

THE EFFECT OF SUPPLEMENTAL GLUTAMINE ON GROWTH PERFORMANCE,
DEVELOPMENT OF THE GASTROINTESTINAL TRACT, AND IMMUNE RESPONSE OF
BROILER CHICKS

by

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(under the direction of Amy B. Batal)

ABSTRACT

Recent controversies involving the use of growth promoting antibiotics in commercial feeds have led to studies evaluating alternatives to antibiotics and one possible alternative is the use of dietary supplemental glutamine. Four studies were conducted to evaluate the effect of supplemental glutamine on growth performance, development of the gastrointestinal tract, and immune response of broiler chicks. Supplementing a standard corn-soybean meal diet with 1% glutamine (old source) for 21 or 40 days (entire experimental period) improved broiler growth performance in all four studies and feed efficiency in the last three studies. Supplementing the diet with 1% glutamine significantly increased villi height, intestinal relative weights, thymus and spleen relative weights, IgA, IgG, IgM and IFN- γ concentrations, and anti-SRBC titers in broiler chicks. Supplementing the diet with 1% glutamine eliminated the weight depression that often accompanies vaccinations and may better protect the gut against an *Eimeria* challenge. Supplemental 1% glutamine in broiler diets enhanced the development of the gastrointestinal tract and immune response to a SRBC challenge, *Eimeria* vaccination, and *Eimeria* challenge. However, improved growth performance was dependent upon the source of glutamine used.

INDEX WORDS: glutamine, broilers, *Eimeria*, gastrointestinal tract, immune response, SRBC

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CHAPTER 1

INTRODUCTION

Today, the poultry industry faces many challenges; among them is the controversy over the use of growth promoting antibiotics. The use of antibiotics as a feed additive is a common practice in livestock production. Low levels of antibiotics are used in poultry diets mainly to reduce disease incidences but they also lead to an increase in growth performance and feed efficiency. The addition of sub-therapeutic levels of antibiotics to poultry diets is beneficial; unfortunately, consumer perceptions are that edible poultry tissues are contaminated with harmful concentrations of drug residues. Consumers and some scientists believe that there may be a link between the agricultural use of antibiotics and antibiotic resistance in humans and regulatory agencies such as the United States Food and Drug Administration, the Centers for Disease Control and Prevention, and the World Health Organization all agree that sub-therapeutic use of antimicrobials in animal feed should be restricted. The use of antibiotics in commercial feeds has been banned in Europe and may be restricted in the U.S. in the future. The possibility of a ban on the use of antibiotics in poultry feeds demands the need for studies to evaluate alternatives. Glutamine supplementation in humans and rats has been used in stressful conditions such as infection, injury, and trauma to improve gut barrier function, enhance the body's ability to mount an immune response, improve immune cell function, and decrease mortality. Supplementation of glutamine in poultry diets may be beneficial to the health of the bird, and thus could help along with other additives to decrease the use of antibiotics.

Another major challenge facing the poultry industry is the control of enteric diseases, such as coccidiosis. Avian coccidiosis is a major parasitic disease of substantial economic significance that is estimated to cost the poultry industry greater than \$800 million in annual losses. Intestinal parasitism is a major stress factor leading to malnutrition and lowering performance and production efficiency of poultry. The weight loss that results from nutrient malabsorption is a major indicator of a coccidial infection and it is this loss in performance, even in sub-clinical levels of the disease that is of economic importance to the producer. Currently, anticoccidial drugs and vaccines are the methods used to control coccidiosis. The search for new methods of control for coccidiosis has been stimulated due to the misuse of anticoccidial drugs that has led to the emergence of drug resistant strains, the many problems of vaccines, and consumer demands of drug and antibiotic free foods. A possible strategy for control of *Coccidia* is the use of dietary supplemental glutamine.

Dietary glutamine supplementation may be a good alternative to the use of antibiotics, coccidiostats, or vaccination, especially during Coccidial infections. During enteric disease challenges, antibiotics keep the cell wall from thickening which would normally occur because of inflammation. Glutamine supplementation may be beneficial to the bird's health status since it is considered an essential amino acid in inflammatory conditions by functioning as an agent to protect the gut barrier thereby reducing inflammation. This maintenance of the gut integrity may help or prevent enteric diseases. Supplementation of glutamine has been shown to stimulate the immune response by increasing lymphocyte proliferation and the number of T-helper cells. The increase in villous length observed in animals fed diets supplemented with glutamine may be the reason for the improvement in feed efficiency observed in animals fed supplemental dietary glutamine. Longer villi enable the animal to better utilize nutrients and could possibly help to

compensate for the depressed weight that occurs during enteric disease challenges. This increase in efficiency of nutrient utilization and improvement in the animal's health may also be valuable in reducing or eliminating weight depression often viewed with vaccination, a common practice in poultry production. Glutamine's immunomodulatory effects and its role in the maintenance of the immune system in a number of animals suggest that dietary glutamine supplementation may help to improve chickens health and performance. Since glutamine supplementation has not been evaluated in poultry diets, the objective of the research presented herein was to evaluate the effect of supplemental glutamine on growth performance, development of the gastrointestinal tract, and immune response of broiler chicks.

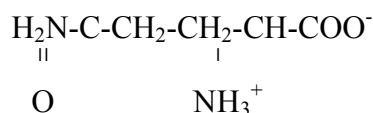
CHAPTER 2

REVIEW OF LITERATURE

I. Characteristics of Glutamine

L-glutamine is the most prevalent amino acid in the bloodstream, accounting for 30 to 35 percent of the plasma amino acid nitrogen and the free amino acid pool in the body (Souba, 1993). Because glutamine contains two ammonia groups, one from its precursor, glutamate, and the other from free ammonia in the bloodstream, one of glutamine's roles is to act as a "nitrogen shuttle," which helps protect the body from high levels of ammonia in the blood. Thus, glutamine can act as a buffer, accepting excess ammonia, and then releasing it when needed to form other amino acids, amino sugars, nucleotides, and urea. The capacity to accept and donate nitrogen makes glutamine the major vehicle for nitrogen transfer between tissues.

Glutamine (Gln)



Glutamine is considered a non-essential (dispensable) amino acid, as most animal cells can readily synthesize it. It is synthesized via activity of the enzyme glutamine synthetase, which is found in high concentration in skeletal muscle, liver, intestine, brain and stomach tissue. It has long been thought that glutamine was not a necessary component of the diet, because of the body's capability to synthesize glutamine, and because of the high relative amount of glutamine in the body compared to other amino acids. In fact, approximately five to ten grams per day of glutamine is consumed in the standard human diet, and under normal circumstances dietary

intake and internal synthesis of glutamine is adequate and balanced with demand. In situations, where a particular tissue has a greater need for glutamine, inter-organ transfer for glutamine usually makes up for the increase in site-specific requirements (Calder, 1994). However, under certain pathological circumstances, such as infection and trauma, certain body tissues may need more glutamine than the amount supplied by the diet and de novo synthesis. During catabolic stress, for instance, intracellular glutamine levels can drop by more than 50 percent, and plasma concentration may fall by 30 percent (Askanazi et al., 1980). It is under these circumstances that supplemental glutamine may become necessary and glutamine is considered an essential amino acid (Newsholme, 2001; Souba, 1992).

Skeletal muscle contains the greatest intracellular concentration of glutamine, comprising up to 60 percent of the total free amino acid pool and has a concentration of approximately 20 mM/g of skeletal muscle in humans (Bergstrom et al., 1974; Lund, 1981). Skeletal muscle is considered the primary storage depot of glutamine and the primary exporter of glutamine to other tissues. Once released from skeletal muscle, glutamine acts as an inter-organ nitrogen transporter (Lund and Williamson, 1985; Newsholme et al., 1989). In times of metabolic stress, glutamine is released from skeletal muscle into circulation, where it is transported to the tissue in need (Calder, 1994). Intracellular skeletal muscle glutamine concentration is affected by various insults, including injury, sepsis, prolonged stress, starvation, and the use of glucocorticoids. Besides skeletal muscle, the lungs are the next largest producer of glutamine.

II. Overview of Glutamine Metabolism

The physiological importance of the amino acid L-glutamine for promoting and maintaining cell function is now widely accepted. The importance of glutamine to cell survival

and proliferation *in vitro* was first reported by Ehrensvar et al. (1949), but was more fully described by Eagle et al. (1966). Glutamine had to be present in 10- to 100-fold excess of any other amino acid in culture and could not be replaced by glutamic acid or glucose for the proliferation of lymphocytes to occur. This work led to the development of the first tissue culture medium that contained essential growth factors, glucose, 19 essential and nonessential amino acids at approximately physiological concentrations, and a high concentration of glutamine (2 mmol/l). It is now known that a large number of tissues and cells in the body utilize glutamine at high rates and that glutamine utilization is essential for their function. These tissues and cells include kidney, intestine, liver, specific neurons in the central nervous system (CNS), cells of the immune system, and pancreatic β -cells (Young and Ajami, 2001; Curi, 2000).

L-glutamine is an important precursor for peptide and protein synthesis, amino sugar synthesis, purine and pyrimidine and thus nucleic acid and nucleotide synthesis, and it also provides a source of carbons for oxidation in some cells. However, the immediate product of glutamine metabolism in most cells is L-glutamate, which is produced by the action of glutaminase, an enzyme found at high concentrations and associated with the mitochondria in cells which readily utilize glutamine. L-glutamate is the most abundant intracellular amino acid (reported concentrations vary between 2 and 20 mM) and is also the most abundant extracellular amino acid *in vivo* (0.7 mM compared to an approximate L-glutamate concentration of 20 μ M). L-glutamate cannot readily cross cell membranes because it has an overall charge of -1 at pH 7.4 and amino acid transporters capable of transporting glutamate into the cell are present at low density in the plasma membrane with the exception of specialized glutamate-metabolizing cells located in the CNS (Matthews and Anderson, 2002). L-glutamate appears to be at the crossroads of amino acid metabolism, where it can donate its amino group for new amino acid synthesis

(transamination) or can lose the amino group, as NH_4^+ , via deamination to α -ketoglutarate. In some tissues and cells such as liver, skeletal muscle or astrocytes, glutamate and NH_4^+ may be combined by the action of glutamine synthetase to produce glutamine. This glutamine is then exported from the cell.

a. *Glutamine/glutamate in the kidney*

Glutamine is quantitatively the most important donor of NH_3 in the kidney. The NH_3 is cleaved from glutamine by the action of phosphate-dependent glutaminase, which is subjected to pH regulation (Gstrunthaler et al., 2000). Ammonia (NH_3) is exported to the lumen of the collecting tubule where it combines with exported H^+ to form NH_4^+ , which is lost in the urine. Hydrogen (H^+) is created from carbonic acid, which dissociates to form HCO_3^- and H^+ . Bicarbonate (HCO_3^-) subsequently enters the circulation where it is important for the maintenance of blood pH. Therefore, glutamine metabolism in the kidney is essential for acid-base buffering in the plasma (Gstrunthaler et al., 2000; Curthoys and Gstrunthaler, 2001). The carbon skeleton of glutamate in the kidney, created by the action of glutaminase, is converted via formation of α -ketoglutarate, succinate, fumarate, malate and oxaloacetate to phosphoenolpyruvate (or malate to pyruvate directly) and then participates in gluconeogenesis. Glucose produced by this pathway provides up to 25 percent of circulating plasma glucose *in vivo* (Stumvoll et al., 1999). Renal gluconeogenesis is especially important in conditions where the blood concentration of ketone bodies increases, causing acidosis. This occurs, for instance, during long periods of hypoglycemia or diabetes. Hepatic gluconeogenesis from amino acids (mainly alanine) is gradually replaced by renal gluconeogenesis. Under these conditions, glucose produced by the kidney can account for up to 50 percent of circulating plasma glucose (Owen et al., 1969).

b. Glutamine/glutamate in the intestine

Glutamine is quantitatively the most important fuel for intestinal tissue. It is metabolized to glutamate by phosphate-dependent glutaminase. Glutamate undergoes transamination with pyruvate generating L-alanine and α -ketoglutarate. The latter metabolite is then oxidized in the tricarboxylic acid (TCA) cycle generating malate, which, by the action of NADP⁺-dependent malic enzyme, generates pyruvate. The NADH and FADH₂ generated via this pathway are used for electron donation to the electron transporting chain in the mitochondria and thus promote ATP synthesis. The L-alanine produced in this pathway is exported to the hepatic portal vein for transport to the liver (Kimura et al., 1988).

Glutamine is recognized as an important dietary component for the maintenance of gut integrity (Neu et al., 2002) and reduces the degree of derangement induced by mechanical intestinal obstruction (Chang et al., 2001). As a result, glutamine administration reduces bacterial translocation (Erbil et al., 1999) and is beneficial to the critically ill and other patients (Newsholme et al., 1987; Boelens et al., 2001). In fact, glutamine has been shown to improve various aspects of nutritional care in medical patients with gastrointestinal disease or cancer, burn victims, postsurgical patients, and low birth weight neonates (Savy, 2000; Gismondo et al., 1998; Neu, 2001). This amino acid also normalizes the AIDS-associated increase in intestinal permeability (Thomson et al., 2001).

c. Glutamine/glutamate in the liver

The liver is the central site for nitrogen metabolism in the body (Haussinger, 1986). Nitrogen is transported from peripheral tissues (principally from muscle and lung) to the central organs as glutamine, plus alanine and aspartate if the glutamine is taken up and metabolized by the intestine (Young and Ajami, 2001). Glutamine can be cleaved by glutaminase to yield

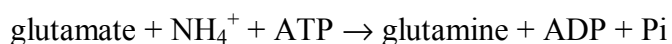
glutamate and NH_3 . The mitochondrial carbamoyl-phosphate synthetase I (CPS I) can then catalyze the following reaction:



The enzyme is allosterically activated by N-acetylglutamate and thus is indirectly regulated by glutamate concentration. Avians lack CPS I.

Glutamine metabolism is partitioned within the liver, where glutamine is taken up by the periportal cells of the liver in which there is a relatively high glutaminase activity (Curthoys and Watford, 1995; Haussinger, 1990). Glutamate that has been produced in the periportal cells may be further metabolized to produce other amino acids by transamination or may enter the TCA cycle as an anaplerotic substrate, which is a substrate that replenishes the intermediates of the TCA cycle or it may enter the pathway of gluconeogenesis via formation of phosphoenolpyruvate from oxaloacetate. Thus, gluconeogenesis from glutamine may be a major consumer of glutamate-derived carbon in the liver, resulting in the formation and export of glucose (De-Souza et al., 2001).

