplemental glutamine [13]. Glutamine supplementation alleviated the adverse effects of necrotic enteritis on performance parameters and intestinal morphology in birds [14].

This study was conducted to evaluate the effects of glutamine supplementation on performance and intestinal immune parameters of broilers during an experimental coccidiosis infection.

MATERIALS AND METHODS

All animal protocols were approved by the Institutional Animal Care and Use Committee at University of Georgia.

Animals, Housing, and Glutamine Supplementation

A total of 75 male Cobb by-product chicks were randomly allocated to 15 Petersime cages with 5 chicks/cage on day of hatch. The 15 cages were randomly assigned one of three glutamine supplementation concentrations of 0, 0.5, and 1.0% in five replications (n=5). The basal mash diet was a corn and soybean meal basal diet (Table 3.1) that had a calculated basal glutamine content of 2.05%. Supplemental glutamine was added at 0, 0.5 and 1.0% and cellulose

was used to replace supplemental glutamine in basal diets. The birds were fed the dietary treatments from d of hatch to 21 d of age with feed and water being provided *ad libitum*. Feed and BW were recorded at 0, 7, 14, and 21 d for calculation of BWG and FCR. On d 21 of the study, one bird per cage (n=5 per treatment) was randomly selected and sacrificed for the following evaluation.

Eimeria Challenge

At 14 d of age, each chicken was orally gavaged with a 20X dose of Coccivac B52® [32]. This dose was chosen to provide a mild coccidiosis infection [15]. On d 7 post-challenge, one bird per cage was randomly selected and scored for gross lesions using the methodology described by Johnson and Reid [16] with scores for lesions in the duodenum, jejunum, and cecum being from 0-4 (0 being no lesions and 4 being the most severe lesions). A 2 cm sample from the Meckel's diverticulum area was collected and examined under microscope to microscore for *E. maxima* lesions. *E. maxima* lesions were microscored in a scale of 0-4, where 0 being 0% of the villi containing *E. maxima*, 1 being 1-25% of the villi containing *E. maxima*, 2 being 26-50% of the villi containing *E. maxima*, 3 being 51-75% of the villi containing *E. maxima*, and 4 being 76-100% of the villi containing *E. maxima*.

Effect of Glutamine Supplementation on CD4+ and CD8+ Cecal Tonsil Cell Percentages

On d 7 post-challenge cecal tonsils were collected from one bird per cage to be analyzed for total CD4+ and CD8+ cell percentages. Each cecal tonsil was homogenized, and the cells were collected using a 0.45 μm cell strainer and then placed in 1 mL of RPMI. The cells were washed with PBS two times by centrifuging at 750 X g for 3 min. The cells were resuspended in wash

buffer (2mM of EDTA and 1.5% FBS in EDTA). 1 X 10_6 cells were added to a 96 well plate. Anti-chicken $CD4^+$ (1:200 dilution) and $CD8^+$ (1:450 dilution) antibodies were added to each well and incubated for 20 min at 4°C [33]. After incubation, the plate was centrifuged for 5 min at 400 X *g,* and the antibody solution was removed. The cells were then washed and resuspended using the buffer previously mentioned. Total $CD4^+$ and $CD8^+$ cell percentages were analyzed using flow cytometry as described previously [17].

Effect of Glutamine Supplementation on Jejunum and Cecal Tonsil mRNA Expression

On d 7 post-challenge, one bird per battery cage was sacrificed and total RNA from jejunum and cecal tonsils were collected. RNA was then reverse transcribed into cDNA using methods described earlier [18]. The mRNA was then analyzed for pro-inflammatory cytokine IFN-γ and anti-inflammatory cytokine IL-10 by real-time PCR using SyBr green after normalizing for β-actin mRNA. Jejunum mRNA was also analyzed for the tight junction proteins, Claudin-1, Claudin-2, and Zonula occluden-1 mRNA content by real-time PCR. Primer sequences and annealing temperatures are described in Table 3.2. Fold change from the reference gene was calculated using the comparative Ct method $2^{(Ct\ Sample\ -\ Housekeeping)}/2^{(Ct\ Reference\ -\ Housekeeping)}$ with Ct being the threshold cycle [19]. Ct was determined by CFX Maestro software when the fluorescence rises exponentially two-fold above background [34]. The reference group was the birds fed 0% glutamine.

Effect of Glutamine Supplementation on Jejunum Villus Height, Crypt Depth, and Villus Height to Crypt Depth Ratio

A 2 cm section of the jejunum, located at Meckel's Diverticulum, was removed from one bird per replicate and placed in 10% formalin solution. The tissues were then dehydrated in a series of graded alcohol and then infiltrated with paraffin overnight at 60°C. Using a microtome, the paraffin blocks were then cross-sectioned into 5 μm sections and mounted on to frosted microscope slides. The slides were warmed to 37°C and stained with hematoxylin and eosin [20]. Each cross-section was viewed at 100x magnification and photographed using a microscope coupled with a camera [35]. Using ImageJ software, three villi per cross section were measured to determine villi height, crypt depth, and the ratio of villi height to crypt depth.

Statistical Analysis

A one-way ANOVA was used to examine the effect of glutamine supplementation on dependent variables [36]. When main effects were significant ($P < 0.05$), differences between means were analyzed by Tukey's multiple comparison test. *Eimeria* lesion scores were analyzed using the Wilcoxon rank sum test.

RESULTS

Effect of Glutamine Supplementation on Production Parameters

Glutamine supplementation did not affect body weight or feed conversion ratio at any time point of the study ($P > 0.05$; Table 3.3).

Effect of Glutamine on Jejunum and Cecal Tonsil mRNA Expression

Birds fed diets supplemented with glutamine had downregulated IFN-γ compared to that of the control group in both jejunum and cecal tonsil. Jejunum IFN-γ mRNA contents were downregulated 11.8- and 3.8-fold in the birds supplemented with 0.5 and 1.0% glutamine, respectively, compared to that in the birds fed 0% supplemental glutamine ($P < 0.05$; Figure 3.1). Cecal tonsil IFN-γ mRNA amounts had 8.1- and 7.9-fold reduction in birds supplemented with 0.5 and 1.0% supplemental glutamine compared to that in the birds fed 0% supplemental glutamine, respectively ($P = 0.01$; Figure 3.1). IL-10 mRNA levels were down-regulated in the jejunum of the birds supplemented with 0.5 and 1.0% having a 25.3- and a 1.5-fold lower content level, respectively ($P < 0.05$; Figure 3.2). Claudin-1 mRNA content for the jejunum was 5.0- and 35.9-fold higher in the birds fed 0.5 and 1.0% supplemental glutamine compared to that in the birds fed 0% supplemental glutamine, respectively ($P = 0.01$; Figure 3.3). The jejunum mRNA content for Claudin-2 was significantly higher in the birds fed 0.5 and 1.0% supplemental glutamine (3.3- and 28.2-fold, $P < 0.01$; Figure 3.3) compared to that in birds fed 0% supplemental glutamine. Zonula occluden-1 mRNA content from the jejunum was 3.6- and 19.8-fold higher in the jejunum of birds given 0.5 and 1.0% glutamine supplementation respectively ($P < 0.01$; Figure 3.3).

Effect of Glutamine on Jejunum Villus Height, Crypt Depth, and Villus Height to Crypt Depth Ratio

Glutamine supplementation at 1.0% improved the jejunum villus height by 22% percent compared to that in the control group ($P > 0.06$; Table 3.4). Crypt depth was reduced in birds fed 0.5 and 1.0% glutamine-supplemented diets by 84 and 117% compared to that in the control

group, respectively ($P < 0.01$; Table 3.4). The birds fed glutamine at 0.5 and 1.0% had increased villus height to crypt depth ratios by 97 and 161% compared to that in the control group, respectively ($P < 0.01$; Table 3.4).

Effect of Glutamine Supplementation on Intestinal Eimeria Lesion Scores

There were no significant differences of supplementing glutamine on intestinal *Eimeria* lesion scores at 7 d post challenge ($P > 0.05$; Table 3.5).

Effect of Glutamine on CD4+ and CD8+ Cecal Tonsil Cell Percentages

There were no significant differences of supplementing glutamine on cecal tonsil CD4+ and CD8+ cell percentages at 7 d post challenge ($P > 0.05$; data not shown).

DISCUSSION

This experiment studied the effects of dietary glutamine supplementation on production parameters, *Eimeria* lesion scores, jejunum histological morphology, and immune parameters of broilers given an experimental coccidiosis infection. Dietary supplementation of glutamine decreased IFN-γ and IL-10 mRNA expression, increased jejunum tight junction protein mRNA expression (Claudin-1, Claudin-2, and Zonula occluden-1), and improved villus heights, crypt depths, and the ratio of villus height to crypt depths in birds during a coccidiosis infection.

Glutamine supplementation decreased IFN-γ and IL-10 mRNA expression in birds that were challenged with an experimental coccidiosis infection. In previous research, IFN-γ has been shown to be elevated in birds challenged with *Eimeria* up to 6 d post infection in genetically resistant birds [8]. *Eimeria* challenged birds have been shown to have higher levels of IFN-γ

expression in their cecal tonsils, 6 d post-challenge, when compared to nonchallenged birds [21]. Decreased IFN-γ mRNA expression in this study may be a result of glutamine supplementation reducing the infection severity by decreasing the gut damage as observed by increased tight junction protein mRNA content. Previous research has documented that glutamine is important for intestinal epithelial cells, lymphocytes, monocytes, macrophages, and neutrophils because glutamine can be used as the primary source of energy [10]. Higher IL-10 mRNA expression has been observed in the intestines of chickens that are susceptible to *E. maxima* infection than chickens that are resistant to *E. maxima* infections. IL-10 has also been shown to be significantly increased in *Eimeria* challenged birds compared to nonchallenged birds [22]. The lower IL-10 mRNA contents in the jejunum of birds supplemented with 0.5% glutamine may provide evidence of decreased coccidiosis infection severity in glutamine supplemented birds.

Glutamine supplementation increased mRNA expression of the tight junction proteins Claudin-1, Claudin-2, and Zonula occluden-1 in *Eimeria* challenged birds. Dietary glutamine supplementation is important for mucosal integrity [23]. Glutamine regulates intestinal tight junctions by the activation of calcium/calmodulin-dependent kinase 2 (CaMKK2) and AMP-activated protein kinase (AMPK) signaling pathways [24]. During a coccidiosis infection, there is substantial damage that occurs to the intestinal cells as well as increases in intestinal permeability due to losses in intestinal barrier function and integrity [4].

Glutamine supplementation decreased jejunum crypt depths and improved villus height to crypt depth ratios in *Eimeria* challenged birds. Birds that were challenged with *E. acervulina* have reduced villus heights and increases in crypt depths [25]. During a coccidiosis infection, villus atrophy occurs which reduces villus lengths and causes an increase in crypt depth [4]. Since the production of villus epithelial cells occurs in the crypt region a decrease in crypt depths

shows a reduction in intestinal cellular turnover [14, 26]. Birds supplemented with glutamine presented longer villus heights as well as a reduction in crypt depth when vaccinated with a coccidiosis vaccine [27]. Previous research demonstrated that supplemental glutamine was able to alleviate the effects of fasting on villus atrophy in chicks that were fasted for 48 h post-hatch [28].

The effects of glutamine supplementation on performance parameters that were observed in other studies were not observed in this study. The inability to see differences in parameters in this study may be due to different rearing systems as well as small sample sizes than those previously mentioned.

In conclusion, dietary glutamine supplementation reduced both pro-inflammatory and anti-inflammatory cytokine mRNA expression and improved intestinal health by increasing tight junction protein mRNA expression and increasing crypt depth and villus height to crypt depth ratios. Therefore, it might be beneficial to supplement glutamine up to 1% during coccidiosis infections in commercial broilers.

CONCLUSIONS AND APPLICATIONS

1. Supplemental glutamine reduced both pro- and anti-inflammatory cytokine mRNA expression
2. Glutamine improved intestinal health through increasing tight junction protein mRNA expression and increasing crypt depth and villus height to crypt depth ratio.
3. Glutamine supplementation may be beneficial during coccidiosis.

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32. Coccivac B52® contains: *E. acervulina*, 2 strains of *E. maxima*, *E. mivati*, and *E. tenella*; Merck Animal Health, Madison, NJ

33. Southern Biotech, Birmingham, AL

34. Biorad, Hercules, CA

35. Leica DC500 camera, Leica Microsystems Inc, Buffalo Grove, IL

36. SAS Studio, SAS Institute Inc., Cary, NC

Table 3.1: Diet blend (%) and calculated nutrient composition of the basal diet.