Glutamine formation and release from the liver occurs mainly in the perivenous region. The hepatocytes in this area are rich in glutamine synthetase (De-Souza et al., 2001). The substrate(s) for glutamine synthesis are glutamate and NH_4^+ . Glutamate may be produced via glucose conversion to α -ketoglutarate and subsequent conversion to glutamate via glutamate dehydrogenase. However, recent data have suggested that arginine catabolism may provide glutamate for the glutamine synthetase reaction (O'Sullivan et al., 1998). The glutamine synthetase reaction is energy requiring and is described below:



Liver glutamine metabolism plays an important role in controlling ammonia levels in venous blood. The synthesis and hydrolysis of glutamine are intermediate steps in urea formation, since the K_M of CPS for ammonia is high (2 mM), which means that CPS has a low affinity for NH_3 , whereas glutamine synthase K_M for ammonia is much lower (0.3 mM). Thus, the liver first removes ammonia present in low concentrations in the blood to form glutamine, which passes through the circulation and reaches the organ again. The key enzymes of urea formation are present in higher amounts in periportal and proximal perivenous hepatocytes, whereas glutamine synthase occurs only in distal perivenous hepatocytes (Gebhardt and Mecke, 1983; Haussinger, 1986; Jungermann and Katz, 1989).

d. Glutamine/glutamate in the central nervous system

The major transmitter at excitatory synapses in the central nervous system (CNS) is glutamate, whereas inhibitory signals are carried by γ -amino butyric acid (GABA) (Raol et al., 2001; Fontana, 2001). The existence of a glutamine/glutamate cycle in the CNS has recently been confirmed (Behar and Rothman, 2001). Glutamine is synthesized from glutamate in the astrocytes, which are nerve cells, so as to return the glutamate that is removed from the synaptic cleft after release from the presynaptic neuron. The neuron will readily convert the astrocyte-derived glutamine to glutamate via glutaminase, to complete the cycle. The cycle is energy dependent since ATP is consumed in the synthesis of glutamine from glutamate. In the human cortex the cycle appears to account for 80% of the energy derived from glucose oxidation (Rothman et al., 1999; Shulman and Rothman, 1998).

e. Glutamine/glutamate in the pancreatic β -cell

Glutamine has been reported to enhance glucose- or leucine-stimulated insulin secretion from pancreatic β -cells (located in the endocrine islets of Langerhans) (Gao et al., 1999;

Tanizawa et al., 2002). Glutamine may act as an anaplerotic substrate in the β -cell, via formation of glutamate and α -ketoglutarate, subsequently stimulating a catalytic enhancement of glucose oxidation (Meglasson et al., 1987). Glutamate is also important in the β -cell as a substrate for the enzyme glutamic acid decarboxylase, which produces the signaling molecule GABA (Rubi et al., 2001). Gamma- amino butyric acid (GABA) production and secretion may be important for the regulation of insulin secretion in intact islets of Langerhans (Winnock et al., 2002). Nutrient metabolism is intimately connected with the process of insulin secretion from the pancreatic β -cell.

f. *Glutamine in skeletal muscle*

Muscle tissue is a major site for glutamine synthesis in the human body and contains over 90 percent of the whole-body glutamine pool. Quantitative studies in humans have demonstrated that, in the postabsorptive state, 60 percent of the amino acids released comprise alanine plus glutamine (Newsholme et al., 1999; Felig, 1975; Newsholme and Parry-Billings, 1990). In resting muscle, six amino acids are metabolized: leucine, isoleucine, valine, asparagine, aspartate and glutamate (Wagenmakers, 1998). These amino acids provide the amino groups and probably the ammonia required for synthesis of glutamine and alanine, which are released in excessive amounts in the postabsorptive state and during ingestion of a protein-containing meal. The release of glutamine from skeletal muscle is also stimulated during stress conditions such as injury and burns (Newsholme et al., 1988; Hammarqvist et al., 2001). Only leucine and isoleucine molecules can be oxidized in muscle after being converted to acetyl-CoA. The other carbon skeletons are used for *de novo* synthesis of TCA cycle intermediates and glutamine. The rate of TCA cycle flux and oxidative metabolism is limited by the concentration of the TCA cycle intermediates.

III. Glutamine and the Gastrointestinal Tract

The gastrointestinal tract has the greatest demand for glutamine in the body (Miller, 1999). The small intestine accounts for the largest uptake of glutamine of any organ, absorbing glutamine from the lumen of the gut as well as from the bloodstream. Epithelial cells lining the small intestine (enterocytes) use glutamine as their principal metabolic fuel (Andrews and Griffiths, 2002). Glutamine depletion can slow enterocyte growth, while adequate glutamine can stimulate growth and cellular proliferation *in vitro* (Newsholme et al., 1989). Glutamine is converted in the mitochondria of intestinal cells to glutamate, then α -ketoglutarate, which is utilized in the Krebs cycle for ATP production (Newsholme et al., 1989). Colonocytes (epithelial cells in the colon) also utilize glutamine; however, they prefer short-chain fatty acids as their primary fuel. Since enterocytes have little glutamine synthetase activity and a great amount of glutaminase, which metabolizes glutamine, they are dependent on a supply of pre-formed glutamine (Ardawi and Newsholme, 1983).

a. Intestinal Morphology

The small intestine mucosa is comprised of a single cell thickness of mostly columnar epithelial cells, with endocrine cells, mucin cells, and Paneth cells interspersed between them. The absorptive capacity of the small intestine is greatly increased by the presence of villi, with corresponding crypts between them (Miller, 1999). In swine, glutamine supplemented at a 1% level in the diet fed for 21 days increased the length of the intestinal villi and reduced atrophy of intestinal villi in weanling pigs (Kitt et al., 2002). Glutamine supplementation has also increased the height of the intestinal villi, but decreased crypt depth in turkey poults (Yi et al., 2001b).

The most mature cells in the small intestine occupy the tip of the villi, while immature cells are at the base of the villi, in the crypts. The immature cells proliferate and migrate to the

tip, where they mature, then are reabsorbed or sloughed off into the lumen. In general, in most animals the entire process takes only three to six days. This high rate of proliferation and turnover is usually well-regulated by nutrient availability, gastrin, growth hormone, bacterial flora, and neuro-regulatory activity. However, the presence of food passing through the gastrointestinal tract seems to be the primary stimulus for regulation of this proliferative response, as it can affect all of the aforementioned regulatory systems (Wilmore, 1997). After seven days of fasting, even with the use of total parenteral nutrition (TPN), gut mass can be reduced by as much as 50 percent (Souba et al., 1988; Souba, 1988). Commercially available TPN solutions, commonly used in trauma and surgical patients, do not contain glutamine. But the supplementation of glutamine in TPN solutions has limited this reduction in gut mass as compared to rats fed a standard TPN solution (with no Gln added) (O'Dwyer et al., 1989).

b. Intestinal Permeability

Most of the research on glutamine and its connection to intestinal permeability has been conducted in conjunction with the use of TPN. Commercially- available TPN solutions, that do not contain glutamine, lead to atrophy in small intestine mucosal villous. In an animal study using Rhesus monkeys, marmosets, rabbits, and rats, glutaminase parenteral infusion significantly decreased glutamine concentration in the bloodstream, accompanied by diarrhea, villous atrophy, mucosal ulceration, and intestinal necrosis (Bakerville et al., 1980). This study emphasizes the importance of glutamine to the integrity of the small intestine, and specifically the need for glutamine uptake from the bloodstream as well as the intestinal lumen. Mucosal atrophy occurred in animals while on TPN and the addition of glutamine to the TPN solution given IV at a level of 2 g Gln/100 mL in Wistar rats, reversed the mucosal atrophy (O'Dwyer et al., 1989). Others have noted similar results of decreased villous atrophy, increased jejunal

weight, and decreased intestinal permeability with the use of glutamine-enriched TPN solutions in surgical patients (Hwang et al., 1987), in Fisher rats (Grant, 1988), in rats with Gln at a 2% level (Barber et al., 1990), in female rats at a 2% level (Li et al., 1994), and in rats receiving 3% alanyl-Gln supplemented TPN (Khan et al., 1999).

One potential consequence of increased intestinal permeability, which is a breakdown in the maintenance between the intestinal enterocytes' tight junctions, is microbial translocation. The derangements in the intestinal mucosa associated with immunosuppression, lack of enteral feedings, trauma, infection, injury, starvation, chemotherapy and other stressors are all associated with the breakdown in the barrier function of the gut (Wilmore et al., 1988). Bacteria, fungi, and their toxins can subsequently translocate across the mucosal barrier into the bloodstream and react with the reticuloendothelial system, which causes infection (Wilmore et al., 1988). Cytokines produced from this reaction stimulate the hypothalamic-pituitary-adrenal axis, resulting in cortisol release from the adrenals (Souba, 1992; Wilmore et al., 1988). Cortisol increases glutaminase activity in intestinal enterocytes, stimulating increased breakdown and utilization of glutamine in the small intestine for gut repair (Wilmore et al., 1988). Cortisol also causes increased proteolysis in other tissues, and release of glutamine from skeletal muscle (Souba, 1992). Although this adaptation response provides metabolic assistance to help heal hyperpermeable gut tissue, severe damage to the mucosa or other tissue utilizing glutamine for healing, or prolonged stress can deplete skeletal muscle glutamine and consequently enterocytes (which are using more glutamine in their stress state) of their vital supply of glutamine, thus causing villus atrophy and a reduction in nutrient absorption and utilization (Souba, 1992; Wilmore et al., 1988). In numerous animal studies, the addition of glutamine or glutamine dipeptides (stable dipeptides of glutamine with alanine or glycine) in experimentally-induced

intestinal hyperpermeability (via radiation, sepsis, chemotherapy, injury, or infection) improves gut barrier function measured by the lactulose:mannitol ratio, as well as immune activity in the gut measured by reduced bacterial counts (Souba et al., 1990; Gianotti et al., 1995; Bai et al., 1996; Pangrahi et al., 1997; Chun et al., 1997; Foitzik et al., 1997). Another consequence of skeletal muscle glutamine depletion is the subsequent depletion of the glutamine-containing tripeptide antioxidant glutathione, which may lead to oxidative damage of the muscle (Hammarqvist et al., 1997).

It is known that glutathione production occurs in the liver; however, supplemental glutamine has been shown to increase gut glutathione production threefold (Cao et al., 1998). Oral glutamine supplementation may increase intestinal glutathione synthetase activity, which can improve antioxidant activity in the gut, as well as augment natural killer (NK) cell activity, which helps eliminate free radicals that can potentially cause infection, thus maintaining the gut barrier function and reducing intestinal permeability (Calder, 1995).

IV. Glutamine and Immune Cells

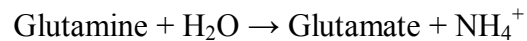
a. Glutamine in cells of the immune system

It is now widely accepted that glutamine is utilized at high rates by isolated cells of the immune system such as lymphocytes, macrophages and neutrophils (Ardawi and Newsholme, 1983; Newsholme et al., 1986; Curi et al., 1997). Although the activity of the first enzyme responsible for the metabolism of glutamine, phosphate-dependent glutaminase, is high in these cells, the rate of oxidation is low. Much of the glutamine is converted to glutamate, aspartate (via TCA cycle activity), lactate, and under appropriate conditions, CO₂. Glutamine has been reported to enhance many functional parameters of immune cells such as T-cell proliferation, B-lymphocyte differentiation, macrophage phagocytosis, antigen presentation and cytokine

production (Newsholme, 2001; Wells et al., 1999; Kew et al., 1999; Yeh et al., 2001; Moinard et al., 1999), plus neutrophil superoxide production and apoptosis (Garcia et al., 1999; Pithon-Curi et al., 2002).

b. *Glutamine metabolism by the immune cells*

The first enzyme in the pathway of glutamine utilization is glutaminase:



The activity of glutaminase is high in all lymphoid organs examined including lymph nodes, spleen, thymus, Peyer's patches, bone marrow (Ardawi and Newsholme, 1985), and from human peripheral blood (Ardawi, 1988; Keast and Newsholme, 1990), in macrophages isolated from the mouse peritoneal cavity (Newsholme et al., 1986) and in rat neutrophils (Curi et al., 1997).

Glutaminase activity increases in the popliteal (located in the leg) lymph node at rest and in response to an immunological challenge (Ardawi and Newsholme, 1982). This increase was also viewed in mesenteric lymph nodes (Ardawi and Newsholme, 1983). Consistent with the high activity of glutaminase, glutamine is utilized at a high rate by cultured resting lymphocytes (Brand, 1985; Ardawi and Newsholme, 1983; Ardawi, 1988; Brand et al., 1989; O'Rourke and Rider, 1989), macrophages (Newsholme et al., 1987; Newsholme and Newsholme, 1988) and neutrophils (Curi et al., 1997). Mitogenic stimulation of lymphocytes by concanavalin A, a T-cell stimulant, increases both glutaminase activity (Brand, 1985) and the rate of glutamine utilization (Brand, 1985; Ardawi and Newsholme, 1983; Ardawi, 1988; O'Rourke and Rider, 1989) in humans and rats. The major products of glutamine utilization by cultured lymphocytes and macrophages are glutamate, aspartate, lactate, and ammonia, although alanine, lactate and pyruvate are also produced and some glutamine ($\leq 25\%$) is completely oxidized to energy (Brand, 1985; Ardawi and Newsholme, 1983; Newsholme et al., 1987; Ardawi, 1988; Brand et al., 1989;

O'Rourke and Rider, 1989; Newsholme et al., 1989). Although pre-surgery the porcine spleen released glutamine, there was net glutamine uptake by the spleen post-surgery which was accompanied by a 7-fold increase in ammonia release (Deutz et al., 1992). Thus, when the immune system is challenged (i.e. by surgery) at least one lymphoid organ (i.e. the spleen) dramatically increases its utilization of glutamine leading to the conclusion that the available Gln is insufficient for that organ in such cases and can lead to a deficiency in Gln and become conditionally essential in such conditions (Newsholme, 2001).

c. Regulation of Immune Cell Functions

The high rate of glutamine utilization by lymphocytes and macrophages and its increase when these cells are challenged suggests that the provision of glutamine might be important to the function of these cells and thus the body's ability to mount an efficient immune response (Calder, 1995). Thirty years ago it was reported that the addition of asparaginase or glutaminase to cultures of lymphocytes prevented the cells from proliferating (Hirsch, 1970; Simberkoff and Thomas, 1970). Furthermore, oral asparaginase treatment in mice and rats leads to immunosuppression (Brambilla et al., 1970; Chakrabaty and Friedman, 1970; Ashworth and MacLennan, 1974; Kafkewitz and Bendich, 1983). The immunosuppression effect of asparaginase was shown to be due to its decrease in ability to hydrolyse glutamine and thus decreasing its availability to the immune system (Ashworth and MacLennan, 1974; Durden and Distasio, 1981; Kafkewitz and Bendich, 1983). These observations suggest that an adequate supply of glutamine is required for the immune system to function optimally.

d. Influence on T-cell Proliferation

Lymphocyte proliferation is the process of cell division in response to a mitogenic stimulus. *In vivo*, the mitogenic stimulus of T-lymphocyte proliferation is most likely to be the

presentation of a processed antigen by an antigen presenting cell. *In vitro* T-lymphocyte can be stimulated to proliferate by using a variety of agents including the mitogens concanavalin A (Con A) and phytohaemagglutinin (PHA). Most commonly lymphocyte proliferation is measured as the incorporation of a radioactively-labeled precursor (i.e. thymidine) into DNA. The proliferative response of rat (Ardawi and Newsholme, 1983; Szondy and Newsholme, 1989), mouse (Griffiths and Keast, 1990; Yaqoob and Calder, 1997) and human (Parry-Billings et al., 1990; Chuang et al., 1990) lymphocytes to T-cell mitogens is dependent upon the availability of glutamine. In the absence of glutamine these cells do not proliferate, but as the glutamine concentration in the culture medium increases lymphocyte proliferation increases. Lymphocyte proliferation increases greatly over the glutamine concentration range between 0.01 and 1 mM and appears to be maximal at normal physiological concentrations. Other amino acids, including glutamate, aspartate and arginine, cannot be substituted for glutamine to support lymphocyte proliferation (Ardawi and Newsholme, 1983; Calder, 1995). However, dipeptides which contain glutamine can act as a replacement for glutamine to support *in vitro* T-lymphocyte proliferation (Brand et al., 1989; Kweon et al., 1991). In response to stimulation of T-cells, there is enhanced transcription of genes for various cytokines and cytokine receptors; among these interleukin-2 (IL-2) and its receptor appear to be particularly important for T-lymphocyte proliferation and for T-lymphocyte-mediated regulation of the activity of other cells of the immune system (macrophages, natural killer cells, and B-lymphocytes) (Calder, 1995). The continued synthesis and secretion of IL-2 are required if activated T-cells are to proliferate (Smith, 1988). As the availability of glutamine increased IL-2 production was enhanced by Con A-stimulated rat (Calder and Newsholme, 1992), mouse (Yaqoob and Calder, 1997) and human (Rohde et al., 1996; Yaqoob and Calder, 1998) lymphocytes. Yaqoob and Calder (1997)

reported that the proportion of CD4⁺ (helper T-cells) lymphocytes increased with increasing concentration of glutamine in the culture medium. These observations suggest that Gln is required for maintenance of the immune system.

e. Influence on Cytokine Production

Despite the large number of *in vitro* studies illustrating the immunoenhancing effect of glutamine, there are relatively few studies evaluating the effectiveness of dietary glutamine *in vivo*. Three animal studies have now reported that enrichment of the diet with glutamine increases *in vivo* T-lymphocyte proliferation (Shewchuk et al., 1997; Yoo et al., 1997; Kew et al., 1999). Shewchuk et al. (1997) reported that Con A-stimulated proliferation of spleen lymphocytes taken from tumor-bearing rats fed diets containing 257g casein plus 20g glutamine/kg was greater than that of those taken from rats fed 257g casein/kg with no added Gln. In a more recent study, spleen lymphocytes from mice fed for two weeks on a diet containing 54.8g glutamine/kg had more proliferation in response to Con A (a T-lymphocyte proliferation stimulant) than those from mice fed on a diet containing 19.6g glutamine/kg (Kew et al., 1999); the glutamine-enriched diet also increased the proportion of CD4⁺ lymphocytes in the spleen and increased the proportion of stimulated lymphocytes bearing the IL-2 receptor.