Diet Blend	%
Corn	58.47
Soybean Meal, 48%	35.15
Soybean Oil	2.27
Monocalcium phosphorus, 21%	1.38
Limestone	1.59
DL-Methionine	0.21
L-Lysine-HCL, 78%	0.14
Salt (NaCl)	0.35
Vitamin premix[1]	0.08
Mineral premix[2]	0.35
Total:	100.0
Calculated Nutrient Composition	
ME, kcal/g	3.05
CP, %	21.44
Calcium, %	0.95
Total Phosphorus, %	0.71
Avail. Phosphorus< %	0.45
Sodium, %	0.16
Chloride, %	0.27
Lysine, %	1.31
Methionine, %	0.56
TSAA, %	0.91
Threonine, %	0.87
Tryptophan, %	0.29
Arginine, %	1.50
Glutamine, %	2.05

[1] Vitamin mix provided the following (per kilogram of diet): thiamin-mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B12 (cobalamin), 12.0g; pyridoxine-HCl, 2.7 mg; D-biotin, 0.11 mg; folic acid, 0.55 mg; menadione sodium bisulfate complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 1,100 IU; trans-reinyl acetate, 2,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.

[2] Trace mineral mix provides the following (per kilogram of diet): manganese (MnSO4.H2O), 101 mg; iron (FeSO4.7H2O), 20 mg; zinc (Zn)), 80 mg; copper (CuSO4.5H2O), 3 mg; iodine (ethylene diamine dihydroiodide), 0.75 mg; magnesium (MgO), 20 mg; selenium (sodium selenite), 0.3 mg.

Table 3.2: Primers and PCR conditions for RTqPCR

Target Gene	Sequence (5'-3')	Annealing Temperature	Reference
IFN-γ-F	TGAGCCAGATTGTTTCGA	54.0°C	[29]
IFN-γ-R	ACGCCATCAGGAAGGTTG		
IL-10-F	CAATCCAGGGACGATGAACT	57.0°C	[17]
IL-10-R	GGCAGGACCTCATCTGTGTAG		
CLAU-1-F	CATACTCCTGGGTCTGGTTGGT	57.5°C	[30]
CLAU-1-R	GACAGCCATCCGCATCTTCT		
CLAU-2-F	CCTGCTCACCCTCATTGGAG	57.5°C	[31]
CLAU-2-R	GCTGAACTCACTCTTGGGCT		
ZO-1-F	TGTAGCCACAGCAAGAGGTG	57.4°C	[28]
ZO-1-R	CTGGAATGGCTCCTTGTGGT		
β-actin-F	ACCGGACTGTTACCAACACC	57.0°C	[17]
β-actin-R	GACTGCTGCTGACACCTTCA		

Table 3.3: Effect of glutamine supplementation on body weight (BW) and feed conversion ratio (FCR) in birds challenged with *Eimeria*.

	Control	0.5%	1.0%	SEM	*P*-value
7 d BW (g)	148.8	146.2	154.3	5.7	0.67
14 d BW (g)	382.6	382.2	402.7	9.2	0.36
21 d BW (g)	681.9	684.1	672.3	17.4	0.93
7 d FCR (g:g)	1.139	1.200	1.145	0.016	0.22
14 d FCR (g:g)	1.346	1.401	1.344	0.019	0.46
21 d FCR (g:g)	1.564	1.583	1.611	0.048	0.84

Birds were fed diets supplemented with glutamine at 0.0, 0.5, and 1.0%. BW was measured and FCR was calculated at 7, 14, and 21 d of age. At 14 d of age, the birds were orally gavaged with a 20x dose of Coccivac B52®. n=5.

Table 3.4: Effect of glutamine supplementation on jejunal villus height, crypt depth, and villus height to crypt depth ratio in birds challenged with *Eimeria*.

	Control	0.5%	1.0%	SEM	*P*-value
Villus Height (μm)	806.15	796.06	982.98	0.29	0.06
Crypt Depth (μm)	248.23$_{a}$	134.64$_{b}$	114.28$_{b}$	0.94	< 0.01
V:C Ratio (μm:μm)	3.44$_{a}$	6.76$_{b}$	8.99$_{c}$	0.54	< 0.01

$_{a\text{-}c}$Means within a row without a common subscript differ significantly ($P < 0.05$).

Birds were fed diets supplemented with glutamine at 0.0, 0.5, and 1.0%. At 14 d of age, the birds were orally gavaged with a 20x dose of Coccivac B52®. On d 21 (7 d post-challenge), jejunal villus height (V), crypt depth (C), and villus height to crypt depth (V:C) ratio was measured. n=5.

Table 3.5: Effect of glutamine supplementation on intestinal *E. acervulina* and *E. maxima* lesion scores.

Eimeria spp.	**Treatment**	**0**	**1**	**2**	**3**	**4**	**Rank Score Means**	**n**	**Chi Sq. *P*-value**
acervulina **Gross Lesion Score**	**Control**	0	2	3	0	0	11.1	5	0.11
	0.5%	1	4	0	0	0	6.4	5	
	1.0%	2	2	1	0	0	6.5	5	
maxima **Microscopic Lesion Score**	**Control**	0	1	1	3	0	9	5	0.77
	0.5%	0	2	1	2	0	7.1	5	
	1.0%	0	1	2	2	0	1.9	5	

At 14 d of age, each chicken was orally gavaged with a 20X dose of Coccivac B52®. 7 d post-challenge one bird per cage was randomly selected and scored for gross lesions in a scale of 0-4 in the duodenum and cecum with 0 being no lesions and 4 being the most severe lesions. 2 two cm samples were taken from the Meckel's diverticulum area to be used for histological examination of *E. maxima* lesions. Histological *E. maxima* lesions were microscored in a scale of 0-4, where 0 being 0% of the villi containing *E. maxima*, 1 being 1-25% of the villi containing *E. maxima*, 2 being 26-50% of the villi containing *E. maxima*, 3 being 51-75% of the villi containing *E. maxima*, and 4 being 76-100% of the villi containing *E. maxima*.

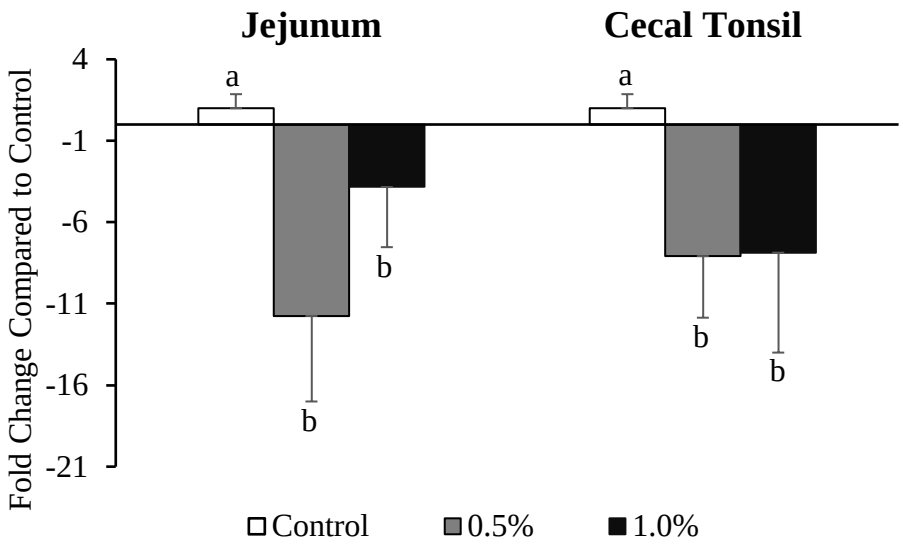

Figure 3.1: *Effect of glutamine supplementation on jejunum and cecal tonsil IFN-γ mRNA expression at 7 d post-challenge*. Birds were fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 14 d of age, the birds were orally gavaged with a 20X dose of Coccivac B52®. At 7 d post challenge, relative IFN-γ mRNA content was analyzed after correcting for β-actin mRNA and normalizing to the mRNA content of the 0% glutamine group. Bars (± SEM) within intestinal location that do not contain a common letter differ significantly ($P < 0.05$). *P*-values: jejunum, $P < 0.05$; cecal tonsil, $P < 0.01$. n=5.

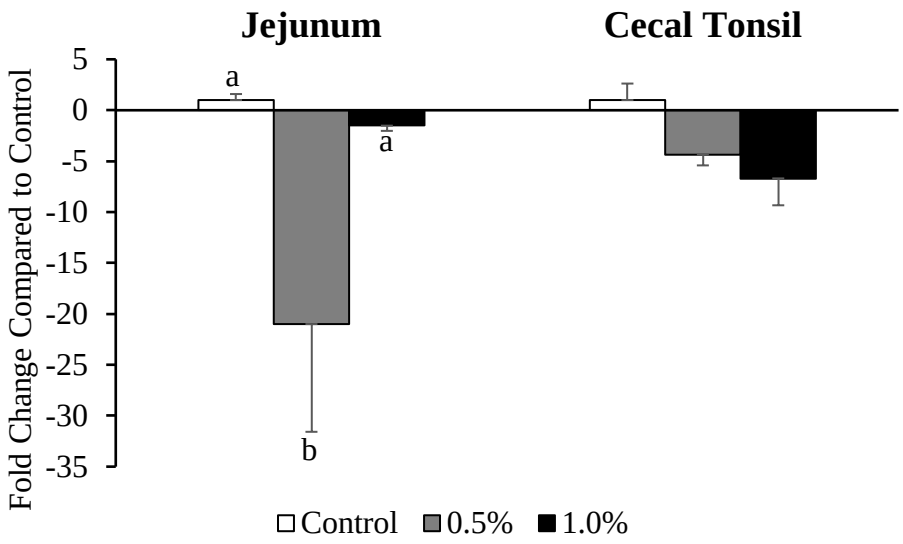

Figure 3.2: *Effect of glutamine supplementation on jejunum and cecal tonsil IL-10 mRNA expression at 7 d post-challenge*. Birds were fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 14 d of age, the birds were orally gavaged with a 20X dose of Coccivac B52®. At 7 d post challenge, relative IL-10 mRNA content was analyzed after correcting for β-actin mRNA and normalizing to the mRNA content of the 0% glutamine group. Bars (± SEM) within intestinal location that do not contain a common letter differ significantly ($P < 0.05$). *P*-values: jejunum, $P < 0.05$; cecal tonsil, $P > 0.05$. n=5.

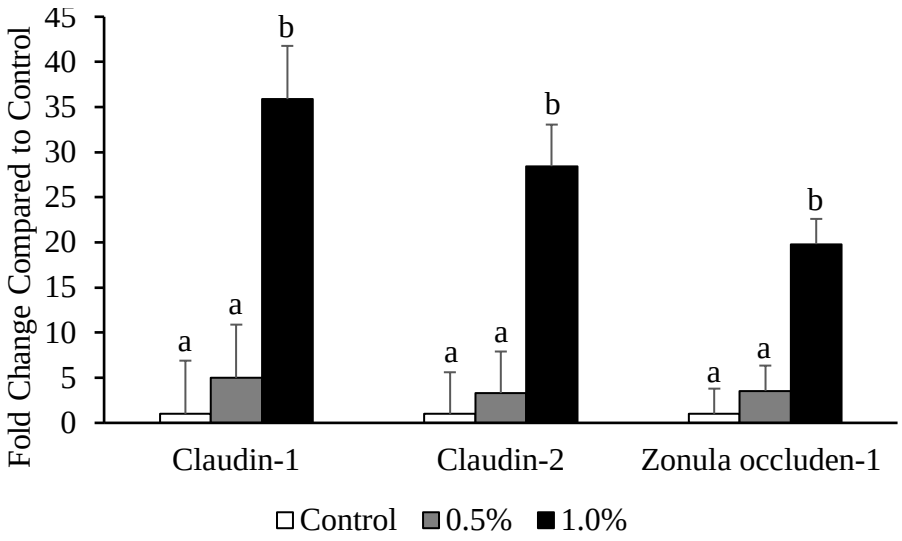

Figure 3.3: *Effect of glutamine supplementation on jejunum Claudin-1, Claudin-2, and Zonula occluden-1 mRNA expression at 7 d post-challenge*. Birds were fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 14 d of age, the birds were orally gavaged with a 20X dose of Coccivac B52®. At 7 d post challenge, relative Claudin-1, Claudin-2, and Zonula occluden-1 mRNA content were analyzed after correcting for β-actin mRNA and normalizing to the mRNA content of the 0% glutamine group. Bars (± SEM) within intestinal location that do not contain a common letter differ significantly ($P < 0.05$). *P*-values: Claudin-1, $P = 0.01$; Claudin-2, $P < 0.01$; Zonula occluden-1, $P < 0.01$. n=5.

CHAPTER 4

EFFECTS OF GLUTAMINE SUPPLEMENTATION ON BROILER PERFORMANCE AND INTESTINAL IMMUNE PARAMETERS DURING AN EXPERIMENTAL NECROTIC ENTERITIS INFECTION

[1]J.H. Oxford, B. Lumpkins, G.F. Mathis, T.T. Ng, and R.K. Selvaraj. Submitted to the *Journal of Applied Poultry Research*.

ABSTRACT

The objective of this study was to evaluate the effects of glutamine (Gln) supplementation on performance and intestinal immune parameters of broilers during an experimental necrotic enteritis (NE) infection. Broilers were randomly distributed to either an unchallenged control (UC, 0% of supplemented Gln), challenged control (CC, 0% of supplemented Gln), or Gln supplemented (0.5% and 1.0%) challenged groups (n=6). On d 14 of the study, each bird in the challenge groups was given an oral gavage of 5,000 *E. maxima* sporulated oocysts. On d 19-21, the challenged birds were given 10_8 CFU of *C. perfringens* via oral gavage (CP; alpha-toxin and NetB positive). At 21 d-of age, gln supplemented at 0.5% increased IL-10 expression compared to the CC by 6.6-fold ($P < 0.05$). Birds fed gln at 1.0% had higher cecal concentrations of CP compared to the UC ($P = 0.02$). An increase in NetB positive CP was observed in the challenge groups compared to the UC group ($P < 0.01$). Intestinal permeability was increased 616% in the CC compared to UC as measured by FITC-dextran ($P < 0.05$). The CC group had 267% higher intestinal permeability compared to the 0.5% Gln group ($P < 0.05$). The 0.5% Gln group had an increase in serum Gln concentration of 135% compared to the CC, and the 1.0% Gln group had an increase of serum Gln of 157% compared to the CC ($P < 0.05$). The results show that during NE, the supplementation of dietary Gln at 0.5% may help reduce the negative effects of NE.

DESCRIPTION OF PROBLEM

Throughout the years, the enteric disease necrotic enteritis, has been controlled using antimicrobial growth promoters (AGPs), however, many poultry producers have moved away from the use of AGPs due to government regulations and consumer preferences (Gadde, et al., 2018). Necrotic enteritis has now become the third most problematic disease in poultry production and has an annual economic cost of around 6 billion dollars (Van Immerseel, et al., 2009; Cervantes, 2017). This disease is multifaceted and occurs when there is a disturbance in the intestinal integrity of birds, mainly due to *Eimeria maxima*. Disturbances in the intestines can lead to excess nutrients for the commensal bacteria, *Clostridium perfringens*, to proliferate and produce the necrosis inducing toxins, alpha-toxin and NetB toxin (Fasina and Lillehoj, 2018).

During necrotic enteritis, severe damage occurs to the intestinal epithelium, which can lead to increased intestinal permeability and in severe cases cause septicemia (Bortoluzzi, et al., 2019). Enterotoxins that are produced by *C. perfringens* target tight junction proteins and cause disturbances in their ability to maintain proper intestinal permeability leading to additional plasma leakage and loss of ion regulation (Awad, et al., 2017). *C. perfringens* has been shown to reduce the expression of pro-inflammatory cytokines like IFN-γ and increase the expression of anti-inflammatory cytokines like IL-10 (Hong, et al., 2012).

In the body, glutamine is one of the most abundant amino acids and is mainly located in the blood and skeletal tissue of humans and animals (Andrews and Griffiths, 2002). Traditionally this amino acid was categorized as non-essential but has recently been shown to be essential in times of enteric challenge (Curi, et al., 2007). Glutamine is used at high rates in prolific cells, such as intestinal epithelial cells and immune cells. Dietary glutamine supplementation has been shown to improve intestinal histomorphology and increased the expression of tight junction

proteins in birds during enteric challenges (Yu, et al., 1999; Xue, et al., 2018; Oxford and Selvaraj, 2019). Supplementation of dietary glutamine increased BW of birds during necrotic enteritis and reduced intestinal necrotic enteritis lesions and has also been shown to reduce the negative effects of coccidiosis vaccines on BW (Mussini, et al., 2012; Luquetti, et al., 2016; Xue, et al., 2018). Furthermore, glutamine supplementation helps reduce intestinal permeability and bacteria translocation during enteric challenges in rodent models (White, et al., 2005). In a leaky gut model conducted in chickens, glutamine supplementation did not improve intestinal permeability, however, the experimental diets that were used in this study were wheat based diets which already contain high concentrations of glutamine (Barekatain, et al., 2019).

The objective of this study was to evaluate the effects of glutamine supplementation on broiler performance and intestinal immune parameters during an experimental necrotic enteritis infection.

MATERIALS AND METHODS

All animal protocols were approved by the Institutional Animal Care and Use Committee at University of Georgia.

Animals, Housing, and Glutamine Supplementation

A total of 144 male Cobb by-product chicks were randomly allocated to 24 Petersime battery cages (67.5 x 67.5 cm) with metal mesh floors supplied with drinkers and feeders (6 chicks/cage on d of hatch). The 24 cages were randomly assigned to either an unchallenged control (UC, 0% of supplemented glutamine), challenged control (CC, 0% of supplemented glutamine), or Gln supplemented challenge groups at either 0.5 or 1.0% (n=6). The basal mash

diet was corn and soybean-meal based diets (Table 4.1) that had a calculated basal glutamine content of 2.05%. Supplemental glutamine was added at 0, 0.5, and 1.0%; cellulose was used in place of glutamine in the basal diets. Feed and water were provided *ad libitum*. Feed intake and BW were recorded at 0, 21, and 28 d for calculation of BWG and FCR. Mortality was recorded daily for the entirety of the study. On d 21, and 28 of the study, birds were sampled for the following parameters.

Necrotic Enteritis Challenge

At 14 d of age, the challenge control (CC), 0.5%, and 1.0% glutamine supplemented birds were orally gavaged with 5,000 sporulated oocysts of *E. maxima*. On d 19, 20, and 21, the birds in the challenge groups were given 10_8 CFU of *C. perfringens* that is alpha-toxin and NetB positive (Miller, et al., 2010). On d 21, three birds per cage were randomly selected and scored for necrotic enteritis lesions on a scale from 0-3. A score of 0 being normal intestines containing no lesions, 1 being increased mucin covering the small intestines, 2 being necrosis in the mucosa of the small intestines, and 3 being when the intestinal mucosa was sloughed and blood was found in the mucosa and luminal contents (Miller, et al., 2010).

Effect of Glutamine Supplementation on Jejunum and Cecal Tonsil mRNA Expression

On d 21 and 28, one bird per cage was sacrificed and total RNA from the jejunum and cecal tonsils were collected. RNA was then reverse transcribed into cDNA using methods described earlier (Selvaraj and Klasing, 2006). The mRNA was then analyzed for pro-inflammatory cytokine IFN-γ and anti-inflammatory cytokine IL-10 by real-time PCR using SyBr green after normalizing for β-actin mRNA. Jejunum mRNA was also analyzed for tight

junction proteins Claudin-1, Claudin-2, and Zonula occluden-1 mRNA content by real-time PCR. Primer sequences and annealing temperatures are described in Table 4.2. Fold change from the reference gene was calculated using the comparative Ct method $2^{(Ct\ Sample - Housekeeping)}/2^{(Ct\ Reference - Housekeeping)}$ with Ct being the threshold cycle (Schmittgen and Livak, 2008). Ct was determined by CFX Maestro software when the fluorescence rises exponentially two-fold above background (Biorad; Hercules, California). The reference group was the unchallenged control.

Effect of Glutamine Supplementation on Cecal Contents DNA expression

On d 21, cecal contents were collected from one bird per cage for *C. perfringens* and NetB toxin analysis. Bacteria DNA was isolated from the samples using methods previously described (Shanmugasundaram, et al., 2013). The sequences and annealing temperatures used for this analysis are described in Table 4.2. The cycle threshold (Ct) value was determined using CFX Maestro software when the fluorescence rises exponentially two-fold above background (Biorad; Hercules, California). The data is represented as mean Ct values with the lower the Ct value being related to a higher level of DNA expression and a higher Ct value meaning a lower level of DNA expression.

Effect of Glutamine Supplementation on Intestinal Permeability

On d 21 of the study, one bird per cage was orally inoculated with 2.2 mg of fluorescein isothiocyanate-dextran (FITC-dextran; 100mg, MW 4,000; Sigma-Aldrich, Canada), two hours later the birds were euthanized, and blood was collected from the heart. FITC-dextran concentration in the blood serum was measured using black plates in a microplate reader at a wavelength of 485 nm and an emission wavelength of 528 nm. FITC-dextran concentration per

mL of serum was calculated based on a standard curve with the higher level of FITC-dextran concentration meaning higher intestinal permeability.

Effect of Glutamine Supplementation on Serum Glutamine Concentration

On d 21, one bird per cage was euthanized and blood was collected from the heart. After coagulation, serum was collected and used for determination of glutamine concentration using the EnzyChrom™ Glutamine Assay Kit from BioAssay Systems, Hayward, CA.

Statistical Analysis

A two-way ANOVA was used to examine the effect of glutamine supplementation and challenge on dependent variables (SAS Studio, SAS Institute Inc., Cary, NC). When main effects were significant ($P < 0.05$), differences between means were analyzed by Tukey's multiple comparison test. Necrotic enteritis lesion scores were analyzed via the Kruskal-Wallis Test.

RESULTS

Effect of Glutamine Supplementation on Production Parameters

There were no significant effects on body weight or feed conversion ratio at any time point of the study ($P > 0.05$; Table 4.3). Challenge significantly increased mortality at 21 and 28 d of age with the UC group having 0% mortality at both timepoints, the CC group having 29.2% mortality for both timepoints, the 0.5% glutamine supplementation group having 25.0% mortality at 21 d and 27.1% mortality at 28 d, and the 1.0% glutamine supplementation group having 37.5% mortality for both timepoints ($P < 0.01$, Table 4.3).

Effect of Glutamine Supplementation on Intestinal Necrotic Enteritis Lesion Scores

There were significant differences in the distribution of necrotic enteritis lesion scores when comparing the treatment groups ($P < 0.01$; Table 4.4). The mean rank scores for intestinal necrotic enteritis lesions was UC having a score of 19.5, CC having a score of 40.2, 0.5% glutamine supplemented group having a score of 43.1, and the 1.0% glutamine supplemented group having a rank score of 43.2. For this analysis the higher rank score means there were more severe lesions observed.

Effect of Glutamine Supplementation on Jejunum and Cecal Tonsil mRNA Expression

The birds that were given supplemental glutamine at 0.5% had higher IL-10 expression in their jejuna with an 8.3-fold increase in IL-10 expression compared to the UC groups at 21 d ($P = 0.04$; Figure 4.1). The birds from the 0.5% glutamine supplemented group also had significantly higher Claudin-1 expression in their jejuna with a 4.5-fold higher mRNA expression compared to the UC at 21 d ($P = 0.01$; Figure 4.2). There were no significant differences between treatment groups for IFN-γ in either jejunum or cecal tonsils, IL-10 in the cecal tonsils, or Claudin-1 and Zonula occluden-1 in the jejunum at 21 d ($P > 0.05$). No significant differences between any treatment were observed at 28 d for any genes of interest ($P > 0.05$).

Effect of Glutamine Supplementation on Cecal Contents DNA expression

Cecal contents *C. perfringens* DNA expression was significantly higher in the 1.0% glutamine supplemented birds compared to that of the UC birds ($P = 0.02$; Figure 4.3). There was a difference of 5.4 Ct values between the UC birds and the 1.0% glutamine supplemented

birds with the 1.0% glutamine supplemented group having a higher load of *C. perfringens*. There was a significant shift in the NetB positive *C. perfringens* population when comparing the challenged groups to the UC, with the CC, 0.5% glutamine supplemented group, and 1.0% glutamine supplemented group having a 10.8, 9.2, and 11.6, respectively, differences in mean Ct values compared to the UC ($P < 0.01$, Figure 4.3).

Effect of Glutamine Supplementation on Intestinal Permeability

The necrotic enteritis challenge increased intestinal permeability as measured by FITC-dextran in all groups compared to that of the UC with the CC having a 616% increase in permeability, the 0.5% glutamine group having a 231% increase, and the 1.0% glutamine group having a 507% increase in permeability compared to the UC (Figure 4.4). The CC group had significantly higher intestinal permeability compared to that of the UC (235.0 vs. 38.1 ng/mL, respectively; $P < 0.05$). The CC and the 0.5% glutamine supplemented groups did not differ significantly (235.0 vs. 193.4 ng/mL, respectively; $P > 0.05$); however, the CC had significantly higher ($P < 0.05$) intestinal permeability compared to the 0.5% glutamine supplemented group with an increase of 267% in intestinal permeability.

Effect of Glutamine Supplementation on Serum Glutamine Concentration

Necrotic enteritis challenge significantly reduced serum glutamine concentrations with the UC having 2.33 μmol/mL of glutamine in the serum and the CC having 1.33 μmol/mL of glutamine in the serum (decrease of 43%; $P < 0.01$, Figure 4.5). When comparing the challenged groups, the 0.5% supplemented glutamine group had an increase in serum glutamine

concentration of 135% compared to the CC, and the 1.0% glutamine supplemented group had an increase of serum glutamine of 157% compared to the CC.

DISCUSSION

This experiment aimed to evaluate the effects of glutamine supplementation on production parameters, necrotic enteritis lesion scores, cytokine gene expression, cecal contents bacteria expression, intestinal permeability, and serum glutamine concentration during an experimental necrotic enteritis infection. Dietary supplementation of 0.5% glutamine increased jejunum IL-10 and Claudin-1 expression, decreased intestinal permeability, and increased serum glutamine concentration at 21 d. Supplementation of glutamine at 1.0% significantly increased mortality at 21 of age, had significantly higher *C. perfringens* expression compared to the UC, and increased serum glutamine concentrations.