Until recently there has been little information about the effect of dietary glutamine on cytokine production. Kew et al. (1999) reported two studies that investigated the effect of increasing the dietary supply of glutamine on *in vivo* production of cytokines by murine macrophages and lymphocytes, respectively. Mice were fed for two weeks on a diet which included 200g casein/kg providing 19.6g glutamine/kg, or a glutamine-enriched diet which provided 54.8g glutamine/kg partly at the expense of casein. The production of all three cytokines investigated (TNF- α , IL-1 β , and IL-6) was greater for LPS-stimulated macrophages

from mice fed the glutamine-enriched diet (Wells et al., 1999). Interleukin-2 production was also significantly greater for Con-A-stimulated spleen lymphocytes from mice fed the glutamine-enriched diet (Kew et al., 1999). These two studies suggest that increasing the amount of glutamine in the murine diet enhances the ability of both macrophages and T-lymphocytes to respond to an immune stimulation, at least in terms of cytokine production. These observations suggest that increasing the oral availability of glutamine could promote immune responses involving macrophage- or T-cell-derived cytokines.

f. Influence on B-lymphocyte Differentiation

The differentiation of B-lymphocytes into antibody synthesizing cells *in vitro* is glutamine dependent and increases greatly over the physiological range of glutamine concentrations (Crawford and Cohen, 1985). Since the expression of IgG (Tsiagbe et al., 1987) and IgA (Muir et al., 2000) is T- helper cell dependent and glutamine increases the number of helper T-cells, then glutamine should increase the concentration of IgG and IgA *in vivo*. Burke et al. (1989) reported that by adding Gln to TPN solutions in rats higher IgA levels were maintained than the other treatment groups that were not supplemented with Gln. IgA is a good marker of intestinal barrier function (Muir et al., 2000) and since glutamine has been shown to increase IgA expression, Gln supplementation should improve barrier function (Burke et al., 1989). The digestive mucosa is continuously exposed to dietary, bacterial, viral, and parasitic antigens (Strobel, 1986). Specific protection against these antigens is achieved mainly by the secretion of IgA, which is synthesized in the gut-associated lymphoid tissue (Piquer, 1990). The increase in IgA concentrations have been related to the increase in the number of lymphoid cells observed in the gallbladder (Leslie et al., 1976) of chickens and small intestine (Piquer, 1990) of turkeys. This suggests that the effect of Gln on the preservation of gut mass includes intestinal

lymphoid tissue as well. IgA functions primarily by preventing the attachment of bacterial to the mucosal cell (Burke et al., 1989). The barrier function of the gut epithelium depends on the presence of IgA, and until IgA is present, the hatchling is more susceptible to oral pathogens (Sell, 1991). These effects of glutamine action cannot be mimicked by glutamate or asparagine (Crawford and Cohen, 1985).

g. Effect on Immune Function and Survival of Infection and Trauma Animal Models

Increased intestinal permeability (disruption in the tight junction between enterocytes) has been associated with infection and trauma from burns (Ziegler et al., 1988), surgery (Roumen et al., 1993), shock (Roumen et al., 1993; Vinnars et al., 1975), and other forms of physical trauma (Houdijk et al., 1998). Injury of any tissue besides the intestines shunts glutamine out of the blood and into the tissues, making less glutamine available for the gut. Glucocorticoid release in acute stress, including surgery, infection, trauma, burns, sepsis, or other severe illness, causes accelerated protein breakdown. Thus, these patients can quickly lose significant amounts of protein, much of it from skeletal muscle, in an attempt to provide adequate amounts of glutamine for tissue healing and maintenance of gut integrity. As glutamine is depleted, wound healing is impaired, intestinal permeability increases, and the risk of microbial translocation, sepsis, and multiple organ failure increases significantly (Biolo et al., 1997; Wilmore, 1991). Maintenance of immune function and gut barrier function following trauma is important, as infection is a major cause of morbidity and mortality in severe trauma cases. It is thought that these infections often are a result of gut hyperpermeability and bacterial translocation to systematic circulation (Newsholme et al., 1988). A recent study conducted by Houdijk et al. (1998) illustrates the need for glutamine supplementation in these patients. Sixty patients with multiple traumas were given glutamine-containing enteral nutrition for at least five

days following their injuries, with dramatic results. Incidence of pneumonia was 17% in the glutamine group, compared to 45% in the control group ($p < 0.02$). Bacteremia occurred in 7% of the glutamine group and 42% of the control group, and only 1 patient (3%) in the glutamine group had sepsis, compared to 8 of the controls (26%). The researchers noted the majority of bacteremia and sepsis cases in the control group involved gram-negative bacteria, while none of the bacteremia and sepsis cases in the glutamine group involved these organisms, which lead them to believe glutamine may have prevented bacterial translocation from the gut (Houdijk et al., 1998). It was also suggested enteral glutamine dosing provided better protection than TPN in trauma patients, with a lower incidence of infections. This has been confirmed by other studies (Moore et al., 1989; Kudsk et al., 1992).

A number of animal studies have been performed to investigate the effect of glutamine supplementation on the body's ability to respond to infection. Glutamine-supplemented parenteral nutrition improved the survival rate (75% vs. 25% in the control group receiving standard parenteral nutrition) in rats following caecal ligation and puncture (Ardawi, 1991). Likewise, intravenous glutamine improved survival rates (92% vs. 55% in the control group) following an intraperitoneal injection of live *Escherichia coli* into rats (Inoue et al., 1993). Parenteral administration of alanyl-glutamine into rats improved survival (86% vs. 44% in the control group) in response to intraperitoneally-infused *E. coli* (Naka, 1996). Suzuki et al. (1993) fed mice for 10 days on diets containing casein or casein supplemented with 20g or 40g glutamine/kg and then inoculated them intravenously with live *Staphylococcus aureus*. Over the following 20 days, during which the mice were maintained on the same diets they had been fed prior to infection, 80% of the control animals died, while mortality was 60% in the 20g glutamine/kg group and 30% in the 40g glutamine/kg group. In addition to enhanced survival,

these studies have shown that glutamine improved nitrogen balance, diminished the sepsis-induced decrease in muscle glutamine concentration, and decreased muscle protein breakdown (Ardawi, 1991), increased plasma glutamine concentration (Inoue et al., 1993), increased intestinal function and/or integrity (Inoue et al., 1993; Naka, 1996), and enhanced muscle protein synthesis (Ardawi, 1991; Naka, 1996). These studies did not measure indices of immune function. However, Yoo et al. (1997) found that proliferation of blood lymphocytes from *E. coli*-infected piglets was significantly higher if the piglets consumed a diet containing 40g glutamine/kg compared with a diet which did not contain supplemental glutamine. Furthermore, infusion of alanyl-glutamine into tumor-bearing rats increased the *in vitro* phagocytic capacity of alveolar macrophages (Kweon et al., 1991), while infusion into septic rats increased *in vitro* proliferation of mitogen-stimulated blood lymphocytes (Yoshida et al., 1992). Glutamine or alanyl-glutamine provided parenterally maintained intestinal integrity in mice given an intranasal inoculation of influenza virus (Li et al., 1998). In an animal model of haemorrhagic shock, standard parenteral nutrition decreased the *in vivo* release of TNF- α and IL-6 by LPS-stimulated gut mononuclear cells and spleen macrophages and was associated with injury to the gut mucosa and bacterial translocation into the mesenteric lymph nodes (Schroder et al., 1998). Inclusion of alanyl-glutamine and glycyl-glutamine in the parenteral regimen improved mucosal structure and prevented the fall in *in vivo* IL-6, but not TNF- α , release (Schroder et al., 1998). These studies indicate that provision of glutamine either parenterally or enterally increases the function of various immune cells and that this might account for the enhanced resistance to infection observed in other studies.

V. Effect of Glutamine on Performance in Animal Models

a. Feed Efficiency

Glutamine supplementation has been shown to increase feed efficiency in animals. Yi et al. (2001b) reported that supplementing the diet with 1% Gln improved feed efficiency (body weight gain:feed intake) of turkey poults the first week after hatch as compared to poults fed a standard corn-soybean meal (SBM) diet. Kitt et al. (2002) reported that the addition of 1% Gln to the diet improved feed efficiency in weanling pigs. Improvements in feed efficiency had been noted in broilers (Yi *et al.*, 2001a) when they were fed a diet supplemented with 1% Gln.

b. Growth

The use of dietary glutamine supplementation on growth performance in different species of animals is ambiguous. Improvements in weight gain have not been reported in swine (Kitt, 2002). Yi et al. (2001b) did report an improvement in body weight gain in turkey poults fed diets supplemented with 1% Gln in the feed, but it was only noted for the first week of age. In fibrosarcoma tumor bearing rats, glutamine supplementation eliminated the weight depression viewed in the other non-glutamine supplemented rats (Fahr et al., 1994). In chickens challenged with coccidiosis, chicks supplemented with 1% Gln had higher body weight gains than those not supplemented with glutamine (Yi et al., 2005).

VI. Nucleotides

The breakdown of glutamine into nucleotides may be the method or mode by which glutamine effects the gastrointestinal tract and immune system. Nucleotides are the building blocks for nucleic acids (DNA and RNA) and are molecules with considerable structural diversity. They are composed of a nitrogenous base linked to a pentose sugar to which at least one phosphate group is attached. Feed or food ingredients containing cellular elements are potential sources of nucleotides. Synthesizing nucleotides *de novo* is energetically costly and requires glutamine. However, nucleotides also have physiological roles in the body such as

being a source of energy (ATP and GTP), cofactors in oxidation and reduction reactions (FAD, NAD⁺, and NADP⁺), serve as physiological regulators (cAMP and cGMP), and carry activated intermediates (UDP-glucose, CMP-sialic acid, and CDP-choline) and acyl groups (CoA). Since nucleotides are one of the end products of glutamine metabolism, they can be substituted for glutamine and still obtain some of the same benefits in intestinal and immune responses that are observed with glutamine supplementation (Domeneghini et al., 2004; McCauley et al., 1998; Yu et al., 2002). Nucleotides have been shown to influence intestinal development and integrity and the development of the immune system. During periods of rapid growth and development, disease challenges, injury, or stress, dietary nucleotide supplementation may be beneficial because of the role of nucleotides in developing and enhancing immunity, maintaining intestinal health, and preserving energy.

a. Effects of nucleotides on the intestinal tract

Dietary nucleotides may enhance the growth and maturation of intestinal epithelial cells as evidenced by an increase formation of mucosal protein, DNA, longer small intestinal villi and increased maltase to lactase enzyme ratio in weanling mice (Carver, 1994) and infants (Uauy et al., 1990). Dietary nucleotides may also stimulate enterocyte differentiation (Sanderson and Youping, 1994). Parenteral supplementation of nucleic acids in rats supports mucosal cell proliferation and function, as demonstrated by increased mucosal weights, protein and DNA contents, villous height, and narrower tight junctions in the jejunum (Kishibuchi et al., 1997; Tsujinaka et al., 1999).

b. Effects of nucleotides on the immune system

Dietary nucleotide supplementation has been associated with both humoral and cellular immunity, but the exact mechanism has not been identified. They contribute to the circulating

pool of nucleosides available to stimulate leukocyte production (Kulkarni et al., 1994; Carver and Walker, 1995). Therefore, there is an elevated need for nucleotides during periods of immunological challenges. Infants fed milk formula fortified with nucleotides had better responses to immunization and higher humoral antibody responses (Fanslow et al., 1988; Pickering et al., 1998) and increased cytokine production (Carver et al., 1991). Similar responses to nucleotide supplementation were reported from *in vivo* experiments with mice (Jyonouchi et al., 1993; Jyonouchi, 1994). Dietary supplementation of purified nucleotides to milk replacers of newborn bull calves challenged with LPS resulted in calves having higher mean IgG levels compared to the unsupplemented control calves (Oliver et al., 2002). Nucleotide supplementation also increased lymphocyte stimulation to PHA and ConA challenges in weanling piglets (Zomborsky-Kovacs et al., 1998).

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CHAPTER 3

THE EFFECT OF SUPPLEMENTAL GLUTAMINE ON GROWTH PERFORMANCE, DEVELOPMENT OF THE GASTROINTESTINAL TRACT, AND HUMORAL IMMUNE RESPONSE OF BROILERS¹

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ABSTRACT Two experiments were conducted to evaluate the effect of supplemental glutamine (Gln) on growth performance, development of the gastrointestinal tract and humoral immune response of broilers. Immediately after hatch six replicate pens of six chicks were randomly assigned to one of seven (Experiment 1) or five (Experiment 2) dietary treatments for 21 d. On Day 4, 7, 14, and 21 twelve chicks per treatment were sacrificed for thymus, spleen, bursa, duodenum, jejunum, ileum, bile, and blood sample collections and weights. In Experiment 1, the effect of 1 or 4% Gln addition to the feed, water, or both was compared to a corn-soybean meal (SBM) control diet. All diets were formulated to be isocaloric and isonitrogenous. Weight gain improved significantly ($P<0.05$) when chicks were fed diets with 1% Gln as compared to chicks fed the control diet (11% average improvement). The addition of 4% Gln to the diet or water depressed ($P<0.05$) growth performance. From Experiment 1, 1% Gln supplementation to the diet was determined to be ample and most practical. Thus in Experiment 2, diets supplemented with 1% Gln were fed for 4, 7, 14, or 21 d after which time chicks were fed the corn-SBM control diet until the experiment was terminated at 21 d. Weight gain improved significantly ($P<0.05$) when chicks were fed diets supplemented with 1% Gln through out the 21 d study. In both experiments, chicks fed diets supplemented with 1% Gln for 21 d had higher concentrations of bile, intestinal, and sera IgA and sera IgG ($P<0.05$). Chicks fed diets with 1% Gln had heavier intestinal relative weights and longer intestinal villi ($P<0.05$) as compared to the chicks fed the corn-SBM diet. Our results indicate that the addition of 1% Gln to the diet of broiler chicks improves growth performance and may stimulate development of the gastrointestinal tract and humoral immune response.