Glutamine supplementation increased jejunum IL-10 expression in birds that were challenged with necrotic enteritis compared to both the UC and CC. Increased IL-10 expression in challenged birds has been documented in previous research and is believed to be a response to tissue damage (Oh and Lillehoj, 2016; Fasina and Lillehoj, 2018). The effects of glutamine supplementation and necrotic enteritis challenge are seen in the jejunum but were not seen in the cecal tonsils; this observation may be due to the site of infection being in the jejunum and not the ceca. In vitro work using macrophages showed that increased glutamine supplementation increased IL-10 mRNA expression and can modulate the inflammatory immune response (de Oliveira, et al., 2017). The response seen in the 0.5% glutamine supplemented birds may show evidence that glutamine supplementation at 0.5% can help reduce tissue damage in challenged birds.

Increased jejunum mRNA expression of Claudin-1 was seen in both the CC and the 0.5% glutamine supplemented groups compared to the UC, however, that 1.0% glutamine supplemented group had no differences in Claudin-1 expression compared to the UC. Previous work has shown that IL-10 expression is correlated with Claudin-1 expression and that when IL-10 was knocked out in mice, the localization of this tight junction protein was interfered (Mazzon, et al., 2002). Increased Claudin-1 expression in the 0.5% glutamine supplemented birds may be a response to increased IL-10 expression observed in these birds. In vitro and in vivo work has shown that glutamine supplementation can increase the expression of Claudin-1 in many different animal models (Kim and Kim, 2017; Oxford and Selvaraj, 2019). Glutamine plays a role in the regulation of tight junctions through the AMP-activated kinase signaling pathway; therefore supplemental glutamine may have an effect on tight junctions during necrotic enteritis (Wang, et al., 2016).

Cecal *C. perfringens* loads were higher in all of the challenged groups compared to the UC; the challenged groups also had a significant shift in NetB positive *C. perfringens* cecal populations. These results show that the experimental model was successful in increasing the amount of NetB positive *C. perfringens* population in the birds, which can also be seen via the observation of necrotic lesions. The 1.0% glutamine supplemented group had the highest *C. perfringens* load of all of the treatment groups. Observed higher concentrations of *C. perfringens* in the 1.0% group may have been observed due to excess luminal glutamine concentrations providing additional substrate for the *C. perfringens* to proliferate. It is known that dietary nutrient levels, when fed above the requirement of the bird, can increase the severity of disease (Umar, et al., 2016). The increases in cecal *C. perfringens* in the 1.0% glutamine supplemented

birds may be an indication that dietary supplementation of glutamine at 1.0% does not provide benefit to the bird during necrotic enteritis.

Intestinal permeability was increased in all of the challenged groups compared to the UC. *C. perfringens* has been shown to impair tight junction function and cause increased intestinal permeability in birds infected with necrotic enteritis (Awad, et al., 2017). Glutamine supplementation at 0.5% reduced intestinal permeability in necrotic enteritis challenged birds compared to the CC. As mentioned above, glutamine has been shown to increase Claudin-1 expression. Claudin-1 is one of the main pore-sealing tight junction proteins, which may explain why reduced permeability was observed in the 0.5% glutamine supplemented group in this study (Awad, et al., 2017). Increased permeability in the 1.0% glutamine supplemented birds may be attributed to increased cecal *C. perfringens* loads. Previous research has shown that glutamine supplementation improves intestinal histomorphology during coccidiosis or necrotic enteritis, which may provide indication of why reduced permeability was seen in the 0.5% glutamine supplemented birds (Xue, et al., 2018; Oxford and Selvaraj, 2019).

Serum glutamine concentrations were reduced during the necrotic enteritis challenge. Previous work evaluating *E. acervulina* challenge on blood amino acid levels showed that glutamine was the most affected amino acid during the challenge (Rochell, et al., 2016). Significant losses in plasma glutamine concentrations have also been reported in humans and other animals during enteric challenges (Kim and Kim, 2017). Glutamine supplementation helped increase serum glutamine concentration in the challenged birds, with the 1.0% glutamine birds having no significant differences in serum concentrations. These results may indicate that challenge increases the glutamine requirement of birds, and that glutamine may be a conditionally essential amino acid during times of stress or challenge.

No effects of glutamine supplementation on BW or FCR were observed in this study. Experimental conditions, sample sizes, and the number of replicates may be the reason that this study was unable to see differences in BW and FCR that were observed in other studies.

This study shows that glutamine supplementation at 0.5% may be beneficial during necrotic enteritis and that it may reduce intestinal permeability through increased IL-10 and Claudin-1 expression and can help alleviate reduced serum glutamine concentrations. Glutamine supplementation at 1.0% had negative effects on mortality and intestinal permeability at 21 d of age, which may be attributed to increased *C. perfringens* loads; therefore, supplementation of glutamine at 1.0% is not recommended during necrotic enteritis. Other necrotic enteritis challenge models have shown that glutamine supplemented at 1.0% is beneficial; the observed differences in this study may be attributed to different challenge models used (Xue, et al., 2018).

CONCLUSIONS AND APPLICATIONS

1. Dietary supplementation of glutamine at 0.5% reduced intestinal permeability through increased IL-10 and Claudin-1 expression and improved serum glutamine concentrations.
2. Supplementation of glutamine at 1.0% provided negative effects to the bird by increasing mortality and intestinal permeability, which may be attributed to increased *C. perfringens* loads.
3. Supplementation of glutamine at 0.5% may be beneficial during necrotic enteritis challenge.

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Table 4.1: Diet blend (%) and calculated nutrient composition of the basal diet.

Diet Blend	%
Corn	58.47
Soybean Meal, 48%	35.15
Soybean Oil	2.27
Monocalcium phosphorus, 21%	1.38
Limestone	1.59
DL-Methionine	0.21
L-Lysine-HCL, 78%	0.14
Salt (NaCl)	0.35
Vitamin premix[1]	0.08
Mineral premix[2]	0.35
Total:	100.0
Calculated Nutrient Composition	
ME, kcal/g	3.05
CP, %	21.44
Calcium, %	0.95
Total Phosphorus, %	0.71
Avail. Phosphorus< %	0.45
Sodium, %	0.16
Chloride, %	0.27
Lysine, %	1.31
Methionine, %	0.56
TSAA, %	0.91
Threonine, %	0.87
Tryptophan, %	0.29
Arginine, %	1.50
Glutamine, %	2.05

[1] Vitamin mix provided the following (per kilogram of diet): thiamin-mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B12 (cobalamin), 12.0g; pyridoxine-HCl, 2.7 mg; D-biotin, 0.11 mg; folic acid, 0.55 mg; menadione sodium bisulfate complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 1,100 IU; trans-reinyl acetate, 2,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.

[2] Trace mineral mix provides the following (per kilogram of diet): manganese (MnSO4.H2O), 101 mg; iron (FeSO4.7H2O), 20 mg; zinc (Zn)), 80 mg; copper (CuSO4.5H2O), 3 mg; iodine (ethylene diamine dihydroiodide), 0.75 mg; magnesium (MgO), 20 mg; selenium (sodium selenite), 0.3 mg.

Table 4.2: Primers and PCR conditions for RTqPCR

Target Gene	Sequence (5'-3')	Annealing Temperature	Reference
IFN-γ-F	TGAGCCAGATTGTTTCGA	54.0°C	(Shanmugasundaram and Selvaraj, 2012)
IFN-γ-R	ACGCCATCAGGAAGGTTG		
IL-10-F	CAATCCAGGGACGATGAACT	57.0°C	(Shanmugasundaram and Selvaraj, 2012)
IL-10-R	GGCAGGACCTCATCTGTGTAG		
CLAU-1-F	CATACTCCTGGGTCTGGTTGGT	57.5°C	(Du, et al., 2016)
CLAU-1-R	GACAGCCATCCGCATCTTCT		
CLAU-2-F	CCTGCTCACCCTCATTGGAG	57.5°C	(Chen, et al., 2016)
CLAU-2-R	GCTGAACTCACTCTTGGGCT		
ZO-1-F	TGTAGCCACAGCAAGAGGTG	57.4°C	(Yi, et al., 2001)
ZO-1-R	CTGGAATGGCTCCTTGTGGT		
CP-F	CGCATAACGTTGAAAGATGG	55.0°C	(Bortoluzzi, et al., 2019)
CP-R	CCTTGGTAGGCCGTTACCC		
NetB-F	CGCTTCACATAAAGGTTGGAAGGC	60.0°C	(Bailey, et al., 2014)
NetB-R	TCCAGCACCAGCAGTTTTTCCT		
β-actin-F	ACCGGACTGTTACCAACACC	57.0°C	(Shanmugasundaram and Selvaraj, 2012)
β-actin-R	GACTGCTGCTGACACCTTCA		

Table 4.3: Effect of glutamine supplementation on body weight (BW), feed conversion ratio (FCR), and mortality in birds challenged with necrotic enteritis.

	Body Weight (g)		FCR (g:g)		Mortality (%)	
	21 d	28 d	21 d	28 d	21 d	28 d
UC	445.7	834.3	1.760	1.664	0.0^{C}	0.0^{B}
CC	383.2	728.9	1.572	1.998	29.2^{AB}	29.2^{A}
0.5%	391.6	659.8	1.565	1.992	25.0^{B}	27.1^{A}
1.0%	412.4	790.7	1.412	2.013	37.5^{A}	37.5^{A}
SEM	13.9	38.0	0.071	0.084	8.1	8.1
P-Value	0.084	0.274	0.092	0.062	<0.001	<0.001

Birds were fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 14 d of age, the challenge control (CC), 0.5% and 1.0% glutamine supplemented birds were orally gavaged 5,000 oocysts of *E. maxima*. On d 19, 20, and 21 the birds were given 10_8 CFU/mL of *C. perfringens* that is alpha toxin and NetB positive. UC: unchallenged control, CC: challenged control. n=6.

Table 4.4: Effect of glutamine supplementation on intestinal necrotic enteritis lesion scores.

	Treatment	0	1	2	3	Rank Score Means	n	Chi Sq. *P*-value
Necrotic Enteritis Lesion Score	**UC**	18	0	0	0	19.5	18	< 0.001
	CC	7	9	2	0	40.2	18	
	0.5%	6	9	2	1	43.1	18	
	1.0%	7	6	4	1	43.2	18	

Birds were fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 14 d of age, the challenge control (CC), 0.5% and 1.0% glutamine supplemented birds were orally gavaged 5,000 oocysts of *E. maxima*. On d 19, 20, and 21 the birds were given 10_8 CFU/mL of *C. perfringens* that is alpha toxin and NetB positive. On d 21, three birds per cage were randomly selected and scored for necrotic enteritis lesions on a scale from 0-3. A score of 0 being normal intestines containing no lesions, 1 being increased mucin covering the small intestines, 2 being necrosis in the mucosa of the small intestines, and 3 being when the intestinal mucosa was sloughed off and blood was found in the mucosa and luminal contents (Miller, et al., 2010). UC: unchallenged control, CC: challenged control. n=18.

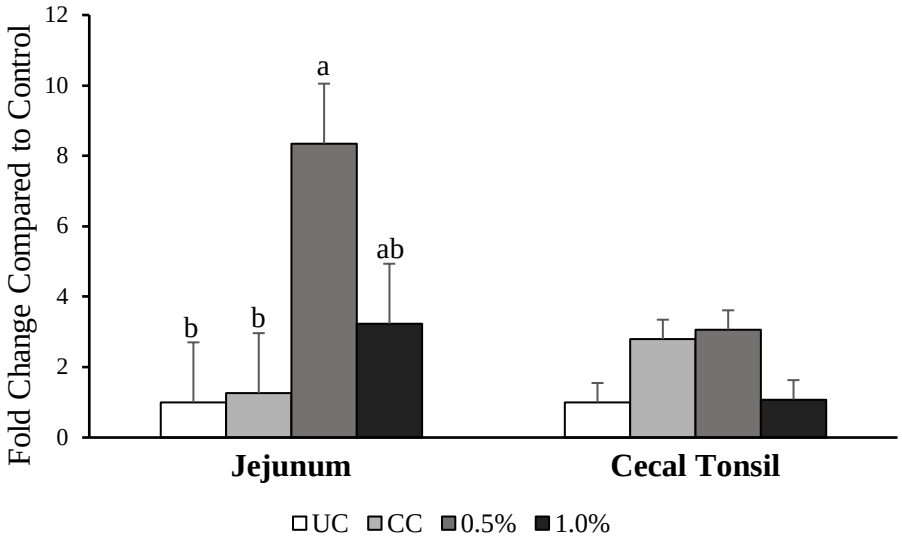

Figure 4.1: *Effect of glutamine supplementation on jejunum and cecal tonsil IL-10 mRNA expression during necrotic enteritis challenge*. Birds were fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 14 d of age, the challenge control (CC), 0.5% and 1.0% glutamine supplemented birds were orally gavaged 5,000 oocysts of *E. maxima*. On d 19, 20, and 21 the birds were given 10_8 CFU/mL of *C. perfringens* that is alpha toxin and NetB positive. On d 21, relative IL-10 mRNA expression was analyzed after correcting for β-actin mRNA and normalizing to the mRNA expression of the unchallenged control group. Bars (± SEM) within intestinal location that do not contain a common letter differ significantly ($P < 0.05$). *P*-values: jejunum, $P = 0.04$; cecal tonsil, $P = 0.09$. UC: unchallenged control, CC: challenged control. n=6.

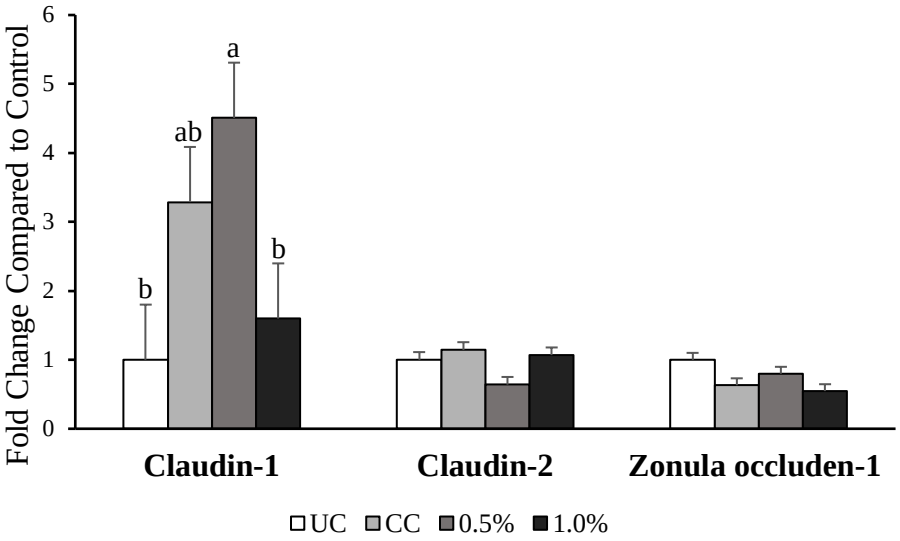

Figure 4.2: *Effect of glutamine supplementation on jejunum Claudin-1, Claudin-2, and Zonula occluden-1 mRNA expression during necrotic enteritis challenge*. Birds were fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 14 d of age, the challenge control (CC), 0.5% and 1.0% glutamine supplemented birds were orally gavaged 5,000 oocysts of *E. maxima*. On d 19, 20, and 21 the birds were given 10_8 CFU/mL of *C. perfringens* that is alpha toxin and NetB positive. On d 21, relative Claudin-1, Claudin-2, and Zonula occluden-1 mRNA expression was analyzed after correcting for β-actin mRNA and normalizing to the mRNA expression of the unchallenged control group. Bars (± SEM) within intestinal location that do not contain a common letter differ significantly ($P < 0.05$). *P*-values: Claudin-1, $P = 0.01$; Claudin-2, $P > 0.05$; Zonula occluden-1, $P > 0.05$. UC: unchallenged control, CC: challenged control. n=6.

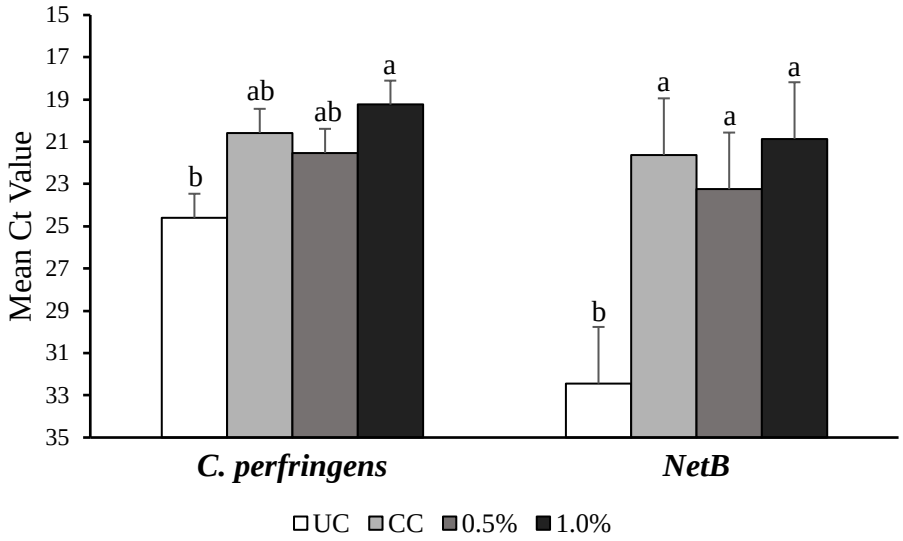

Figure 4.3: *Effect of glutamine supplementation on cecal contents C. perfringens and NetB DNA expression during necrotic enteritis challenge*. Birds were fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 14 d of age, the challenge control (CC), 0.5% and 1.0% glutamine supplemented birds were orally gavaged 5,000 oocysts of *E. maxima*. On d 19, 20, and 21 the birds were given 10_8 CFU/mL of *C. perfringens* that is alpha toxin and NetB positive. On d 21, *C. perfringens* and NetB DNA content was analyzed and reported as 40 minus the mean cycle threshold value (Ct value) for each treatment. Bars (± SEM) within intestinal location that do not contain a common letter differ significantly ($P < 0.05$). *P*-values: *C. perfringens*, $P = 0.02$; NetB, $P < 0.01$. UC: unchallenged control, CC: challenged control. n=6.

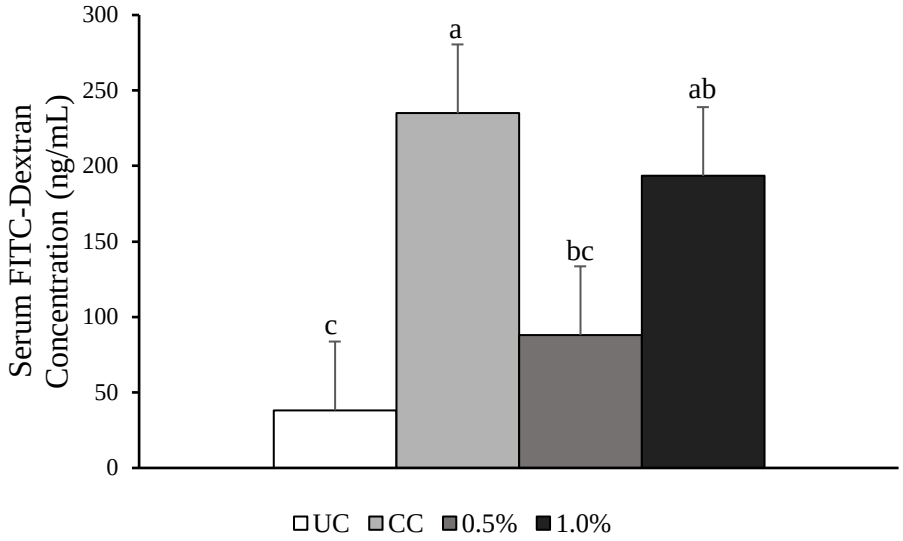

Figure 4.4: *Effect of glutamine supplementation on intestinal permeability during necrotic enteritis challenge*. Birds were fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 14 d of age, the challenge control (CC), 0.5% and 1.0% glutamine supplemented birds were orally gavaged 5,000 oocysts of *E. maxima*. On d 19, 20, and 21 the birds were given 10_8 CFU/mL of *C. perfringens* that is alpha toxin and NetB positive. On d 21, one bird per cage was orally inoculated with 2.2 mg of FITC-dextran, two hours later the birds were euthanized, and blood was collected from the heart. FITC-dextran concentration in the blood serum was measured using a microplate reader at a wavelength of 485 nm and an emission wavelength of 528 nm. FITC-dextran concentration per mL of serum was calculated based on a standard curve with the higher level of FITC-dextran concentration meaning higher intestinal permeability. Bars (± SEM) that do not contain a common letter differ significantly ($P < 0.05$). *P*-value: $P = 0.02$. UC: unchallenged control, CC: challenged control. n=6.

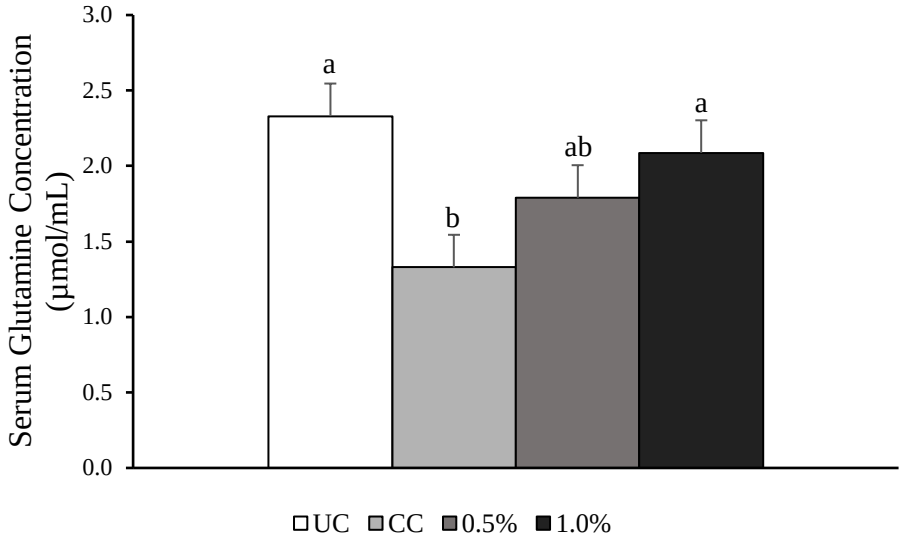

Figure 4.5: *Effect of glutamine supplementation on serum glutamine concentration during necrotic enteritis challenge*. Birds were fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 14 d of age, the challenge control (CC), 0.5% and 1.0% glutamine supplemented birds were orally gavaged 5,000 oocysts of *E. maxima*. On d 19, 20, and 21 the birds were given 10_8 CFU/mL of *C. perfringens* that is alpha toxin and NetB positive. On d 21, one bird per cage was euthanized, and blood was collected from the heart for analysis of serum glutamine concentration. Bars (± SEM) that do not contain a common letter differ significantly ($P < 0.05$). *P*-value: $P < 0.01$. UC: unchallenged control, CC: challenged control. n=6.

CHAPTER 5

EFFECTS OF GLUTAMINE SUPPLEMENTATION ON BROILER IMMUNITY ACQUISITION WHEN GIVEN A NON-ATTENUATED COCCIDIOSIS VACCINE

[1]J.H. Oxford and R.K. Selvaraj. To be submitted to the *Journal of Applied Poultry Research*.

ABSTRACT

The objective of this study was to evaluate the effects of glutamine supplementation on broiler immunity acquisition when given a non-attenuated coccidiosis vaccine. Cobb by-product male chicks were randomly assigned to one of four treatments (24-floor pens, 6 pens/treatment with 20 birds each), an unchallenged control (UC), or a challenged glutamine supplementation level of 0% (challenged control; CC), 0.5, or 1.0% (n=6). All of the chicks in the study were vaccinated with Coccivac B52® on d of hatch. To evaluate immunity to coccidiosis, the birds in the challenge groups were gavaged with a 25X dose of Coccivac B52® on d 21. At 7 d post-challenge, cecal tonsil IFN-γ mRNA expression was similar for the 0.5 and 1.0% glutamine groups to that of the UC (1.2- and 1.4-fold higher expression, respectively, $P > 0.05$), while the CC had a 2.4-fold higher level of expression compared to the UC ($P < 0.05$). Glutamine supplementation at 0.5% increased jejunum IL-10 expression by 1.6-fold of that of the CC at 7 d post-challenge ($P < 0.05$). At 7 d post-challenge, serum anticoccidial IgG content was significantly higher in the CC compared to the UC, 0.5% glutamine supplemented, and 1.0% glutamine supplemented groups, 149, 160, and 174%, respectively ($P < 0.05$). This study showed supplementation of glutamine in coccidiosis vaccinated birds reduced cecal tonsil IFN-γ mRNA expression, increased jejunum IL-10 mRNA expression, and reduced serum anticoccidial IgG content during challenge. These results indicate that glutamine supplementation may be beneficial when giving a coccidiosis vaccine.

DESCRIPTION OF PROBLEM

Coccidiosis is considered to be one of the most impactful diseases in commercial poultry production and is caused by Apicomplexa intestinal parasites of the genus *Eimeria* (McDonald and Shirley, 2009). This disease has traditionally been prevented through the use of anticoccidial drugs. Due to consumer preference, commercial poultry producers have started to move away from the use of ionophores, because of their classification as antibiotics, and have begun to use coccidiosis vaccines more frequently. The use of coccidiosis vaccines relies on adequate "cycling" of the *Eimeria* through the bird and back into the litter where the oocyst will be consumed by the birds again; it usually requires 2-4 cycles through the bird to provide adequate immunity to the parasite (Joyner and Norton, 1973). When administered and managed correctly, coccidiosis vaccines can provide good immunity and prevent coccidiosis; however, even when done properly, vaccines can have negative effects on performance in chickens (Mussini, et al., 2012). In the past, antibiotics have been provided in the feed when giving coccidiosis vaccines to help reduce the incidences of necrotic enteritis; but many producers are following antibiotic free production guidelines (Williams, 2005).