Key words: Glutamine, gastrointestinal tract, broiler chicks, immune response

INTRODUCTION

L-Glutamine (Gln) is the most prevalent amino acid in the bloodstream, accounting for 30 to 35 percent of the amino acid nitrogen in the plasma, and in the free amino acid pool in the body. Because Gln contains two ammonia groups, one from its precursor, glutamate, and the other from free ammonia in the bloodstream, Gln acts as a “nitrogen shuttle,” which helps protect the body from high levels of ammonia (Labow, 2001). Thus, Gln can act as a buffer, accepting excess ammonia, and then releasing it when needed to form other amino acids, amino sugars, glucose, proteins, nucleotides, glutathione, and urea (Rennie, 2001; Souba, 1993). This capacity to accept and donate nitrogen makes Gln the major vehicle for nitrogen transfer between tissues. Glutamine is the principle metabolic fuel for small intestine enterocytes, lymphocytes, macrophages, and fibroblasts (Andrews and Griffiths, 2002; Cynober, 1999) and is considered an essential amino acid in some species under inflammatory conditions such as infection and injury (Newsholme, 2001). Calder (1999) reported that in culture Gln is utilized at a high rate by cells of the immune system and is required to support optimal lymphocyte proliferation and cytokine production by lymphocytes and macrophages. Glutamine is also the precursor for the net synthesis of arginine, which has been shown to increase thymus and spleen size in mice (Adjei *et al.*, 1994) and increase cytokine production and enhance lymphocyte proliferation (Reynolds *et al.*, 1988).

Many benefits have been observed due to glutamine supplementation in the diet of humans and rats and little research has been done with swine and poultry. Yi *et al.* (2001b) reported that supplementing the diet with 1% Gln improved the body weight gain and feed efficiency (body weight gain:feed intake) of turkey poults during the first

week post hatch as compared to poult fed a standard corn-soybean meal (SBM) diet. Kitt *et al.* (2002) reported that the addition of 1% Gln to the diet improved the feed efficiency in weanling pigs. Glutamine supplementation increased intestinal villous height in poult (Yi *et al.*, 2001a) and weanling pigs (Kitt *et al.*, 2002). Glutamine supplementation has been reported to stimulate gut mucosal proliferation in rats (Inoue *et al.*, 1993). It has also been observed that supplementing with 1.5% Gln in a total parenteral nutrition (TPN) diet maintains gut integrity (Naka, 1996), which is important in preventing bacterial infections and Gln has been shown to prevent intestinal hyperpermeability and bacterial translocation in mice during an immunological challenge (Adjei *et al.*, 1994). During stressful conditions, intestinal permeability increases allowing bacteria to enter the bloodstream thus causing infection (Adjei *et al.*, 1994) and Gln has also been shown to decrease the incidence of infection in surgery and trauma patients (Newsholme, 2001; Medina, 2001; Andrew and Griffiths, 2002).

To date little research has been conducted on the use of glutamine supplementation in poultry diets. Therefore, two studies were conducted to determine the effect of Gln supplementation on growth performance, development of the gastrointestinal tract and humoral immune response of broiler chicks.

MATERIALS AND METHODS

Experimental Birds and Diets

Two studies were conducted with Cobb 500 by-product male chicks obtained from a local hatchery and immediately placed in Petersime battery cages² with wire-mesh floors in an environmentally controlled room. Chicks were weighed and randomly allotted to pens such that each pen of chicks had a similar initial weight distribution.

Chicks were maintained on a 24 hour constant lighting schedule and the room temperature was maintained at 80 to 75 degrees F. Chicks had *ad libitum* access to feed and water. The treatment diets were formulated to meet the NRC (1994) recommendations and were fed from 0 to 21 d of age. The experimental diets were formulated to be isonitrogenous and isocaloric with 22.5% crude protein, 3,150 kcal TME/kg, a constant fat level of 5%, and sand as a filler (Table 3.1). Lys and Thr were added to the 4% Gln diet because of the large change in SBM inclusion (2.72% difference in SBM between control and 1% Gln diets and 8.84% difference between the 1% Gln and 4% Gln diets). Group body weight and feed intakes were measured on d 4, 7, 14, and 21. Weight gain and feed efficiency (gain:feed) were calculated for each pen. Calculations were adjusted for mortalities when appropriate.

Experiment 1 Experiment 1 was conducted to evaluate the effects of Gln supplementation, the optimal level (1 or 4% Gln) and route of administration (addition to the feed, water, or both), and to determine if the positive benefits observed in humans and rats can be viewed in poultry. Four hundred and twenty chicks were randomly divided into 7 treatment groups of 6 replicates of 10 birds each. The treatment groups were as follows: 1) Control, a standard corn-SBM diet, 2) corn-SBM diet supplemented with 1% Gln, 3) 1% Gln added to standard city drinking water, the water treatments were mixed every 7 d and fed the control diet, 4) corn-SBM diet supplemented with 1% Gln and 1% Gln added to city drinking water, 5) corn-SBM diet supplemented with 4% Gln, 6) 4% Gln added to standard city drinking water, the water treatments were mixed every 7 d and fed the control diet, and 7) corn-SBM diet supplemented with 4% Gln and 4% Gln added

² Petersime Incubator Co., Gettysburg, OH 45328.

to city drinking water. Due to the problem of Gln precipitating out of solution in the water treatments and low water intake, the water treatments were discontinued at d 14.

Experiment 2 Experiment 2 was conducted to determine how long the 1% Gln needed to be supplemented in the diet in order to achieve the improvement in growth performance, enhancement in development of the gastrointestinal tract and antibody concentrations. Three hundred chicks were randomly divided into 5 treatment groups of 6 replicates of 10 birds each. The chicks were fed a standard corn-SBM control diet or a corn-SBM diet supplemented with 1% Gln. The experimental treatments were as follows: 1) Control, a corn-SBM diet fed until 21 d of age, 2) a corn-SBM diet supplemented with 1% Gln fed for 4 d after which time the chicks were fed the control corn-SBM diet until 21 d of age, 3) a corn-SBM diet supplemented with 1% Gln fed for 7 d then the control diet was fed from d 8 to 21, 4) the corn-SBM diet supplemented with 1% Gln fed for 14 d after which time the control corn-SBM diet was fed until 21 d of age, and 5) a corn-SBM diet supplemented with 1% Gln fed for the entire 21 d experimental period.

Sampling

In Experiments 1 and 2, twelve chicks per treatment (two chicks per pen) were randomly selected on d 0, 4, 7, 14, and 21 for sampling of blood, organ weights, and intestinal measurements. Chicks were weighed and killed by cervical dislocation, and then the abdominal cavity was opened. The thymus, spleen, and bursa were removed and weighed. The thymus weight was determined as the five lobes located bilaterally on the sides of the esophagus. For intestinal weight measurements, the small intestine was removed and divided into three segments: duodenum (from gizzard to entry of the bile

and pancreatic ducts), jejunum (from entry of the ducts to yolk stalk), and ileum (from yolk stalk to ileocecal junction). The ileum was flushed with 10 to 20 mL of deionized water and the empty weight was recorded. Because morphologic analysis of the duodenum and jejunum was to be determined, these segments were flushed with 20 mL of physiological saline solution, and the empty weight was recorded. Organ weights were expressed on a relative (grams/100 grams BW) body weight and an absolute basis. For morphologic analysis, approximately 5 cm of the middle portion of the duodenum and jejunum (the apex of the duodenum and midway between the point of entry the bile ducts and Meckel's diverticulum of the jejunum) was excised and fixed in 10% formalin. Six cross sections of 70% ethanol-preserved segments for each duodenal and jejunal sample were then prepared for staining with hematoxylin and eosin using standard paraffin embedding procedures (Uni *et al.*, 1995). A total of 4 intact, well-oriented villi were selected in six replicates for each intestinal cross section (24 measurements for each intestinal sample with 288 measurements per treatment). Villus height was measured from the tip of the villi to the villus crypt junction. Morphological indices were determined using computer-aided light microscope (16x magnification of the objective lens) image analysis³.

Blood, bile, and jejunum samples were collected from 2 birds per pen (twelve birds per treatment) on d 7, 14, and 21 in Experiments 1 and 2. Blood was obtained by jugular venipuncture from each bird. Blood samples were centrifuged at 1000 X g for 10 minutes at room temperature and the serum fraction was frozen and stored at -20 C until analyzed. The birds were killed by cervical dislocation, and bile and jejunal samples were obtained. Bile was aspirated from the gall bladder with a 25-gauge needle coupled

³ Image-Pro Plus Version 3.0, Media Cybernetics, Silver Spring, MD 20910.

to a 3-mL syringe and then stored at -20 C until IgA analysis was conducted. The jejunum, i.e., the portion of the small intestine between the opening of the pancreobiliary ducts and the yolk stalk, was excised from each bird. Ten centimeters of the middle portion of the jejunum was separated and stored at -20 C until prepared for analysis. At the time of analysis, the jejunal samples were thawed at room temperature, 2 g of jejunal sample was weighed, 20 mL of deionized water was added, and it was homogenized for 30 seconds with a mechanical homogenizer⁴ at a speed setting of 5. An aliquot (5 mL) of the sample was centrifuged at 20,000 x g for 30 minutes. The supernatant was obtained and stored at -20 C until analyzed for IgA concentration.

Analysis of Ig in Serum, Bile, and Intestine

Serum samples for all treatment and age groups were analyzed for IgA and IgG at the same time to avoid variation that may occur with analyses done at different times. Serum, bile, and jejunal IgA and serum IgG were determined using a double antibody technique ELISA kit⁵. Absorbance was measured at 450 nm. The absorbance of the control wells were adjusted to zero prior to measuring absorbance in the samples. Because absorbance units are linearly related to the logarithm of the Ig concentration (Piquer *et al.*, 1991), we considered that the absorbance measurements obtained could be used as estimates of Ig concentrations. Therefore, no standard curve was used to calculate Ig concentration.

Statistical Analysis

All the data were subjected to ANOVA procedures for completely randomized designs using the GLM procedure of SAS® (SAS Institute, 1990). Statistical

⁴ VirTis, Gardiner, NY 12525.

⁵ Bethyl Laboratories, Inc., Montgomery, TX 77356.

significances of differences among treatment group means were determined using Duncan's multiple range test (Duncan, 1955). Single degree of freedom orthogonal contrasts were performed to compare the effects of 1% Gln supplementation in the feed vs. the control diet in Experiment 1 and in Experiment 2 to compare the effect of Gln supplementation for any length of period vs. the control. A probability level of $P \leq 0.05$ was used to determine statistical significance.

RESULTS

Experiment 1 By d 14, body weight gain significantly increased in birds that were fed diets supplemented with 1% Gln when compared to the control birds fed the standard corn-SBM diet ($P < 0.0001$) (Table 3.2). Weight gain had increased 11% by 21 d of age in the birds fed diets supplemented with 1% Gln as compared to the birds fed the control diet. Although the cumulative (0-21 d) weight gain of birds fed diets supplemented with 1% Gln was not statistically different for the birds fed the control diet, it is numerically greater and when a single degree of freedom orthogonal contrast was performed they were significantly different ($P < 0.0001$). Due to the problem of Gln precipitating out of solution in the water treatments and the low water intake observed in these groups, the water treatments were discontinued at d 14; therefore, data for these treatments are not displayed in the tables. Body weight gain was significantly depressed in birds fed diets supplemented with the 4% level of Gln ($P < 0.0001$). Gln supplemented at either the 1 or 4% level in the feed, water, or both did not consistently affect feed efficiency (gain:feed) (Table 3.3). The thymus (0.51g vs. 0.35g) and spleen (0.13g vs. 0.08g) relative weights of broilers were significantly heavier when 1% Gln was supplemented in the feed and water as compared to the birds fed the control corn-SBM

diet ($P < 0.05$) (Data not shown). The duodenum (1.56 to 1.66g vs. 1.30g) and jejunum (2.41 to 2.45g vs. 2.07g) relative weights were significantly heavier with the addition of 1% and 4% Gln supplementation in the feed, water or both as compared to the control birds ($P < 0.05$) (Data not shown). Gln supplementation in the feed, water, or both did not affect the chick's bursa weight. Villi length in the duodenum and jejunum (Table 3.4) were significantly longer in the birds fed diets supplemented with Gln, with the birds fed the diet with 4% Gln having the longest villi ($P < 0.0001$). The birds fed diets supplemented with 1% Gln had significantly higher IgA concentrations in the serum (Table 3.4) and bile (Data not shown) than the controls birds ($P < 0.05$). By d 21, the birds fed diets supplemented with 4% Gln had significantly lower IgA concentrations in the serum ($P < 0.001$) as compared to the birds fed the control diet and birds fed diets supplemented with 1% Gln. The d 21 IgA intestinal concentrations were significantly higher in the birds fed the 1% Gln supplemented diet than the birds fed the control corn-SBM diet ($P < 0.01$). The birds fed diets supplemented with 1% Gln had significantly higher IgG concentrations in the serum by 21 d of age than the birds fed the control diet ($P < 0.03$).

Experiment 2 There was no significant benefit from 1% Gln supplementation in the feed on chick performance until d 14 (Table 3.5). Overall, birds fed diets supplemented with 1% Gln for at least 14 d had significantly better body weight gain as compared to the birds fed the control diet, with the birds fed 1% Gln for 21 d having the largest gain ($P < 0.02$). The body weight gain difference was 10.9% between the birds fed the control diet and the birds fed diets supplemented with 1% Gln for 21 d. There was no improvement in feed efficiency due to the addition of Gln (Table 3.6). The birds fed

diets supplemented with 1% Gln for 21 d had significantly heavier duodenum (1.37g vs. 1.06g) and jejunum (2.05g vs. 1.83g) relative weights as compared to the birds fed the control diet ($P<0.05$) (Data not shown). There was no benefit on the thymus, spleen, and bursa weights due to the addition of Gln. The supplementation of Gln in the diet of broilers for any length of time yielded significantly longer villi in the duodenum and jejunum (Table 3.7) as compared to the villi length of the control birds ($P<0.0001$). The birds fed diets supplemented with 1% Gln for 7 d or more had significantly higher IgA concentrations in the serum and bile (Data not shown) than the control birds ($P<0.05$). Although the chicks fed diets supplemented with Gln had higher IgA concentrations, when Gln supplementation was discontinued, the concentrations began to decline to those of the control birds. The d 21 intestinal IgA concentrations were higher in the birds fed diets supplemented with 1% Gln for any length of time compared to that of the control birds ($P<0.01$). The birds fed diets supplemented with 1% Gln had significantly higher IgG concentrations in the serum compared to the control birds ($P<0.04$).

DISCUSSION

Significant improvements in body weight gain were observed when 1% Gln was supplemented in the feed for 21 d as compared to the birds fed the corn-SBM diet (an average 11% increase). This finding was surprising since improvements in weight gain had not been reported in swine (Kitt *et al.*, 2002) or other species due to Gln supplementation. Yi *et al.* (2001b) did report an improvement in body weight gain in turkey poults fed diets supplemented with 1% Gln, but it was only noted for the first wk of age. An improvement in feed efficiency was not observed, however improvements in feed efficiency had been noted in swine (Kitt *et al.*, 2002) and turkey poults (Yi *et al.*,

2001b) when they were fed a diet supplemented with 1% Gln. The weight depression observed in chicks fed diets supplemented with 4% Gln may indicate a toxic effect when supplemented at 4% in the feed. However, the reduction in weight gain of the birds fed the diet supplemented with 4% Gln may be due to the large decrease in SBM in the 4% Gln diet (26.7% SBM) vs. the control diet (38.3% SBM). Although the diets were formulated to meet or exceed the NRC (1994) total amino acid recommendations, the control diet and the diet with only 1% Gln had much higher levels of all the indispensable amino acids (except for the total sulfur amino acids) than the levels in the 4% Gln diet, which may be the main reason for the lower growth performance of broilers fed the diet supplemented with 4% Gln.

The birds fed diets supplemented with Gln had significantly longer intestinal villi than the intestinal villi of birds fed the control corn-SBM diet. Interestingly, the microvilli of enterocytes increase in length during the first week of life (Chambers and Gray, 1979). If the intestinal villi height can be increased early in the chick's life, then the chick may be able to utilize nutrients more efficiently earlier in life and thus have improved growth performance. An important correlation is the relationship between growth rate and gut organ development. Lilja (1983) reported that avian species with a high growth rate capacity were characterized by a rapid early development of the digestive organs and liver. Birds with faster growth rates were reported by Nitsan *et al.* (1991) to secrete high levels of digestive enzymes, implying that initial growth is only limited by the early development of the digestive organs. By reducing the time for development of the digestive organs, growth improvements could be achieved. Increased villi height has been proposed to increase performance by improving nutrient absorption

(Coates *et al.*, 1954 and Izat *et al.*, 1989). The increase in villi height that was observed might indicate that the birds fed diets supplemented with 1% Gln may have had greater nutrient absorption and utilization as increases in villi height results in more surface area for nutrient utilization. The increase in surface area might also explain the significantly heavier intestinal relative weights ($P < 0.05$) and improved weight gain that was observed due to Gln supplementation.