One of the birds main response to *Eimeria* is cell mediated and causes increases in $CD4^+$ and $CD8^+$ T cell populations (Lillehoj and Trout, 1996; Walston, et al., 2016). During the immune response, IFN-γ expression is increased and is believed to help reduce the coccidiosis infection (Tang, et al., 2018). Following infection, increased IL-10 expression has been shown to induce cellular repair and tissue damage during the recovery process (Shivaramaiah, et al., 2014). Little work has shown the effects of antibody production on *Eimeria* infection, but it is hypothesized that antibodies are mainly used during immunization rather than infection (Rose and Long, 1971).

The amino acid, glutamine, is found at high concentrations in the body and is considered to be conditionally essential during times of stress or enteric challenge (Newsholme, et al., 2002). Luquetti, et al. (2016), demonstrated that glutamine supplementation at 1.0% in coccidiosis vaccinated birds helped improve intestinal morphology and reduce adverse effects on feed conversion during immunity acquisition. Glutamine supplementation in coccidiosis vaccinated birds has also been shown to alleviate losses in body weight associated with vaccination (Mussini, et al., 2012). Furthermore, glutamine supplementation has been shown to help improve performance parameters, intestinal morphology, and reduce lesions in enteric challenge models in chickens (Xue, et al., 2018; Oxford and Selvaraj, 2019).

This study aimed to evaluate the effects of dietary glutamine supplementation on immunity acquisition in birds given a non-attenuated coccidiosis vaccine.

MATERIALS AND METHODS

All animal protocols were approved by the Institutional Animal Care and Use Committee at University of Georgia.

Animals, Housing, and Glutamine Supplementation

A total of 480 male Cobb500 chicks were randomly allocated to 24-floor pens (1.22x1.52 m) with 20 chicks/pen on d of hatch. The 24 pens were randomly assigned to one of four treatments, an unchallenged control (UC) or a challenged glutamine supplementation level of 0% (challenged control; CC), 0.5, or 1.0% in six replications (n=6). There were two dietary phases, a starter diet fed from 0-21 d and a grower diet fed from 21-35 d. The basal mash diets were corn and soybean meal based that had a calculated basal glutamine content of 2.05% for the starter

diet and 2.00% for the grower (Table 1). Supplemental glutamine was added at 0, 0.5, and 1.0% with cellulose being used in place of supplemental glutamine in the basal diets. Feed and water were provided *ad libitum*. Feed and BW were recorded at 0, 14, 21, 28, and 35 d for the calculation of BWG and FCR. On d 28 and 35 of the study, birds were sampled for the following parameters.

Eimeria Challenge

At 21 d of age, each chicken in the CC, 0.5, and 1.0% glutamine supplementation groups was orally gavaged with a 25X dose of Coccivac B52® (Merck Animal Health, Madison, NJ). This dose was chosen to provide a mild coccidiosis infection with lesion scores from 1-2 (Wang, et al., 2018). On d 7 post-challenge, four birds per pen were randomly selected and scored for gross lesions using the methodology described by Johnson and Reid (1970) with scores for lesions in the duodenum, jejunum, and cecum being from 0-4 (0 being no lesions and 4 being the most severe lesions). Mucosal scrapings were taken from the jejunum around Meckel's diverticulum and placed on microscope slides to be used for microscopic examination of *E. maxima* scores. *E. maxima* was microscored in a scale of 0-4, where 0 being no visible oocyst, 1 being 1-20 *E. maxima* oocysts per 10X field of view, 2 being 21-50 *E. maxima* oocysts per 10X field of view, 3 being 51-100 *E. maxima* oocysts per 10X field of view, and 4 being more than 100 *E. maxima* oocysts per 10X field of view (Goodwin, et al., 1998). The objective of the *Eimeria* challenge at 21 d of age was to test *Eimeria* infection immunity.

Effect of Glutamine Supplementation on Fecal Eimeria Oocyst Shedding

On d 7, 14, 21, and 28, fecal samples were collected from individual pens in airtight plastic bags and stored at 4◦C until further analysis. On the d of analysis, samples were homogenized, and *Eimeria* oocysts were enriched using a salt flotation technique described previously (Levine, et al., 1960). *Eimeria* oocysts were diluted and counted using the McMaster counting chamber (Chalex Corporation, Ketchum, ID).

Effect of Glutamine Supplementation on Jejunum and Cecal Tonsil mRNA Expression

On d 7 and 14 post-challenge, one bird per pen was sacrificed, and total RNA from the jejunum and cecal tonsils were collected. RNA was then reverse transcribed into cDNA using methods described earlier (Selvaraj and Klasing, 2006). The mRNA was then analyzed for pro-inflammatory cytokine IFN-γ, anti-inflammatory cytokine IL-10, and mucus production related chemokine MUC2 by real-time PCR using SyBr green after normalizing for β-actin mRNA. Jejunum mRNA was also analyzed for tight junction proteins Claudin-1, Claudin-2, and Zonula occluden-1 mRNA content by real-time PCR. Primer sequences and annealing temperatures are described in Table 2. Fold change from the reference gene was calculated using the comparative Ct method $2^{(Ct\ Sample - Housekeeping)}/2^{(Ct\ Reference - Housekeeping)}$ with Ct being the threshold cycle (Schmittgen and Livak, 2008). Ct was determined by CFX Maestro software when the fluorescence rises exponentially two-fold above background (Biorad, Hercules, CA). The reference group was the birds fed 0% glutamine that were not challenged (UC).

Effect of Glutamine Supplementation on CD4+ and CD8+ Cecal Tonsil Cell Percentages

On d 7 post-challenge, cecal tonsils were collected from one bird per pen to be analyzed for total CD4+ and CD8+ cell percentages. Each cecal tonsil was homogenized, and the cells were collected using a 0.45 μm cell strainer and then placed in 1 mL of RPMI. The cells were washed with PBS two times by centrifuging at 750 X g for 3 min. The cells were resuspended in wash buffer (2mM of EDTA and 1.5% FBS in EDTA). 1 X 10_6 cells were added to a 96 well plate. Anti-chicken CD4+ (1:200 dilution) and CD8+ (1:450 dilution) antibodies were added to each well and incubated for 20 min at 4°C (Southern Biotech, Birmingham, AL). After incubation, the plate was centrifuged for 5 min at 400 X *g*, and the antibody solution was removed. The cells were then washed and resuspended using the buffer previously mentioned. Total CD4+ and CD8+ cell percentages were analyzed using flow cytometry as described previously (Shanmugasundaram and Selvaraj, 2012).

Effect of Glutamine Supplementation on Serum Anticoccidial IgG Content

At d 14, 21, and 28, serum was collected from one bird in each pen (n=6) and stored at −20°C until further use. Serum anticoccidial IgG content was determined by ELISA as described previously with minor modifications (Garcia, et al., 2008). The optimal dilutions of sera, antigen, and conjugates were standardized using checkerboard titrations. Flat-bottomed, high-binding 96-well plates (Greiner Bio-One, Monroe, NC) were coated (100 μL/well) with the *Eimeria* antigen (10 μg/mL) diluted in 0.1 *M* carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed three times with PBS-Tween 20 (0.05% Tween 20 in PBS, pH 7.4) and blocked using 8% nonfat dry milk in PBS-Tween 20 (200 μL/well) for 1 h 30 min at 37°C. The plates were washed three times with PBS-Tween 20. The serum samples were diluted to 1:20

with 5% nonfat dry milk in PBS-Tween 20 and added to the 96-well plates in duplicates (100 μL/well). The plates were incubated at 37°C for 90 min. The plates were then washed with PBS-Tween 20. HRP-labeled anti-chicken IgG (Southern Biotech, Birmingham, AL) was diluted to 1:8,000 in 5% nonfat dry milk-PBS-Tween 20 and added to the 96-well plates (100 μL/well). The plates were then incubated at 37°C for 30 min. The plates were washed with PBS-Tween 20, and the substrate 3,3,5,5-tetramethylbenzidine solution (eBioscience, San Diego, CA) was added to the wells (100 μL/well). The reaction was stopped after 10 min using 1 *N* HCl (100 μL/well), and the optical density was read at 450 nm using a microplate ELISA reader.

Statistical Analysis

A two-way ANOVA was used to examine the effect of glutamine supplementation and challenge on dependent variables (SAS Studio, SAS Institute Inc., Cary, NC). When main effects were significant ($P < 0.05$), differences between means were analyzed by Tukey's multiple comparison test. *Eimeria* lesion scores and microscopic scores were analyzed using the Kruskal-Wallis Test.

RESULTS

Effect of Glutamine Supplementation on Production Parameters

There were no significant differences observed between treatments at any time point for body weight or feed conversion ratio ($P > 0.05$; Table 5.3).

Effect of Glutamine Supplementation on Jejunum and Cecal Tonsil mRNA Expression

At 7 d post-challenge, the challenged control birds had significantly higher IFN-γ mRNA expression found in their cecal tonsils compared to that of the other treatment groups with the CC being 2.4-fold higher than the UC ($P < 0.05$; Figure 5.1). The challenged glutamine supplemented groups did not differ from the unchallenged control group in IFN-γ mRNA expression in the cecal tonsils and were 1.2- and 1.4-fold higher for the 0.5% and 1.0% glutamine supplemented groups, respectively ($P > 0.05$; Figure 5.1). There were no significant differences among all treatments for IFN-γ mRNA expression in the jejunum at 7 d post-challenge ($P > 0.05$; Figure 5.1). There were significant differences observed between treatments for IL-10 in jejunum of birds 7 d post-challenge ($P < 0.05$; Figure 5.2). The 0.5% glutamine supplemented birds had 1.4-fold higher IL-10 mRNA expression compared to the unchallenged control. The challenged control and 1.0% glutamine did not differ significantly from the unchallenged control and were 0.9- and 1.1-fold different from the unchallenged control, respectively. There were no observed significant differences in IL-10 mRNA expression in the cecal tonsils between any of the treatment groups at 7 d post-challenge ($P > 0.05$; Figure 5.2). There were significant differences observed for Claudin-2 jejunum mRNA expression ($P < 0.05$; Figure 5.3). The 1.0% glutamine supplemented birds had 1.5-fold higher Claudin-2 mRNA compared to that of the unchallenged control and the 0.5% glutamine supplemented groups. There were no significant differences observed between treatment groups at 7 d post-challenge for MUC2, Claudin-1, or Zonula occluden-1 mRNA expression ($P > 0.05$; data not shown). There were also no differences among treatments observed for any of the genes of interest at 14 d post-challenge ($P > 0.05$; data not shown).

Effect of Glutamine Supplementation on CD4+ and CD8+ Cecal Tonsil Cell Percentages

There were significant differences in the ratios of CD8+ to CD4+ cells from the cecal tonsils of the birds in this study ($P < 0.05$; Table 4). The 0.5% glutamine supplemented birds had significantly lower CD8+ to CD4+ cell ratios compared to that of the challenged control and the 1.0% glutamine supplemented group with a ratio of 0.75 compared to 1.03 and 1.04, respectively. There were no differences observed for CD8+ to CD4+ ratios at 14 d post-challenge ($P > 0.05$; data not shown).

Effect of Glutamine Supplementation on Serum Anticoccidial IgG Content

Glutamine supplementation significantly affected serum anticoccidial IgG content at 28 d of age (7 d post-challenge), with the challenged control group having 149, 160, and 174% higher OD values over the unchallenged control, 0.5% glutamine supplemented birds, and the 1.0% glutamine supplemented birds, respectively ($P < 0.05$; Figure 5.5). There were no significant differences in serum anticoccidial IgG content between treatments at either 14 or 21 d of age ($P > 0.05$; Figure 5.5).

Effect of Glutamine Supplementation on Intestinal Eimeria Lesion Scores and Microscopic E. maxima Scores

There were no significant differences observed for gross lesions or microscopic scores between treatments 7 d post-challenge ($P > 0.05$; Table 5.4).

Effect of Glutamine Supplementation on Fecal Eimeria Oocyst Shedding

There were no differences in fecal *Eimeria* oocyst shedding at any timepoint when comparing the treatment groups ($P > 0.05$; data not shown).

DISCUSSION

The objective of this experiment was to evaluate the effects of glutamine supplementation on production parameters and immunity acquisition in broilers vaccinated with a live non-attenuated coccidiosis vaccine. Glutamine supplementation reduced IFN-γ mRNA expression in the cecal tonsils of birds while increasing IL-10 expression in the jejunum of birds at 7 d post-challenge. Supplementation of glutamine at 1.0% increased jejunum Claudin-2 expression at 7 d post-challenge while supplementation of glutamine at 0.5% reduced the ratio of $CD8^+$ to $CD4^+$ cells in the cecal tonsils of birds 7 d post-challenge. Dietary glutamine supplementation also reduced serum anticoccidial IgG contents to that of the unchallenged birds at 7 d post-challenge.

Glutamine supplementation reduced cecal tonsil IFN-γ mRNA expression in vaccinated birds that were challenged. In work conducted with birds genetically resistant to *Eimeria* species, there was increased expression of IFN-γ found in the cecal tonsils of the bird's post-challenge (Rothwell, et al., 2004). It is known that in mice, IFN-γ producing T cells (Th1) are used for resistance to primary and secondary infections to *Eimeria* (Allen and Fetterer, 2002). In a study conducted by Oxford and Selvaraj (2019), non-immunized birds challenged with an experimental coccidiosis infection and supplemented with glutamine had decreased IFN-γ expression in the jejunum and cecal tonsils at 7 d post-challenge. Decreased levels of IFN-γ mRNA expression in the cecal tonsils may indicate that glutamine supplemented birds had developed immunity to the *Eimeria* challenge; therefore, the infections were cleared before 7 d post-challenge.

Increased expression of IL-10 observed in the jejunum of 0.5% glutamine supplemented birds may coincide with the reduced ratio of $CD8^+$ to $CD4^+$ cells in the cecal tonsils of these birds. $CD4^+CD25^+$ cells have been shown to have increased expression of IL-10 which may explain the observations observed in this study; it is important to note that we did not specifically gate for $CD4^+CD25^+$ cells (Kim, et al., 2019). IL-10 has been shown to be associated with mucosal tissue repair during recovery from coccidiosis; therefore, increased levels of IL-10 may show evidence of the birds moving from a pro-inflammatory state to an anti-inflammatory state (Shivaramaiah, et al., 2014). These observations may provide evidence that the birds supplemented with glutamine at 0.5% may have overcome the challenge before the other groups and have moved towards a recovery state.

Increases in Claudin-2 expression were seen in the 1.0% glutamine supplemented group. A similar observation was observed in previous work during a coccidiosis infection; however in said study differences, were observed for all tight junction proteins measured (Oxford and Selvaraj, 2019). A potential reason that differences may have not been observed for all tight junction proteins evaluated in this study may be due to the birds being immune to the challenge. The challenged groups in this study had similar *Eimeria* gross lesion scores, *E. maxima* microscopic scores, and oocyst production to that of the vaccinated unchallenged control group showing that the coccidiosis vaccine was able to develop protective immunity by 21 d, when the challenge was given.

The glutamine supplemented groups had similar serum anticoccidial IgG concentrations to that of the unchallenged control group and were significantly lower than the challenged control group. It is believed that the primary immune response to *Eimeria* infections is a cellular-based immune response rather than humoral (Lillehoj and Trout, 1996). There are differing

opinions on the importance of the humoral immune response to *Eimeria* challenges, with some believing that there is little to no role of the humoral immune response (Wallach, 2010; Min, et al., 2013). Reduced antibody concentrations in the glutamine supplemented groups may indicate that these birds are overcoming challenge faster than that of the challenge control group. This hypothesis may be reflected by the 0.5% glutamine supplemented birds having reduced IFN-γ levels observed in their cecal tonsils.

There were no differences observed in gross lesion scores, *E. maxima* microscopic scores, fecal oocysts production, or performance parameters between all of the treatment groups; this observation provides an indication that the birds may have achieved immunity by the time of challenge. No differences observed in fecal oocyst shedding may indicate that the supplementation of glutamine did not negatively affect the coccidiosis vaccine cycling. The inability to see differences in performance parameters that were observed in previous studies may be attributed to experimental design, rearing conditions, or sample sizes.

In conclusion, 0.5% supplementation of glutamine in coccidiosis vaccinated birds reduced cecal tonsil IFN-γ mRNA expression, increased jejunum IL-10 mRNA expression, and reduced serum anticoccidial IgG content during the challenge and did not inhibit immunity acquisition or cycling of the coccidiosis vaccine. These results indicate that glutamine supplementation does not interfere with immunity acquisition and may be beneficial when giving a coccidiosis vaccine.

CONCLUSIONS AND APPLICATIONS

1. Dietary glutamine supplementation did not interfere with immunity acquisition in birds vaccinated with a non-attenuated coccidiosis vaccine.

2. Supplementation of glutamine may improve clearance or resistance to *Eimeria* challenge.
3. Supplementation of glutamine may be beneficial when giving birds a coccidiosis vaccine.

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Coccivac B52® contains: *E. acervulina*, 2 strains of *E. maxima*, *E. mivati*, and *E. tenella*; Merck Animal Health, Madison, NJ.

Biorad, Hercules, CA

Chalex Corporation, Ketchum, ID

Southern Biotech, Birmingham, AL

Greiner Bio-One, Monroe, NC

eBioscience, San Diego, CA

SAS Studio, SAS Institute Inc., Cary, NC

Table 5.1: Diet blend (%) and calculated nutrient composition of the starter and grower basal diets.

Diet Blend %	Starter	Grower
Corn	58.47	64.66
Soybean Meal, 48%	35.15	29.63
Soybean Oil	2.27	1.82
Monocalcium phosphorus, 21%	1.38	1.27
Limestone	1.59	1.40
DL-Methionine	0.21	0.24
L-Lysine-HCL, 78%	0.14	0.18
Salt (NaCl)	0.35	0.43
Vitamin premix[1]	0.08	0.08
Mineral premix[2]	0.35	0.30
Total:	100	100
Calculated Nutrient Composition		
ME, kcal/g	3.05	3.09
CP, %	21.44	19.32
Calcium, %	0.95	0.84
Total Phosphorus, %	0.71	0.67
Avail. Phosphorus< %	0.45	0.42
Sodium, %	0.16	0.20
Chloride, %	0.27	0.33
Lysine, %	1.31	1.19
Methionine, %	0.56	0.57
TSAA, %	0.91	0.89
Threonine, %	0.87	0.78
Tryptophan, %	0.29	0.25
Arginine, %	1.5	1.33
Glutamine, %	2.05	2.00

[1] Vitamin mix provided the following (per kilogram of diet): thiamin-mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B12 (cobalamin), 12.0g; pyridoxine-HCl, 2.7 mg; D-biotin, 0.11 mg; folic acid, 0.55 mg; menadione sodium bisulfate complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 1,100 IU; trans-reinyl acetate, 2,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.

[2] Trace mineral mix provides the following (per kilogram of diet): manganese (MnSO4.H2O), 101 mg; iron (FeSO4.7H2O), 20 mg; zinc (Zn)), 80 mg; copper (CuSO4.5H2O), 3 mg; iodine (ethylene diamine dihydroiodide), 0.75 mg; magnesium (MgO), 20 mg; selenium (sodium selenite), 0.3 mg.

Table 5.2: Primers and PCR conditions for RTqPCR

Target Gene	Sequence (5'-3')	Annealing Temperature	Reference
IFN-γ-F	TGAGCCAGATTGTTTCGA	54.0°C	(Shanmugasundaram and Selvaraj, 2012)
IFN-γ-R	ACGCCATCAGGAAGGTTG		
IL-10-F	CAATCCAGGGACGATGAACT	57.0°C	(Shanmugasundaram and Selvaraj, 2012)
IL-10-R	GGCAGGACCTCATCTGTGTAG		
CLAU-1-F	CATACTCCTGGGTCTGGTTGGT	57.5°C	(Du, et al., 2016)
CLAU-1-R	GACAGCCATCCGCATCTTCT		
CLAU-2-F	CCTGCTCACCCTCATTGGAG	57.5°C	(Chen, et al., 2016)
CLAU-2-R	GCTGAACTCACTCTTGGGCT		
ZO-1-F	TGTAGCCACAGCAAGAGGTG	57.4°C	(Bortoluzzi, et al., 2019)
ZO-1-R	CTGGAATGGCTCCTTGTGGT		
MUC2-F	CAGCACCAACTTCTCAGTTC	54.5°C	(Bortoluzzi, et al., 2019)
MUC2-R	TCTGCAGCCACACATTCTTT		
β-actin-F	ACCGGACTGTTACCAACACC	57.0°C	(Shanmugasundaram and Selvaraj, 2012)
β-actin-R	GACTGCTGCTGACACCTTCA		

Table 5.3: Effect of glutamine supplementation on body weight (BW) and feed conversion ratio (FCR) in birds vaccinated with coccidiosis vaccine and challenged with *Eimeria*.

	Body Weight (g)				Feed Conversion Ratio (g:g)			
	14 d	21 d	28 d	35 d	14 d	21 d	28 d	35 d
UC	294.1	648.4	1146.8	1783.1	1.464	1.601	1.651	1.835
CC	287.8	647.9	1134.7	1744.7	1.428	1.639	1.701	1.820
0.5%	299.2	665.7	1158.5	1783.0	1.414	1.548	1.633	1.855
1.0%	320.4	682.6	1188.9	1892.6	1.363	1.570	1.650	1.883
SEM	7.1	8.3	11.6	31.9	0.021	0.020	0.015	0.014
P-Value	0.160	0.676	0.599	0.201	0.098	0.529	0.655	0.842

Birds were fed diets supplemented with glutamine at 0.0, 0.5, and 1.0%. BW was measured and FCR was calculated at 14, 21, 28, and 35 d of age. All birds were vaccinated with a coccidiosis vaccine at d of hatching by course spray. At 21 d of age, the CC (0%), 0.5%, and 1.0% glutamine supplemented birds were orally gavaged with a 25X dose of Coccivac B52®. UC: unchallenged control, CC: challenged control. n=6.

Table 5.4: Effect of glutamine supplementation on intestinal *E. acervulina*, *E. maxima*, and *E. tenella* lesion scores and *E. maxima* microscopic scores.

Eimeria spp.	Treatment	0	1	2	3	4	Rank Score Means	n	Chi Sq. *P*-value
acervulina	**UC**	21	1	2	0	0	42.5	24	0.604
Gross Lesion Score	**CC**	18	3	3	0	0	48.4	24	
	0.5%	16	7	1	0	0	51.0	24	
	1.0%	16	5	3	0	0	52.1	24	
maxima	**UC**	24	0	0	0	0	46.5	24	0.250
Gross Lesion Score	**CC**	22	2	0	0	0	50.5	24	
	0.5%	22	2	0	0	0	50.5	24	
	1.0%	24	0	0	0	0	46.5	24	
tenella	**UC**	22	1	1	0	0	45.5	24	0.565
Gross Lesion Score	**CC**	22	1	0	1	0	45.6	24	
	0.5%	19	4	0	1	0	51.3	24	
	1.0%	19	3	0	2	0	51.6	24	
maxima	**UC**	23	1	0	0	0	44.0	24	0.384
Microscopic Score	**CC**	19	5	0	0	0	52.0	24	
	0.5%	21	3	0	0	0	48.0	24	
	1.0%	20	4	0	0	0	50.0	24	

At 21 d of age, each chicken was orally gavaged with a 25X dose of Coccivac B52®. 6 d post-challenge four birds per pen were randomly selected and scored for gross lesions in a scale of 0-4 in the duodenum, jejunum, and cecum with 0 being no lesions and 4 being the most severe lesions (Johnson and Reid, 1970). Mucosal scrapings were taken from the Meckel's diverticulum area and placed on microscope slides to be used for microscopic examination of *E. maxima* scores. *E. maxima* was microscored in a scale of 0-4, where 0 being no visible oocyst, 1 being 1-20 *E. maxima* oocysts per 10X field of view, 2 being 21-50 *E. maxima* oocysts per 10X field of view, 3 being 51-100 *E. maxima* oocysts per 10X field of view, and 4 being more than 100 *E. maxima* oocysts per 10X field of view (Goodwin, et al., 1998). UC: unchallenged control, CC: challenged control. n=24.

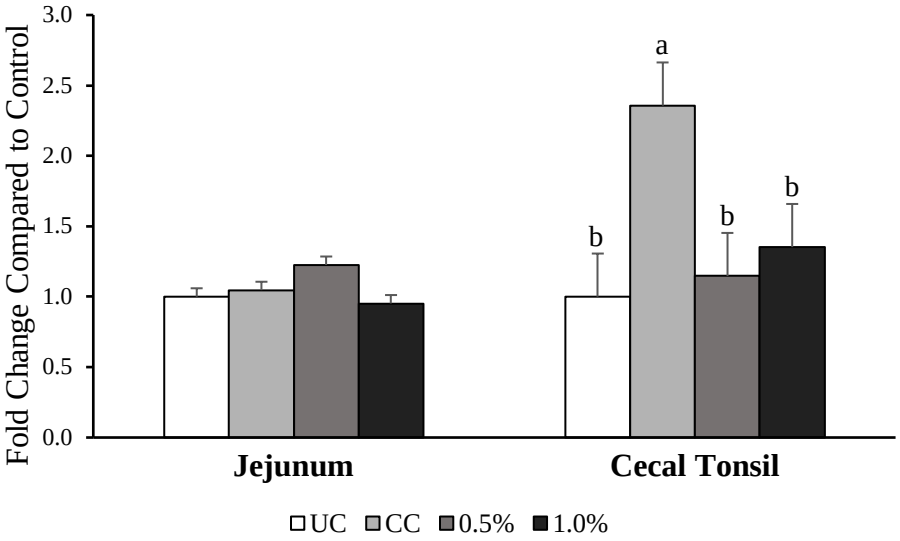

Figure 5.1: *Effect of glutamine supplementation on jejunum and cecal tonsil IFN-γ mRNA expression at 7 d post-challenge and challenged with Eimeria*. Birds were given coccidiosis vaccination at d of hatch and fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 21 d of age, the birds were orally gavaged with a 25X dose of Coccivac B52®. At 7 d post challenge, relative IFN-γ mRNA expression was analyzed after correcting for β-actin mRNA and normalizing to the mRNA expression of the unchallenged control group. Bars (± SEM) within intestinal location that do not contain a common letter differ significantly ($P < 0.05$). *P*-values: jejunum, $P > 0.05$; cecal tonsil, $P = 0.03$. UC: unchallenged control, CC: challenged control. n=6.