Higher IgA concentrations in the serum, bile, and intestines observed in the birds fed diets supplemented with Gln supports evidence reported by Burke *et al.* (1989) that rats fed diets supplemented with Gln maintained higher serum IgA levels than the other treatment groups that were not fed diets with Gln supplementation. The digestive mucosa is continuously exposed to dietary, bacterial, viral, and parasitic antigens (Strobel, 1986). Specific protection against these antigens is achieved mainly by the secretion of IgA, which is synthesized in the gut-associated lymphoid tissue (Piquer *et al.*, 1991). The increase in IgA concentrations has been related to the increase in the number of lymphoid cells observed in the gallbladder (Leslie *et al.*, 1976) of chickens and small intestine (Piquer *et al.*, 1990) of turkeys. This suggests that the effect of Gln on the preservation of gut mass includes intestinal lymphoid tissue as well. IgA functions primarily by preventing the attachment of bacterial to the mucosal cell (Burke *et al.*, 1989). The barrier function of the gut epithelium depends on the presence of IgA, and until IgA is present, the hatchling is more susceptible to oral pathogens (Sell, 1991). The role of the gut as a barrier is to prevent the spread of intraluminal bacteria in systemic organs and tissues. This may indicate that the birds fed diets supplemented with 1% Gln had better gut barrier function since the birds had higher IgA concentrations in the

intestines and thus may be more resistant to infection. However, these statements must be further studied and evaluated.

Gln supplementation has been shown to increase the proportion of CD4⁺ (T-helper):CD8⁺ (T cytotoxic/suppressor) cells (Yeh, 2001; Kew *et al.*, 1999), which suggests that the supplementation of Gln stimulates the proliferation of CD4⁺ cells in preference to CD8⁺ cells. IgG expression is T helper cell dependent (Singh, 1996) and is indicative of T-helper cell response (Mathers *et al.*, 2004). Since IgG levels did increase in birds fed diets supplemented with Gln, this may indicate that Gln is important for the synthesis of the IgG antibodies or perhaps required for thymus-derived (T)-cell helper function and response. The data we compared here indicated that alterations of total IgG production induced by dietary Gln in chicks without an antigenic challenge might reflect the potential of specific antibody IgG production when chicks are challenged with an antigen. However, further investigations are required.

Immune tissue development is the basis of immune functionality. The supplementation of Gln in diets fed to chicks significantly promoted the growth of the spleen and thymus (in Experiment 1), but had no effect on the bursa weight. The increase in immune tissue weight resulting from Gln supplementation correlated with the functionality of thymus and spleen in terms of IgA and IgG production. The results of this experiment give insights into a potential dietary method to modulate chicken immune responses toward improving chicken performance under a given condition. For example, the inflammatory response is the first line of defense against novel pathogens, but cells and mediators of the inflammatory responses have been implicated in the pathology of many poultry diseases, including coccidiosis (Trout and Lillehoj, 1993). Modification of

antibody production and activity by dietary Gln supplementation may provide an avenue to strengthen chick immunity and protection against various pathogens. However, long-term effects of immunomodulation induced by Gln supplementation on the resistance of chickens to commercially relevant infectious challenges and chick performance remain to be investigated.

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TABLE 3.1. Composition of the dietary¹ treatments (as-fed basis), Experiments 1 and 2

Ingredients	Control	1% Glutamine	4% Glutamine
	%		
Corn	51.90	52.90	56.70
Soybean meal	38.28	35.56	26.72
Poultry fat	5.00	5.00	5.00
Limestone	1.49	1.50	1.52
Dicalcium P	1.75	1.75	1.83
Vitamin ² premix	0.25	0.25	0.25
Mineral ³ premix	0.08	0.08	0.08
Salt	0.30	0.30	0.30
DL-Met	0.17	0.21	0.32
L-Lys-HCL	---	---	0.20
L-Thr	---	---	0.10
Sand	0.78	1.45	2.98
L-Glutamine	---	1.00	4.00

¹ Calculated Composition 22.5% CP, 3,200 kcal TME/kg, 0.90% total sulfur amino acids, and at least 1.10% total lysine.

² Vitamin Premix supplied (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), 12.0 µg; 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-retinyl acetate, 5,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.

³ Mineral Premix supplied (per kilogram of diet): manganese (MnSO₄·H₂O), 60 mg; iron (FeSO₄·7H₂O), 30 mg; zinc (ZnO), 50 mg; copper (CuSO₄·5H₂O), 5 mg; iodine (ethylene diamine dihydroiodide), 1.5 mg; selenium (Na₂SeO₃), 0.3mg.

TABLE 3.2. Effect of Gln supplementation on body weight gain (g/chick) of broilers, Experiment 1

Treatment ¹	Days of Age				
	0-4	4-7	7-14	14-21	0-21 ³
Corn-SBM	46 ^a	63 ^a	237 ^b	360 ^{ab}	706 ^{ab}
1% Gln in Feed	49 ^a	61 ^a	261 ^a	400 ^a	771 ^a
1% Gln in Water	40 ^{bc}	57 ^b	232 ^{bc}	---	---
1% Gln in Feed + Water	38 ^c	57 ^b	237 ^b	---	---
4% Gln in Feed	45 ^{ab}	53 ^b	215 ^c	321 ^b	634 ^{bc}
4% Gln in Water	29 ^d	40 ^c	196 ^d	---	---
4% Gln in Feed + Water	30 ^d	38 ^c	174 ^e	---	---
Pooled SEM	1.8	2.1	6.8	22.2	26.8
P-value	0.0001	0.0001	0.0001	0.03	0.0001

^{a-c}Means within columns having the same superscript do not differ significantly (P < 0.05).

¹Means represent 6 pens per treatment; 10 chicks per pen (60 chicks per treatment).

²Results are not shown for the water treatments after 14 d as these treatments were detrimated at d 14.

³Using single degree of freedom contrast the control and 1% Gln in the feed are significantly different (P < 0.05).

TABLE 3.3. Effect of Gln supplementation on the feed efficiency (Gain:Feed)² of broilers, Experiment I

Treatment ¹	Days of Age				
	0-4	4-7	7-14	14-21	0-21
Corn-SBM	1070 ^{ab}	820 ^{ab}	610	520	590
1% Gln in Feed	1070 ^{ab}	780 ^{bc}	680	540	610
1% Gln in Water	1090 ^a	850 ^{ab}	680	----- ³	-----
1% Gln in Feed + Water	1060 ^{ab}	850 ^{ab}	640	-----	-----
4% Gln in Feed	1030 ^b	720 ^c	640	500	580
4% Gln in Water	1110 ^a	890 ^a	630	-----	-----
4% Gln in Feed + Water	1110 ^a	750 ^c	650	-----	-----
Pooled SEM	20.0	20.0	30.0	40.0	20.0
P-value	0.04	0.003	0.48	0.62	0.55

^{a-c}Means within columns having the same superscript do not differ significantly (P < 0.05).

¹Means represent 6 pens per treatment; 10 chicks per pen (60 chicks per treatment).

²Gain:Feed = weight gain (g)/feed intake (kg) .

³Results are not shown after 14 d for the water treatments as these treatments were terminated at d 14 of age.

TABLE 3.4. Effect of Gln supplementation on villous height and humoral immune response of broilers, Experiment I

Treatment ¹	Duodenal Villi Height	Jejunual Villi Height	Serum IgA concentrations	Intestinal IgA concentrations	Serum IgG concentrations
Corn-SBM	651.49 ^c	526.02 ^c	0.531 ^c	---	1.578 ^b
1% Gln in Feed	762.63 ^b	693.72 ^b	0.816 ^a	---	1.997 ^a
4% Gln in Feed	921.34 ^a	743.55 ^a	0.734 ^b	---	1.035 ^c
Pooled SEM	34.06	14.61	0.02	---	0.11
P-value	0.0001	0.001	0.01	---	0.02
			<u>D-14</u>		
Corn-SBM	706.57 ^b	481.36 ^c	0.772 ^c	---	1.697 ^b
1% Gln in Feed	934.09 ^a	697.88 ^b	1.213 ^a	---	1.782 ^a
4% Gln in Feed	990.07 ^a	779.80 ^a	0.902 ^b	---	1.283 ^c
Pooled SEM	69.84	25.31	0.04	---	0.02
P-value	0.0001	0.0001	0.001	---	0.01
			<u>D-21</u>		
Corn-SBM	738.64 ^b	447.00 ^c	1.853 ^b	2.784 ^b	1.829 ^c
1% Gln in Feed	907.56 ^a	749.59 ^b	2.808 ^a	3.509 ^a	2.506 ^a
4% Gln in Feed	936.61 ^a	783.67 ^a	1.276 ^c	2.455 ^b	2.065 ^b
Pooled SEM	50.22	27.83	0.05	0.03	0.08
P-value	0.0001	0.0001	0.001	0.01	0.03

^{a-c}Means within columns and age period having the same superscript do not differ significantly (P < 0.05).

¹Means represent 6 pens per treatment; 10 chicks per pen (60 chicks per treatment).

TABLE 3.5. Effect of Gln supplementation fed for varying lengths of time on body weight gain (g/chick) of broilers, Experiment 2

Treatment ¹	Days of Age				
	0-4	4-7	7-14 ²	14-21	0-21
Corn-SBM	45	80	265	350 ^b	739 ^b
1% Gln for 4 days	47	74	275	346 ^b	742 ^b
1% Gln for 7 days	52	81	276	367 ^{ab}	775 ^{ab}
1% Gln for 14 days	51	79	275	386 ^{ab}	791 ^a
1% Gln for 21 days	51	78	275	401 ^a	805 ^a
Pooled SEM	2.8	2.0	6.3	12.8	15.3
P-value	0.29	0.16	0.71	0.03	0.02

^{a-b}Means within columns having the same superscript do not differ significantly (P < 0.05).

¹Means represent 6 pens per treatment; 10 chicks per pen (60 chicks per treatment).

²Using single degree of freedom contrast the control vs. 1% Gln fed for any length of time are significantly different (P < 0.05).

TABLE 3.6. Effect of Gln supplementation fed for various lengths of time on the feed efficiency (Gain:Feed)² of broilers, Experiment 2

Treatment ¹	Days of Age				
	0-4	4-7	7-14	14-21	0-21
Corn-SBM	880	860	590 ^b	600	630
1% Gln for 4 days	890	830	640 ^{ab}	590	630
1% Gln for 7 days	890	870	700 ^a	570	650
1% Gln for 14 days	910	870	670 ^a	610	670
1% Gln for 21 days	890	860	630 ^{ab}	620	650
Pooled SEM	20.0	20.0	20.0	20.0	20.0
P-value	0.68	0.21	0.01	0.66	0.32

^{a-b}Means within columns having the same superscript do not differ significantly (P < 0.05).

¹Means represent 6 pens per treatment; 10 chicks per pen (60 chicks per treatment).

²Gain:Feed = weight gain (g)/feed intake (g).

TABLE 3.7. Effect of Gln supplementation fed for various lengths of time on villous height and humoral immune response of broilers, Experiment 2

Treatment ¹	Duodenal		Jejunal Villi Height	Serum IgA concentrations		Intestinal IgA concentrations		Serum IgG concentrations	
	Villi Height	Villi Height		concentrations	concentrations	concentrations	concentrations	concentrations	concentrations
Corn-SBM	778.27 ^c	607.37 ^c	0.625 ^c	---	---	1.241 ^c	---	---	1.241 ^c
1% Gln for 4 days	838.58 ^b	609.34 ^c	0.993 ^d	---	---	1.332 ^d	---	---	1.332 ^d
1% Gln for 7 days	982.79 ^a	836.99 ^b	1.358 ^b	---	---	1.487 ^c	---	---	1.487 ^c
1% Gln for 14 days	911.28 ^{ab}	934.55 ^a	1.116 ^c	---	---	1.585 ^b	---	---	1.585 ^b
1% Gln for 21 days	968.05 ^a	959.32 ^a	1.483 ^a	---	---	1.727 ^a	---	---	1.727 ^a
Pooled SEM	20.10	32.52	0.04	---	---	0.03	---	---	0.03
P-value	0.003	0.001	0.05	---	---	0.05	---	---	0.05
Corn-SBM	855.82 ^c	672.29 ^c	0.897 ^d	---	---	1.574 ^c	---	---	1.574 ^c
1% Gln for 4 days	1055.82 ^a	775.43 ^d	1.249 ^c	---	---	1.885 ^b	---	---	1.885 ^b
1% Gln for 7 days	925.83 ^b	826.62 ^c	1.774 ^b	---	---	1.963 ^b	---	---	1.963 ^b
1% Gln for 14 days	1031.29 ^a	948.79 ^b	1.714 ^b	---	---	1.942 ^b	---	---	1.942 ^b
1% Gln for 21 days	1046.85 ^a	1000.95 ^a	2.570 ^a	---	---	2.192 ^a	---	---	2.192 ^a
Pooled SEM	23.34	17.06	0.15	---	---	0.02	---	---	0.02
P-value	0.002	0.004	0.001	---	---	0.05	---	---	0.05
Corn-SBM	745.48 ^c	591.06 ^c	1.293 ^c	1.905 ^c	---	2.290 ^c	1.905 ^c	---	2.290 ^c
1% Gln for 4 days	934.98 ^b	729.56 ^d	1.430 ^c	2.832 ^b	---	2.589 ^b	2.832 ^b	---	2.589 ^b
1% Gln for 7 days	1023.20 ^{ab}	980.99 ^b	1.940 ^b	3.392 ^a	---	2.741 ^b	3.392 ^a	---	2.741 ^b
1% Gln for 14 days	1026.74 ^{ab}	940.57 ^c	1.683 ^b	3.418 ^a	---	2.639 ^b	3.418 ^a	---	2.639 ^b
1% Gln for 21 days	1091.01 ^a	1048.75 ^a	2.914 ^a	3.267 ^a	---	3.475 ^a	3.267 ^a	---	3.475 ^a
Pooled SEM	34.66	21.22	0.07	0.08	---	0.05	0.08	---	0.05
P-value	0.0001	0.001	0.02	0.01	---	0.04	0.01	---	0.04

^{a-b}Means within columns and age period having the same superscript do not differ significantly (P < 0.05).

¹Means represent 6 pens per treatment; 10 chicks per pen (60 chicks per treatment).

²Gain:Feed = weight gain (g)/feed intake (g).

CHAPTER 4

THE EFFECT OF SUPPLEMENTING GLUTAMINE, GLUTAMIC ACID, HISTIDINE, AND ASPARAGINE ON GROWTH PERFORMANCE, DEVELOPMENT OF THE GASTROINTESTINAL TRACT, AND IMMUNE RESPONSE OF BROILERS⁶

⁶ Bartell, S.M. and A.B. Batal. Will be submitted to *Poultry Science*.

ABSTRACT An experiment was conducted to evaluate the effect of supplemental glutamine (Gln) as compared to similar amino acids (AA) on growth performance, development of the gastrointestinal tract and immune response of broilers. Immediately after hatch six replicate pens of ten chicks were randomly assigned to one of eight dietary treatments for 21 d. All diets were formulated to be isocaloric and isonitrogenous. The birds were fed either a corn-soybean meal (SBM) control diet, a diet supplemented with 0.05% bacitracin dimethylene salicylate, 1% Gln (original source), 1% Gln (new source), 2% Gln (original), 1% His, 1% Glu, or 1% Asn. His, Glu, and Asn were supplemented to determine if the benefits observed in previous studies due to Gln supplementation could be duplicated with other similar AA. The three AA that were evaluated along with Gln were chosen because His is most similar to Gln in its manufacturing process, Glu is thought to be interchangeable with Gln in the body, and Asn is the most similar to Gln in chemical structure. On D 0, 7, and 21 two chicks per pen were sacrificed for duodenum, jejunum, bile, and blood sample collections. On d 14, chicks were injected with 0.05% SRBC and on d 21 blood was collected for the determination of hemagglutination titers. Weight gain and feed efficiency improved significantly ($P < 0.001$) in broilers fed a diet supplemented with 1% Gln (original) as compared to all other dietary treatments. Supplementing the diet with 1% Gln (new), 2% Gln, and 1% His significantly depressed weight gain ($P < 0.05$) as compared to the other treatments. Villi length and concentrations of bile, intestinal and serum IgA, serum IgG, serum IgM, and hemagglutination titers to SRBC were significantly higher ($P < 0.05$) in chicks fed diets supplemented with Gln (either source) as compared to all other dietary treatments. The performance and immune responses observed in the birds fed diets supplemented with Gln and not with Glu

indicate that Glu cannot be substituted for Gln. The results indicate that the supplementation of dietary Gln in broiler diets improves growth performance, may enhance the development of the gastrointestinal tract and immune response, and can not be duplicated by the supplementation of His, Glu, or Asn. However, the enhancement in performance appears to be source dependent.

Key Words: Glutamine, gastrointestinal tract, broiler chicks, SRBC, immune response

INTRODUCTION

L-Glutamine (Gln) is the principle metabolic fuel for small intestine enterocytes, lymphocytes, macrophages, and fibroblasts (Andrews and Griffiths, 2002; Cynober, 1999) and is considered an essential amino acid in some species under inflammatory conditions such as infection and injury (Newsholme, 2001). Calder (1999) reported that in culture Gln is utilized at a high rate by cells of the immune system and is required to support optimal lymphocyte proliferation and cytokine production by lymphocytes and macrophages. Glutamine is also the precursor for the net synthesis of arginine, which has been shown to increase thymus and spleen size in mice (Adjei *et al.*, 1994) and increase cytokine production and enhance lymphocyte proliferation (Reynolds *et al.*, 1988).

Many benefits have been observed due to glutamine supplementation in the diet of humans and rats, and some in swine and poultry. Yi *et al.* (2001b) reported that supplementing a turkey poult diet with 1% Gln vs. poult fed a standard corn-soybean meal (SBM) diet improved body weight gain and feed efficiency (body weight gain:feed intake) during the first week post hatch. The performance improvements have also been reported in broiler chicks (Bartell and Batal, 2004; Yi *et al.*, 2005). Kitt *et al.* (2002) reported that the addition of 1% Gln to the diet improved feed efficiency in weanling pigs. Glutamine supplementation also increases the intestinal villous height in chicks (Bartell and Batal, 2004; Yi *et al.*, 2001a; Yi *et al.*, 2005) and weanling pigs (Kitt *et al.*, 2002). It has been demonstrated that dietary Gln supplementation increases concentrations of serum, bile, and intestinal IgA, serum IgG (Bartell and Batal, 2004), and serum IFN- γ and IL-2 (Yi *et al.*, 2005). Studies have shown that Gln stimulates gut mucosal proliferation in rats (Inoue *et al.*, 1993). It has been observed that

supplementing with 1.5% Gln in a total parenteral nutrition (TPN) diet in rats maintains gut integrity (Naka, 1996), which is important in preventing bacterial infections.

Glutamine supplementation has been shown to prevent intestinal hyperpermeability and bacterial translocation in mice during an immunological challenge (Adjei *et al.*, 1994).

Glutamine has also been shown to decrease the incidence of infection in surgery and trauma patients (Newsholme, 2001; Medina, 2001; Andrew and Griffiths, 2002). Since our previous studies with Gln supplementation were conducted in non-challenged chicks, an objective in this study was to evaluate the response of chicks fed diets supplemented with Gln and challenged with SRBC, a T-cell dependent antigen. If an increase in anti-SRBC titers is observed, then it could possibly be indicative of chick performance response to an enteric disease challenge, such as coccidiosis (Parmentier *et al.*, 2001).

Based on the immune response trends observed in previous studies conducted on unchallenged chicks (Bartell and Batal, 2004), it was questioned if the immune benefits viewed could be due to the manufacturing process of Gln. Glutamine is manufactured by bacterial fermentation and if some residue (such as a microbe's cell wall) remains from the production of Gln, this could be the reason for the immune stimulus (Kusumoto, 2001). Since a chemically synthesized form of Gln was unattainable, various amino acids (AA) were compared to Gln to determine if the same performance effects and immune trends could be obtained with the supplementation of other similar AA. By comparing the other AA, it still does not determine if any bacterial residue is there, but one can suspect that if it is there in Gln, it should be there after the fermentation of these other AA. However, the benefits could still be due to the specific bacteria used to produce Gln.

Therefore, the objectives of the study were to evaluate the response of SRBC challenged chicks fed diets supplemented with Gln and to evaluate if the effects of supplementing His, Glu, and Asn yielded the same immune benefits observed with dietary Gln supplementation in previous studies.

MATERIALS AND METHODS

Experimental Birds and Diets

Four hundred and eighty Cobb 500 by-product male chicks were obtained from a local hatchery, randomly divided into 8 treatment groups of 6 replicates of 10 birds each, and immediately placed in Petersime battery cages⁷ with wire-mesh floors in an environmentally controlled room. Chicks were weighed and randomly allotted to pens such that each pen of chicks had a similar initial weight distribution. Chicks were maintained on a 24 hour constant lighting schedule and room temperature was maintained at 80 to 75 degrees F. Chicks had *ad libitum* access to feed and water. The experimental diets were formulated to meet the NRC (1994) recommendations and to be isonitrogenous and isocaloric with 22.5% crude protein, 3,150 kcal TME/kg, and a constant fat level of 5% (Table 4.1). Group body weight and feed intakes were measured on d 7, 14, and 21. Weight gain and feed efficiency (gain:feed) were calculated for each pen. Calculations were adjusted for mortalities when appropriate.

The treatment groups were as follows: 1) Control, a standard corn-SBM diet, 2) corn-SBM diet with 0.05% bacitracin dimethylene salicylate (BMD), 3) corn-SBM diet supplemented with 1% Gln (original source), 4) corn-SBM diet supplemented with 2% Gln (original), 5) corn-SBM diet supplemented with 1% Gln (new source), 6) corn-SBM diet supplemented with 1% His, 7) corn-SBM diet supplemented with 1% Glu, and 8)

corn-SBM diet supplemented with 1% Asn. Since synthetically produced Gln could not be obtained, different AA were evaluated to compare the performance and immune benefits observed previously when Gln was supplemented to the diet. Unless otherwise stated, Gln without notation to source refers to the Gln from the original source that was used and evaluated in previous studies (Bartell and Batal, 2004). Bacitracin dimethylene salicylate (BMD), a commonly used growth promoting antibiotic, was used to compare the chick's immune response to those fed diets supplemented with Gln. A 2% level of Gln was used to determine if the benefits would be greater as the level of Gln was increased. Another source of Gln was used to determine if there is variation among Gln sources, since it was originally thought that this new source of Gln was synthetic. Histidine (His) was evaluated because it is the most similar to Gln in the bacterial fermentation manufacturing process, utilizing the same starting substrate of glucose and having similar chemical properties. Glutamic acid (Glu) is the acid form of Gln and is interchangeable with Gln in the body, thus it would be of benefit to substitute Glu for Gln, since it is cheaper and commercially available. Asparagine (Asn) is the most similar to Gln in its chemical structure, only lacking a methyl group.

SRBC Immunization and Hemagglutination Assay

The birds were immunized with SRBC as previously described by Gross (1978). Briefly, 30 mL of blood was collected from a single sheep. Antigen was prepared by washing the sheep red blood cells and suspending them in a saline concentration of 0.05%. On d 14, a 0.05% SRBC solution was administered intravenously into 3 chicks per pen (18 birds per treatment) at a dose of 0.1 mL in the brachial vein of each bird.

⁷ Petersime Incubator Co., Gettysburg, OH 45328.

On d 21 (7 days after antigen challenge), a 1.0 mL sample of blood was collected from the brachial vein of each SRBC challenged bird and placed in a heparinized microfuge tube. The blood samples were microfuged at 10,000 rpm for 2 min and the plasma fractions were removed. The hemagglutination assay was run as previously described by Gross (1978). Briefly, 50 μ L of saline was added to each well of a 96 well microtiter plate. Fifty μ L of the plasma sample was added to the appropriately labeled well in the top row. The diluted plasma samples were carefully mixed and 50 μ L was transferred to the well directly below and this dilution procedure was repeated for all the wells in a column. Fifty μ L of the 0.05% SRBC solution was added to each well of the microtiter plate. The contents of each well were mixed and the plate was incubated overnight at 37 C. The following day, the antibody titer was determined by identifying the first “negative” well (the first well in which a distinct button of red blood cells forms at the bottom of the well). The hemagglutination antibody titers were expressed as \log_2 of the reciprocal of the lowest dilution which produced agglutination by the assay. The mean of the titers were determined for each treatment group.

Sampling

On d 0, 7, and 21 twelve chicks per treatment (two chicks per pen) and on d 21 twelve SRBC challenged chicks per treatment (two chicks per pen) were randomly selected for sampling of blood, organ weights, and intestinal measurements. Chicks were weighed and killed by cervical dislocation, and then the abdominal cavity was opened. The thymus, spleen, and bursa were removed and weighed. The thymus weight was determined as the five lobes located bilaterally on the sides of the esophagus. For intestinal weight measurements, the small intestine was removed and divided into three

segments: duodenum (from gizzard to entry of the bile and pancreatic ducts), jejunum (from entry of the ducts to yolk stalk), and ileum (from yolk stalk to ileocecal junction). The ileum was flushed with 10 to 20 mL of deionized water and the empty weight was recorded. Because morphologic analysis of the duodenum and jejunum was to be determined, these segments were flushed with 20 mL of physiological saline solution, and the empty weight was recorded. Organ weights were expressed on a relative (grams/100 grams BW) body weight and an absolute basis. For morphologic analysis, approximately 5 cm of the middle portion of the duodenum and jejunum (the apex of the duodenum and midway between the point of entry the bile ducts and Meckel's diverticulum of the jejunum) was excised and fixed in 10% formalin. Six cross sections of 70% ethanol-preserved segments for each duodenal and jejunal sample were then prepared for staining with hematoxylin and eosin using standard paraffin embedding procedures (Uni *et al.*, 1995). A total of 4 intact, well-oriented villi were selected in six replicates for each intestinal cross section (24 measurements for each intestinal sample with 288 measurements per treatment). Villus height was measured from the tip of the villi to the villus crypt junction. Morphological indices were determined using computer-aided light microscope (16x magnification of the objective lens) image analysis⁸.

Blood, bile, and jejunum samples were collected from 2 birds per pen (12 birds per treatment) on d 7 and 21 and from 2 SRBC challenged birds per pen (12 birds per treatment) on d 21. Blood was obtained by jugular venipuncture from each bird. Blood samples were centrifuged at 1000 X g for 10 min at room temperature and the serum fraction was frozen and stored at -20 C until analyzed. The birds were killed by cervical dislocation, and bile and jejunal samples were obtained. Bile was aspirated from the gall

⁸ Image-Pro Plus Version 3.0, Media Cybernetics, Silver Spring, MD 20910.

bladder with a 25-gauge needle coupled to a 3-mL syringe and then stored at -20 C until IgA analysis was conducted. The jejunum, i.e., the portion of the small intestine between the opening of the pancreobiliary ducts and the yolk stalk, was excised from each bird. Ten centimeters of the middle portion of the jejunum was separated and stored at -20 C until prepared for analysis. At the time of analysis, the jejunal samples were thawed at room temperature, 2 g of jejunal sample was weighed, 20 mL of deionized water was added, and it was homogenized for 30 s with a mechanical homogenizer⁹ at a speed setting of 5. An aliquot (5 mL) of the sample was centrifuged at 20,000 x g for 30 min. The supernatant was obtained and stored at -20 C until analyzed for IgA concentration.

Analysis of Ig in Serum, Bile, and Intestine

Serum samples for all treatment and age groups were analyzed for IgA, IgG and IgM at the same time to avoid variation that may occur with analyses done at different times. Serum, bile, and jejunal IgA and serum IgG and IgM were determined by using a double antibody technique ELISA kit¹⁰. Absorbance was measured at 450 nm. The absorbance of the control wells were adjusted to zero prior to measuring absorbance in the samples. Because absorbance units are linearly related to the logarithm of the Ig concentration (Piquer *et al.*, 1991), we considered that the absorbance measurements obtained could be used as estimates of Ig concentrations. Therefore, no standard curve was used to calculate Ig concentration.

Statistical Analysis

All the data were subjected to ANOVA procedures for completely randomized designs using the GLM procedure of SAS® (SAS Institute, 1990). Statistical

⁹ VirTis, Gardiner, NY 12525.

¹⁰ Bethyl Laboratories, Inc., Montgomery, TX 77356.

significances of differences among treatment group means were determined using Duncan's multiple range test (Duncan, 1955). A probability level of ($P \leq 0.05$) was used to determine statistical significance.

RESULTS

Body weight gain increased significantly in birds that were fed diets supplemented with 1% Gln, regardless of SRBC challenge, when compared to the birds fed the control corn-SBM diet and all other dietary treatments ($P < 0.0001$) (Tables 4.2 and 4.3). By d 14, body weight gain was significantly depressed in birds fed diets supplemented with 1% Gln (new source), 2% Gln, and 1% His ($P < 0.0001$) (Data not shown). However, by 21 d of age the birds challenged with SRBC and fed diets supplemented with 2% Gln or 1% Gln (new source) were not significantly different than the birds fed the control corn-SBM diet (Table 4.3). However, the unchallenged birds were still significantly smaller. There was no improvement in growth performance when 0.05% BMD, 1% Glu, or 1% Asn were supplemented in the control diet. Overall, the birds challenged with SRBC were larger than the non-challenged birds regardless of treatment. Birds fed diets supplemented with 1% Gln had the best feed efficiency (body weight gain:feed intake ratio) ($P < 0.0001$) (Table 4.2) as compared to the other treatment groups. Birds fed diets supplemented with 2% Gln, 1% Gln (new source), 1% His, and 1% Glu had a lower gain:feed ratio as compared to the birds fed diets supplemented with 0.05% BMD, 1% Gln, 1% Asn, and birds fed the control diet ($P < 0.0001$).

The relative spleen weights were significantly heavier in chicks fed diets supplemented with Gln (either source), regardless of challenge, as compared to the other dietary treatments (0.18 g average of birds fed Gln supplemented diets, regardless of

amount or source vs. 0.12 g average of birds fed the control diet) ($P < 0.05$) (Data not shown). The duodenum (1.48 g vs. 1.25 g) and jejunum (2.27 g vs. 1.98 g) relative weights were significantly heavier when 1% and 2% Gln were supplemented in the feed, regardless of challenge or source, as compared to the other treatments ($P < 0.05$). The thymus relative weights were significantly heavier when Gln was supplemented in the diet, regardless of source or amount, of the SRBC challenged birds as compared to the control and the other AA treatments (0.48 g to 0.36 g) ($P < 0.05$). Dietary Gln supplementation (either source) did not affect the chick's bursa weights.

Villi length in the duodenum and jejunum were significantly longer in the unchallenged (Table 4.4) and SRBC challenged (Table 4.5) birds fed diets supplemented with Gln (either source), with the birds fed diets supplemented with the 2% level of Gln having the longest villi as compared to the other treatment groups ($P < 0.01$). The birds fed diets supplemented with 1% Glu had longer villi as compared to the birds fed the corn-SBM control diet or a diet supplemented with 0.05% BMD, 1% His, or 1% Asn, but shorter villi than the birds fed a diet supplemented with Gln (either source). There was no benefit of supplementing the diet with BMD, 1% His, or 1% Asn on the villi length in broilers. Overall, the challenged birds had shorter villi as compared to the non-challenged birds.