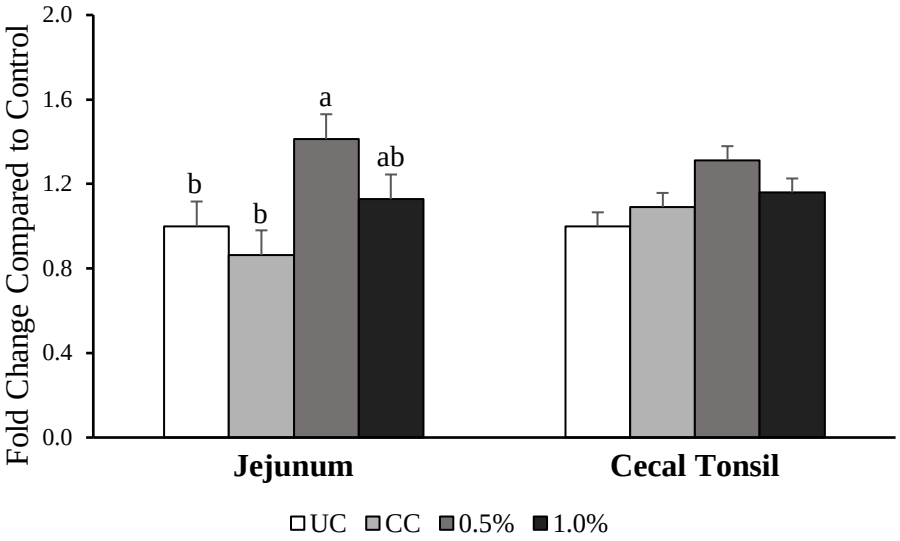

Figure 5.2: *Effect of glutamine supplementation on jejunum and cecal tonsil IL-10 mRNA expression at 7 d post-challenge and challenged with Eimeria*. Birds were given coccidiosis vaccination at d of hatch and fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 21 d of age, the birds were orally gavaged with a 25X dose of Coccivac B52®. At 7 d post challenge, relative IL-10 mRNA expression was analyzed after correcting for β-actin mRNA and normalizing to the mRNA expression of the unchallenged control group. Bars (± SEM) within intestinal location that do not contain a common letter differ significantly ($P < 0.05$). *P*-values: jejunum, $P = 0.01$; cecal tonsil, $P > 0.05$. UC: unchallenged control, CC: challenged control. n=6.

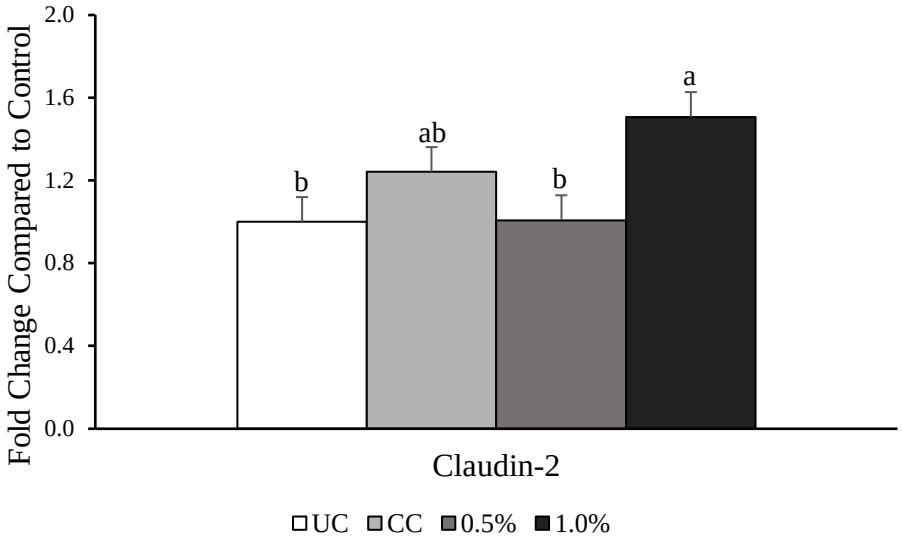

Figure 5.3: *Effect of glutamine supplementation on jejunum Claudin-2 mRNA expression at 7 d post-challenge and challenged with Eimeria*. Birds were given coccidiosis vaccination at d of hatch and fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 21 d of age, the birds were orally gavaged with a 25X dose of Coccivac B52®. At 7 d post challenge, relative Claudin-2 mRNA expression were analyzed after correcting for β-actin mRNA and normalizing to the mRNA expression of the 0% glutamine group. Bars (± SEM) within intestinal location that do not contain a common letter differ significantly ($P < 0.05$). *P*-value: $P < 0.05$. UC: unchallenged control, CC: challenged control. n=6.

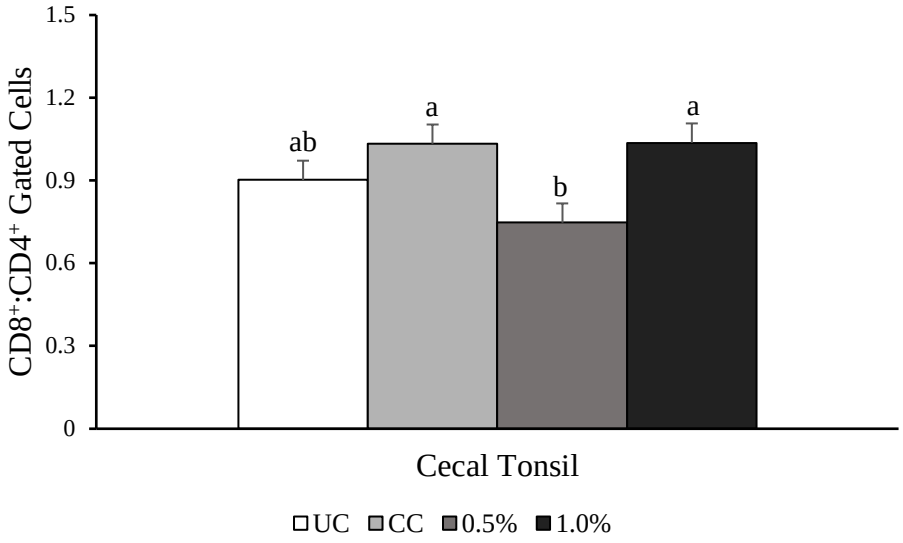

Figure 5.4: *Effect of glutamine supplementation on cecal tonsil CD8+ to CD4+ cell ratios post-vaccination and challenged with Eimeria*. Birds were given coccidiosis vaccination at d of hatch and fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 21 d of age, the birds were orally gavaged with a 25X dose of Coccivac B52®. On d 28, post-vaccination, cecal tonsils were collected from one bird per pen for analysis of CD4+ and CD8+ T cells using flow cytometry. Results are reported ratio of CD8+ to CD4+ T cells with bars (± SEM) that do not contain a common letter differ significantly ($P < 0.05$). *P*-values: $P < 0.05$. UC: unchallenged control, CC: challenged control. n=6.

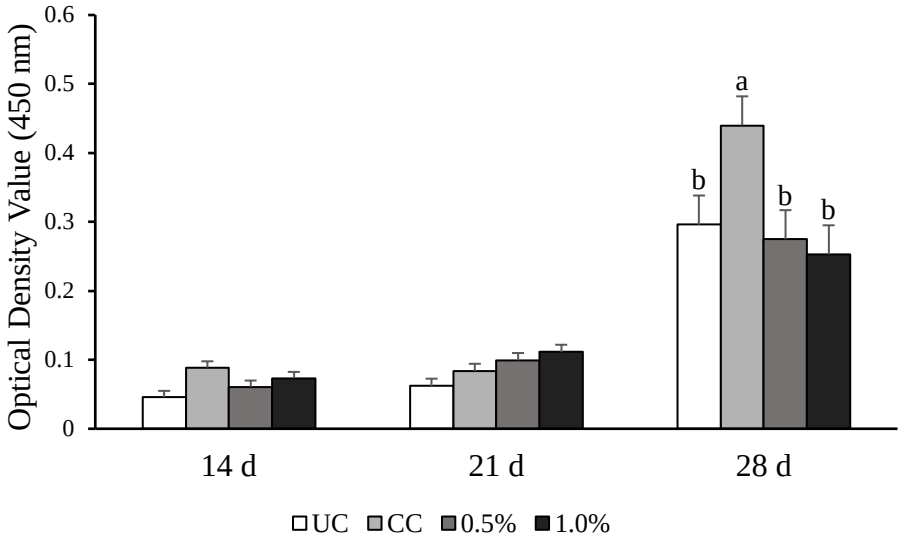

Figure 5.5: *Effect of glutamine supplementation on serum anti-coccidial IgG antibody titers at 14, 21, and 28 days post-vaccination and challenged with Eimeria*. Birds were given coccidiosis vaccination at d of hatch and fed basal diets supplemented with glutamine at 0 (control), 0.5, and 1.0%. At 21 d of age, the birds were orally gavaged with a 25X dose of Coccivac B52®. At 14, 21, and 28 d post-vaccination, blood samples were collected from one bird per pen for anti-coccidial IgG titers using ELISA. Results are reported as average optical density values (OD) values with bars (± SEM) within time periods that do not contain a common letter differ significantly ($P < 0.05$). *P*-values: 14 d, $P > 0.05$; 21 d, $P > 0.05$; 28 d, $P < 0.05$. UC: unchallenged control, CC: challenged control. n=6.

CHAPTER 6

CONCLUSION

Enteric diseases like coccidiosis and necrotic enteritis have become very problematic in the poultry industry. In the past, these diseases were prevented using antibiotic growth promoters like Virginiamycin. With the current direction of the poultry industry moving away from antibiotic growth promotors, disease prevention, and reduction using alternative feed additives will become increasingly more common. Moving forward, nutrition will be at the forefront of disease prevention, with optimal diets being a necessity.

The first study in this work evaluated the effects of dietary glutamine supplementation during an experimental coccidiosis infection. In this study, glutamine supplementation reduced both pro- and anti-inflammatory mRNA expression 7 d post-infection. Glutamine supplementation also improved jejunum histological morphology and increased tight junction protein mRNA expression. In this work, it was concluded that glutamine supplementation may be beneficial during coccidiosis.

The second study of this work evaluated the effects of dietary glutamine supplementation during an experimental necrotic enteritis infection. It was observed that when glutamine was supplemented at 0.5%, there was reduced intestinal permeability, measured by FITC-dextran, via increased IL-10 and Claudin-1 mRNA expression and improved serum glutamine concentrations during necrotic enteritis. Glutamine supplementation at 1.0% produced adverse effects on intestinal permeability leading to higher mortality. Supplementation at 1.0% also provided increased ceca *C. perfringens* and is not recommended for use when a necrotic enteritis infection

may occur in the field. Overall this study showed that glutamine supplementation at 0.5% may be beneficial during a necrotic enteritis infection.

The third study evaluated the effects of glutamine supplementation on immunity acquisition in birds vaccinated with a non-attenuated coccidiosis vaccine. In this study, supplementation of glutamine reduced cecal tonsil IFN-γ mRNA expression while increasing jejunum IL-10 mRNA expression and decreasing serum anticoccidial IgG content in birds that were vaccinated and challenged with *Eimeria*. Glutamine supplementation had no negative effects on coccidiosis lesion scores or coccidiosis fecal oocyst counts, indicating that there were no adverse effects of glutamine supplementation on immunity acquisition. This study concluded that glutamine supplementation did not interfere with immunity acquisition and may help immune birds overcome potential field challenges.

From this research, one of the big messages is that amino acids and other nutrients that have been traditionally considered as non-essential may be essential during times of stress and disease. In necrotic enteritis challenged birds, serum glutamine concentrations significantly decreased, providing evidence of a loss in total glutamine levels for the bird. Glutamine has been identified as conditionally essential in humans and other mammals during stress and challenge, and with more research, we may conclude that glutamine is conditionally essential in poultry during enteric challenges. There is still much research that is required on glutamine supplementation in birds with future research being aimed at other nutritional models and studies evaluating glutamine digestibility in feed ingredients.