The IgA concentrations in the serum were significantly higher in the non-challenged (Table 4.4) and SRBC challenged (Table 4.5) birds fed diets supplemented with Gln (either source) as compared to all of the other treatment groups ($P < 0.05$). The serum IgA levels increased as the birds aged. The serum IgA concentrations were higher in the SRBC challenged birds relative to the non-challenged birds, with the same trends

among the treatment groups. The IgA concentrations in the bile at d 21 were significantly higher in the unchallenged and SRBC challenged (Data not shown) birds fed the 1 (either source) and 2% Gln supplemented diet than the non-challenged and challenged birds fed all the other treatment diets ($P<0.05$). The SRBC challenged chicks fed diets supplemented with Gln had higher IgA concentrations in the bile relative to the non-challenged chicks fed dietary supplemental Gln. Whereas, the chicks fed all the other diets had similar bile IgA concentrations, regardless of challenge. The IgA intestinal concentrations in the non-challenged and SRBC challenged birds fed dietary supplemental Gln (either source) were significantly higher relative to the non-challenged and challenged chicks fed the other dietary treatments ($P<0.05$). The challenged chicks fed the control diet and the diets supplemented with 1% His, 1% Glu, and 1% Asn had lower d 21 intestinal IgA concentrations relative to the challenged chicks fed dietary supplemental BMD. The birds fed diets supplemented with 1% His had the lowest intestinal IgA concentrations, regardless of challenge, as compared to all the other treatment groups. The IgA concentrations in the intestines increased when Gln was supplemented in the diet and increased to d 7 with all the other treatments but then decreased to d 21 (compared to d 7). The intestinal IgA concentrations were higher in the SRBC challenged chicks relative to the non-challenged chicks.

The serum IgG concentrations were significantly higher in the non-challenged chicks (Table 4.4) and SRBC challenged chicks (Data not shown) fed diets supplemented with Gln (either source) relative to the other treatment groups, regardless of challenge ($P<0.05$). On d 21, the non-challenged and SRBC challenged birds fed diets supplemented with 1% His had lower serum IgG concentrations as compared to the other

treatment groups. The IgG concentrations increased in all treatments as the birds aged except for the birds fed diets supplemented with 1% His which had lower IgG levels at d 21 as compared to d 7. The serum IgG concentrations were higher in the SRBC challenged chicks relative to the non-challenged chicks fed diets supplemented with Gln (either source). Whereas, the chicks fed all of the other experimental diets had similar IgG concentrations, regardless of challenge.

The serum IgM concentrations were significantly higher in the non-challenged chicks and SRBC challenged chicks (Data not shown) fed diets supplemented with Gln (either source) relative to the other treatment groups, regardless of challenge ($P < 0.05$). The non-challenged and SRBC challenged birds fed supplemental 1% His had lower serum IgM concentrations as compared to the other treatment groups, regardless of challenge. The serum IgM concentrations were similar in the non-challenged chicks as compared to the SRBC challenged chicks fed the same treatment diet.

Challenged birds fed supplemental dietary Gln (either source) had significantly higher hemagglutination titers (Table 4.5) in response to SRBC as compared to the other dietary treatment groups with the birds fed diets supplemented with 2% Gln having the highest titer levels ($P < 0.02$). There was no statistical difference in the hemagglutination titers in response to SRBC among the Gln sources and there was no statistical difference in the hemagglutination titers among all the other dietary treatment groups.

DISCUSSION

Significant improvements in body weight gain were observed when 1% Gln was supplemented in the feed (12% increase). The body weight gains observed in this study were higher than the body weight gains previously reported in other studies (Bartell and

Batal, 2004), possibly being due to the increase in the body weight gain in the SRBC challenged chicks vs. the non-challenged chicks. An improvement in growth performance has not been previously reported due to the 1% supplementation of Glu, His, or Asn was not observed in this study. In a previous study, in which a 4% level of Gln supplementation was used, it was suggested that the depression in growth performance may be due to the large difference in the amount of SBM between the control and 4% Gln diets (Bartell and Batal, 2004). Since a depression in growth also occurred with the dietary supplementation of 2% Gln, it may be concluded that the depression is more likely due to a toxic effect rather than a difference in the amount of SBM in the diet because the change in SBM in 1% vs. 2% supplemented diet was minimal (decrease of 3%). Birds fed diets supplemented with 1% His (total 1.61% His) were not only smaller, but also experienced leg problems, suggesting that feeding a 1% His level above the NRC (1994) recommendation may be toxic, although no reports indicating a toxic level of His could be found. Birds fed diets supplemented with 1% Gln (new source) had significant depressed weight gain, which was surprising because a difference among Gln sources was not expected and may indicate that the improved growth performance in chicks due to 1% dietary Gln supplementation may be affected by where the Gln is produced.

Due to a problem of feed waste in previous studies (Bartell and Batal, 2004), grates were added to the feeders to minimize waste. As a result, an improvement in feed efficiency was observed when 1% Gln was supplemented to the diet, which had not been observed in our previous studies (Bartell and Batal, 2004). This result is consistent with findings of improvements in feed efficiency noted in swine (Kitts *et al.*, 2002), turkey

poult (Yi *et al.*, 2001b), and chicks (Yi *et al.*, 2005) when they were fed a diet supplemented with 1% Gln.

The birds fed diets supplemented with 1% or 2% Gln from either source, regardless of challenge, had significantly longer intestinal villi. Increased villi height has been proposed to increase performance by improved nutrient absorption (Coates *et al.*, 1954; Izat *et al.*, 1989). The increase in villi height may indicate that the birds fed diets supplemented with Gln could have had greater nutrient absorption and utilization. This increase in villi length might explain the heavier intestinal relative weights, improved weight gain, and feed efficiency that occurred due to Gln supplementation. Since there was not an increase in villi length or intestinal relative weights in the chicks supplemented with the other AA, it may explain why there was not an improvement observed in growth performance or feed efficiency in these treatments. However, an improvement in villi length was viewed with Gln (new source) and Glu supplementation although it did not result in an enhancement in performance.

The digestive mucosa is continuously exposed to dietary, bacterial, viral, and parasitic antigens (Strobel, 1986) and is protected by a barrier of the gut epithelium which is dependent upon the presence of IgA that functions primarily by preventing the attachment of bacterial to the mucosal cell (Burke *et al.*, 1989). Higher IgA concentrations in the serum, bile, and intestines observed in the birds fed diets supplemented with Gln, regardless of challenge or source, as compared to the other treatment groups supports previous findings that the addition of dietary Gln in broilers and rats helps to maintain higher IgA levels than viewed with other treatment groups without Gln supplementation (Bartell and Batal, 2004; Burke *et al.*, 1989). The increase

in IgA concentrations may indicate that chicks fed diets supplemented with Gln in the diet can better illicit an immune response against pathogens present in the gut. The role of the gut as a barrier is to prevent the spread of intraluminal bacteria in systemic organs and tissues (Sell, 1991). This indicates that the birds fed diets supplemented with 1% Gln (either source) may have a better gut barrier function when challenged with enteric diseases, since the birds had a higher IgA concentration in the intestines and the levels were higher in the challenged chicks vs. the non-challenged birds. Since supplementing the other AA had no effect on IgA levels, this may indicate that there is no improvement in gut barrier function and these birds would not perform as well as birds fed diets supplemented with Gln in response to an enteric disease challenge.

IgG expression is T-helper cell dependent (Singh, 1996) and is indicative of T-helper cell response (Mathers and Cuff, 2004). Since IgG levels did increase in non-challenged and SRBC challenged birds fed diets supplemented with Gln (either source), this may indicate that Gln and not the other AA stimulate the synthesis of the IgG antibodies or perhaps are required for thymus-derived (T)-cell helper function and response. The data compared here indicated that alterations of total IgG production induced by dietary Gln in the non-challenged chicks and chicks subjected to an antigenic challenge might reflect the potential of the chick's ability to illicit IgG antibody production when presented with a vaccination or disease challenge. However, further investigations are required. The birds fed diets supplemented with 1% His had lower IgG concentrations as compared to the other treatment groups giving further evidence that 1% His above the NRC (1994) recommendation (total 1.61% His) may be toxic. This

observation supports Tsiagbe's *et al.* (1987) conclusion that depressed IgG levels indicate a toxic effect.

The IgM concentrations increased any Gln supplementation from either source as compared to the birds fed the other dietary treatments, regardless of challenge. This is consistent with the fact that IgM expression requires T-helper cell action (Isakson *et al.*, 1983) and Gln has been shown to increase the number of T-helper cells (Kew *et al.*, 1999; Yeh, 2001). The increase in the IgM concentrations possibly indicates that the birds fed diets supplemented with Gln have more T-lymphoproliferative cells, which react efficiently with SRBC, a T-cell dependent antigen (Sarker *et al.*, 1999). The birds fed diets supplemented with Gln may have more B-cells for multiplication and differentiation as well as T-cells which react specifically to SRBC for antibody production. However, further investigation of the T-cell subsets and T-cell receptor along with B-cell number would be helpful to understand the immune mechanism.

The hemagglutination titers increased in response to the challenge with SRBC when birds are fed diets supplemented with 1% or 2% Gln from either source. This increase in the titers possibly indicates that birds fed diets supplemented with Gln may be better able to handle a disease or vaccine challenge, since resistance of poultry to diseases may be improved by selecting birds for the immune response to the SRBC antigen (Siwek *et al.*, 2004). It was not surprising to note an increase in the antibody concentrations and hemagglutination titers, since the antibodies in the serum are mainly produced by antibody-forming cells located in the spleen and immune tissue development is the basis of immune functionality (Al-Garib *et al.*, 2000; Russell and Ezeifeke, 1995), which may explain the increase in spleen size observed in birds fed dietary supplemental

Gln. This may also explain why there was no increase in spleen, thymus, or bursa weights, antibody concentrations, and hemagglutination titers observed with the dietary supplementation of BMD, His, Glu, or Asn. The present study indicates that modulation of antibody responses of chickens to SRBC was possible via the diet.

The results of this experiment give insights into a potential dietary method to modulate chicken immune responses toward improving chicken performance under a given condition. For example, the inflammatory response is the first line of defense against novel pathogens, but cells and mediators of the inflammatory responses have been implicated in the pathology of many poultry diseases, including coccidiosis (Trout and Lillehoj, 1993). Modification of antibody production and activity by dietary Gln supplementation may provide an avenue to strengthen the chicken's immunity and protect against various pathogens. However, long-term effects of immunomodulation induced by Gln supplementation on the resistance of chickens to commercially relevant infectious challenges and chick performance remains to be investigated.

In summary, performance and immune benefits viewed with dietary Gln supplementation could not be duplicated by supplementing the diet with His, Glu, or Asn. This suggests that benefits observed with dietary Gln supplementation to the diet was not due to the diet being deficient in non-essential amino acids or nitrogen. The lack of improvement in weight gain and antibody titers in the birds fed diets with 0.05% BMD was possibly due to the birds being placed in "clean" battery cages, where the birds were not challenged with disease antigens. The birds fed diets supplemented with Glu could not mimic the performance and immune results of the birds fed Gln. This finding is consistent with previous studies (Burrin *et al.*, 1991; Ewtushik, 2000; Newsholme *et al.*,

2003) in which the same effects were not observed with Glu as with Gln. This is possibly due to the fact that Glu may not be converted into Gln during immune stress or if it is, it is rapidly further metabolized (Pow, 1993) and it can not readily cross cell membranes, which means that Glu cannot cross the blood-brain barrier and be transported to other cellular tissues and act as Gln (Newsholme *et al.*, 2003). The improvement in villi length that occurred with Glu supplementation was a surprise since such an observation could not be found in previous reports. His could not mimick the performance and immune results that occur with Gln supplementation and was thought to be toxic supplemented at the 1% level (total 1.61% His). Since His is manufactured similarly to Gln, it would lend evidence to the statement that bacterial fermentation is not a factor in the benefits observed with Gln supplementation, but that was more likely due to the toxic effect of His. Although, residual bacteria as the reason for improved growth and immune responses cannot be ruled out. Further studies are needed to eliminate residual bacteria as a factor for improvements observed in this study. The supplementation of 2% Gln, 1% Gln (new source), BMD, His, Glu, or Asn to the diet could not replicate the same benefits in growth performance or enhancement of the immune response that are observed with 1% Gln supplementation. Thus, dietary supplementation of 1% Gln enhances performance, development of the gastrointestinal tract, and immune system in broilers. However, the improvement in performance seems to be source dependent.

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TABLE 4.1. Composition of the experimental diets¹ (as-fed basis)

Ingredients	Control	BMD	%				
			1% Gln	2% Gln	1% His	1% Glu	1% Asn
Corn	51.90	51.90	52.90	54.30	53.80	51.70	53.40
Soybean meal	38.28	38.28	35.56	32.75	34.37	37.05	35.22
Poultry fat	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Limestone	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Dicalcium P	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Vitamin premix ²	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Mineral premix ³	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30
DL-Met	0.17	0.17	0.25	0.25	0.22	0.19	0.21
L-Lys-HCL	---	---	---	0.01	---	---	---
Sand	0.72	0.67	1.40	1.76	1.68	1.13	1.24
BMD ⁴	---	0.05	---	---	---	---	---
L-Glutamine ⁵	---	---	1.00	2.00	---	---	---
L-Histidine ⁶	---	---	---	---	1.00	---	---
L-Glutamic Acid ⁷	---	---	---	---	---	1.00	---
L-Asparagine ⁸	---	---	---	---	---	---	1.00

¹ Calculated Composition 22.5% CP, 3,200 kcal TME/kg.

² Vitamin premix supplied (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), 12.0 µg; 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-retinyl acetate, 5,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.

³ Mineral premix supplied (per kilogram of diet): manganese (MnSO₄·H₂O), 60 mg; iron (FeSO₄·7H₂O), 30 mg; zinc (ZnO), 50 mg; copper (CuSO₄·5H₂O), 5 mg; iodine (ethylene diamine dihydroiodide), 1.5 mg; selenium (Na₂SeO₃), 0.3mg.

⁴ BMD= bacitracin dimethylene salicylate (supplied 387 g per gram of diet).

⁵ Provides 19.2% N

⁶ Provides 27.1% N

⁷ Provides 9.52% N

⁸ Provides 21.2% N

TABLE 4.2. The effect of supplementing amino acids on weight gain and feed efficiency (gain:feed) of broilers at 21 d of age

Treatment ¹	Body Wt. Gain (g/chick)	Gain:Feed (g/kg)
Corn-SBM ²	665 ^b	730 ^{bc}
BMD ³	667 ^b	740 ^b
1% Glutamine	736 ^a	820 ^a
2% Glutamine	607 ^{cd}	680 ^d
1% Glutamine (New source) ⁴	619 ^c	690 ^d
1% Histidine	579 ^d	700 ^{cd}
1% Glutamic Acid	663 ^b	730 ^{bc}
1% Asparagine	658 ^b	710 ^{cd}
Pooled SEM	11.1	10.0

^{a-d}Means within columns having the same superscript do not differ significantly (P < 0.05).

¹Means represent 6 pens per treatment; 10 chicks per pen (60 chicks per treatment).

²SBM=soybean meal.

³BMD= bacitracin dimethylene salicylate.

⁴The term “new” refers to birds that were fed diets supplemented with 1% Gln from a new source vs. the original Gln used here and in previous studies.

TABLE 4.3. The effect of supplementing amino acids on the 14 to 21 d body weight gain (g/chick) of SRBC challenged vs. unchallenged broilers

Treatment ¹	Challenged Birds	Unchallenged Birds
Corn-SBM ²	380 ^{bc}	367 ^b
BMD ³	378 ^{bc}	367 ^b
1% Glutamine	446 ^a	426 ^a
2% Glutamine	388 ^{bc}	329 ^{cd}
1% Glutamine (New source) ⁴	396 ^b	345 ^{bc}
1% Histidine	351 ^c	309 ^d
1% Glutamic Acid	400 ^b	363 ^b
1% Asparagine	392 ^b	357 ^{bc}
Pooled SEM	12.7	9.8
p-value	0.0002	0.0001

^{a-d}Means within columns having the same superscript do not differ significantly (P < 0.05).

¹Means represent 6 pens per treatment; 3 challenged chicks per pen (18 chicks per treatment) and 5 unchallenged chicks per pen (30 chicks per treatment).

²SBM=soybean meal.

³BMD= bacitracin dimethylene salicylate.

⁴The term “new” refers to birds that were fed diets supplemented with 1% Gln from a new source vs. the original Gln used here and in previous studies.

TABLE 4.4. Effect of supplementing various amino acids on d 21 villous height and humoral immune response of unchallenged broilers

Treatment ¹	Duodenal Villi Height	Serum IgA Concentrations	Intestinal IgA Concentrations	Serum IgG Concentrations
Corn-SBM ²	335.8 ^c	0.808 ^b	0.505 ^b	1.579 ^b
BMD ³	387.1 ^c	0.845 ^b	0.495 ^b	1.539 ^b
1% Glutamine	557.1 ^a	1.806 ^a	1.310 ^a	1.971 ^a
2% Glutamine	585.2 ^a	1.784 ^a	1.144 ^a	1.953 ^a
1% Glutamine (New source) ⁴	517.9 ^a	1.694 ^a	1.380 ^a	1.874 ^a
1% Histidine	352.9 ^c	0.790 ^b	0.290 ^c	1.090 ^c
1% Glutamic Acid	441.5 ^b	0.890 ^b	0.506 ^b	1.494 ^b
1% Asparagine	386.3 ^c	0.700 ^b	0.540 ^b	1.426 ^b
Pooled SEM	17.83	0.23	0.06	0.11

^{a-c}Means within columns and age period having the same superscript do not differ significantly (P < 0.05).

¹Means represent 6 pens per treatment; 5 chicks per pen (30 chicks per treatment).

²SBM=soybean meal.

³BMD= bacitracin dimethylene salicylate.

⁴The term “new” refers to birds that were fed diets supplemented with 1% Gln from a new source vs. the original Gln used here and in previous studies.

TABLE 4.5. Effect of supplementing various amino acids on d 21 villous height and humoral immune response of SRBC challenged broilers

Treatment ¹	Duodenal Villi Height	Serum IgA Concentrations	Intestinal IgA Concentrations	Log ₂ Hemagglutination Titer
Corn-SBM ²	366.0 ^c	0.864 ^b	1.622 ^b	6 ^c
BMD ³	355.4 ^c	0.980 ^b	1.789 ^b	7 ^c
1% Glutamine	488.2 ^a	2.862 ^a	2.473 ^a	18 ^b
2% Glutamine	503.0 ^a	2.671 ^a	2.259 ^a	26 ^a
1% Glutamine (New source) ⁴	470.5 ^a	2.685 ^a	2.132 ^a	15 ^b
1% Histidine	338.8 ^c	1.029 ^b	1.259 ^c	3 ^c
1% Glutamic Acid	409.4 ^b	1.003 ^b	1.541 ^b	5 ^c
1% Asparagine	364.3 ^c	0.884 ^b	1.655 ^b	6 ^c
Pooled SEM	12.54	0.54	0.09	0.11

^{a-c}Means within columns and age period having the same superscript do not differ significantly (P < 0.05).

¹Means represent 6 pens per treatment; 3 chicks per pen (18 chicks per treatment).

²SBM=soybean meal.

³BMD= bacitracin dimethylene salicylate.

⁴The term “new” in the legend refers to birds that were fed diets supplemented with 1% Gln from a new source vs. the original Gln used here and in previous studies.

CHAPTER 5

THE EFFECT OF SUPPLEMENTAL GLUTAMINE ON GROWTH PERFORMANCE,
DEVELOPMENT OF THE GASTROINTESTINAL TRACT, AND IMMUNE RESPONSE OF
BROILERS VACCINATED AND CHALLENGED WITH *EIMERIA ACERVULINA* AND
*EIMERIA MAXIMA*¹¹

¹¹ Bartell, S.M. and A.B. Batal. Will be submitted to *Poultry Science*.

ABSTRACT An experiment was conducted to evaluate the effect of dietary glutamine (Gln) supplementation on growth performance, development of the gastrointestinal tract and immune response of broilers vaccinated and challenged with *Eimeria*. There were 12 experimental treatments in a 3x2x2 factorial design (dietary treatment x vaccination x coccidia challenge). Immediately after hatch, sixteen replicate pens of thirty-five chicks were randomly assigned to one of three dietary treatments, 1) corn-soybean meal control diet, 2) diet supplemented with 1% Gln fed for the starter period (0 to 19 d), after which time the birds were fed the control corn-SBM diet from 19 to 40 d, and 3) a diet supplemented with 1% Gln was fed for the entire experimental period (0 to 40 d). All diets were formulated to be isocaloric and isonitrogenous. Immediately posthatch, half of the birds were vaccinated for *Eimeria*. On d 19, half of the vaccinated and half of the non-vaccinated birds were challenged with *Eimeria*. On d 0, 19, 32 and 40 twelve chicks per treatment were sacrificed for duodenum, jejunum, bile, and blood sample collections. On d 25, five birds per pen were sacrificed for small intestine lesion scores. Overall, weight gain and feed efficiency improved significantly ($P<0.001$) in the vaccinated, non-vaccinated, challenged, or non-challenged birds fed diets supplemented with 1% Gln as compared to the non-challenged, non-vaccinated chicks fed the control diet. Vaccinated birds fed diets supplemented with 1% Gln performed better than the vaccinated control birds and performed better than non-vaccinated control birds ($P<0.05$). Challenged birds fed diets supplemented with 1% Gln performed better than the challenged control birds and performed the same as the non-challenged control birds ($P<0.05$). The chicks fed diets supplemented with 1% Gln had lower intestinal lesion scores ($P<0.05$) and less incidences of necrotic enteritis than the control birds. Concentrations of bile, intestinal

and serum IgA and serum IgG, IgM, and IFN- γ were higher in chicks fed diets with 1% Gln for 40 d ($P<0.05$). Breast yield was larger in the birds fed diets supplemented with 1% Gln ($P<0.001$) as compared to the birds fed the control corn-SBM diet. The results indicate that 1% dietary Gln supplementation in broiler diets improves growth performance and may stimulate development of the gastrointestinal tract and immune system when challenged or vaccinated with *Eimeria*.

Key Words: Glutamine, gastrointestinal tract, broiler chicks, *Eimeria*, immune response

INTRODUCTION

Avian coccidiosis is a major parasitic disease of substantial economic significance and it is estimated to cost the poultry industry greater than \$800 million in annual losses (Lillehoj *et al.*, 2003). Intestinal parasitism is a major stress factor leading to malnutrition and lowering performance and production efficiency of livestock, especially chickens. Avian coccidiosis is an infectious disease caused by any of the species of *Eimeria* individually or in combination. Two of the commonly occurring species of *Eimeria* in poultry are *E. aceruvlina* and *E. maxima*. The different species of *Eimeria* attack different areas of the gastrointestinal tract, *E. aceruvlina* attacks the upper region (duodenum) and *E. maxima* attacks the mid region (jejunum) of the small intestine. *Eimeria* species invade the intestinal mucosa and induce a degree of epithelial cell damage and inflammation (Finlay *et al.*, 1993), often times leading to secondary infections, such as necrotic enteritis (Parish, 1961). Extensive damage leads to diarrhea, dehydration, clinical illness, and often mortality (Cook, 1988). Nutrition, microflora, pathogens, and other factors affect the maintenance of the digestive tract and its associated immune system. The gut wall is a primary line of defense between the environment and the internal physiology of the animal. The breakdown in the mucosa of the gut opens a pathway for toxins and limits the transport and passage of nutrients into the host, resulting in reduced weight gain (Duffy *et al.*, 2005). The reduced weight gain caused by nutrient malabsorption is a major indicator of a coccidiosis infection and it is this loss in performance, even in sub clinical infection levels of the disease, that is of economic importance to the producer (Ruff and Wilkins, 1990). Currently, anticoccidial

drugs and vaccines are the methods used to control coccidiosis (McDougald, 1990). The continuous misuse of anticoccidial drugs that have led to the emergence of drug resistant strains (Long, 1982), the many problems of vaccines (McDougald, 1990), and consumer demands of drug and antibiotic free foods (Duffy *et al.*, 2005) have stimulated the search for new methods of control. A possible strategy for the control of *Coccidia* is the use of use of supplemental glutamine.

L-glutamine (Gln) is an important precursor for the synthesis of amino acids, nucleotides, proteins, and many other biologically important molecules (Souba, 1993). Glutamine is the main energetic substrate for rapidly proliferating cells such as intestinal enterocytes and activated lymphocytes and macrophages (Calder and Yaqoob, 1999), and is considered a conditionally essential amino acid in some species under inflammatory conditions such as infection and injury (Newsholme, 2001). Glutamine is known for its role in the differentiation of epithelial cells and is essential for the integrity of mucosal surfaces (Calder and Yaqoob, 1999). Coccidial infections led to keratinization of the secretory mucosal epithelia, thereby compromising the physical barrier and modulating intestinal epithelial signals involved in inflammatory responses (Kagnoff, 1996; Kagnoff *et al.*, 1997). Glutamine is used at a high rate by isolated cells of the immune system in culture and is required to support optimal lymphocyte proliferation and cytokine production by lymphocytes and macrophages (Calder and Yaqoob, 1999). The immune response of broiler chicks infected with *Eimeria* includes generation of superoxide and nitric oxide free radicals at infection sites (Allen, 1997). Glutamine may play a role in eliminating those free radicals because it is also a precursor for antioxidant glutathione synthesis (Wu, 1998). Studies have shown that following a coccidial infection, both cell-

mediated and humoral immunity play a role in resistance (Guo *et al.*, 2003). T-cells limit oocyst production (Lillehoj *et al.*, 1989; Martin and Lillehoj, 1993; Martin *et al.*, 1994; Lillehoj, 1998) and antibodies in the serum and intestine may play a part in resistance against coccidial infections (Wallach *et al.*, 1994). Based on previous studies that have shown that supplementing 1% Gln in chick diets significantly improves chick performance, improves feed efficiency (body wt. gain:feed intake), increases villi height (Bartell and Batal, 2004; Bartell and Batal, 2005; Yi *et al.*, 2005), and increases concentrations of serum, bile, and intestinal IgA, serum IgG, and serum IFN- γ and IL-2 (Bartell and Batal, 2005; Yi *et al.*, 2005), Gln supplementation may be beneficial in helping the bird be more resistant to Coccidial infections. Glutamine's immunomodulatory effects, its role in maintenance of the immune system and the high antibody responses to SRBC when chicks are fed diets supplemented with Gln (Bartell and Batal, 2005), which has been shown to be indicative of chicks being more resistant to *Eimeria* species (Gross *et al.*, 1980) suggest that Gln supplementation may be a good strategy for the control of enteric diseases such as Coccidia. Therefore, the objective of this study was to evaluate the effect of supplemental glutamine on growth performance, development of the gastrointestinal tract, and immune response of broilers vaccinated and challenged with *E. acervulina* and *E. maxima*.

MATERIALS AND METHODS

Experimental Birds and Diets

A study was conducted with Cobb 500 by-product male chicks obtained from a local hatchery and immediately placed in floor pens in an environmentally controlled room. Chicks were weighed and randomly allotted to pens such that each pen of chicks

had a similar initial weight distribution. Chicks were maintained on a 24 hour constant lighting schedule. The room temperature was maintained at 80 to 75 degrees F. Chicks had *ad libitum* access to feed and water. The dietary treatments (Trt) were formulated to meet or exceed the NRC (1994) recommendations and were fed from 0 to 40 d of age. The experimental diets were formulated to be isonitrogenous and isocaloric with 22.5% crude protein, 3,100 kcal TME/kg, 0.9% total sulfur amino acids (TSAA), 1.2% Lys, and a constant fat level of 5% during the starter period (0 to 19 d), 22.0% crude protein, 3,150 kcal TME/kg, 0.72% TSAA, 1.1% Lys, and a constant fat level of 3.5% during the grower period (19 to 32 d), and 18.0% crude protein, 3,200 kcal TME/kg, 0.6% TSAA, 0.85% Lys, and a constant fat level of 3% during the finisher period (32 to 40 d) (Table 5.1). Group body weight and feed intakes were measured on d 0, 19, 25, 32, and 40. Weight gain and feed efficiency (gain:feed) were calculated for each pen. Calculations were adjusted for mortalities when appropriate.

One thousand six hundred and eighty chicks were randomly divided into 12 treatment groups of 4 replicates of 35 birds each in a 3x2x2 factorial design. There were 3 dietary treatments with half of the birds being vaccinated or half non-vaccinated, and half challenged with *Eimeria* or half not challenged. The treatments groups are represented in Table 5.2. The chicks were placed on one of three experimental diets consisting of a control corn-SBM diet fed for the entire experiment, a corn-SBM diet supplemented with 1% Gln fed only during the starter period (0 to 19 d) after which time the chicks were fed the control corn-SBM diet, and a corn-SBM diet supplemented with 1% Gln fed for the entire experimental period (d 0 to 40). On d 0, half of the birds (treatments 4 to 6 and 10 to 12) were vaccinated orally with 100 μ L consisting of 500

viable sporulated oocysts of both *E. aceruvlina* and *E. maxima*. Half of the vaccinated and half of the unvaccinated birds (treatments 7 to 12) were orally challenged with 1 mL of 250,000 viable sporulated oocysts of *E. aceruvlina* and 100,000 oocytes of *E. maxima* on d 19 posthatch. On d 40, 10 birds per pen (40 chicks per treatment) were processed for carcass yields.

Sampling

On d 0, 25, 32 and 40 twelve chicks per treatment (three chicks per pen) were randomly selected for sampling of intestinal measurements. Chicks were weighed and killed by cervical dislocation, and then the abdominal cavity was opened. For intestinal samples, the small intestine was removed and divided into three segments: duodenum (from gizzard to entry of the bile and pancreatic ducts), jejunum (from entry of the ducts to yolk stalk), and ileum (from yolk stalk to ileocecal junction). The ileum was flushed with 10 to 20 mL of deionized water. Because morphologic analysis of the duodenum and jejunum was to be determined, these segments were flushed with 20 mL of physiological saline solution. For morphologic analysis, approximately 5 cm of the middle portion of the duodenum and jejunum (the apex of the duodenum and midway between the point of entry the bile ducts and Meckel's diverticulum of the jejunum) was excised and fixed in 10% formalin. Six cross sections of 70% ethanol-preserved segments for each duodenal and jejunal sample were then prepared for staining with hematoxylin and eosin using standard paraffin embedding procedures (Uni *et al.*, 1995). A total of 4 intact, well-oriented villi were selected in six replicates for each intestinal cross section (24 measurements for each intestinal sample with 288 measurements per treatment). Villus height was measured from the tip of the villi to the villus crypt

junction. Morphological indices were determined using computer-aided light microscope (40x magnification) image analysis¹².

Blood, bile, and jejunum samples were collected from 3 birds per pen (twelve birds per treatment) on d 19, 25, 32, and 40. Blood was obtained by jugular venipuncture from each bird. Blood samples were centrifuged at 1000 X g for 10 min at room temperature and the serum fraction was frozen and stored at -20 C until analyzed. The birds were killed by cervical dislocation, and bile and jejunal samples were obtained. Bile was aspirated from the gall bladder with a 25-gauge needle coupled to a 3-mL syringe and then stored at -20 C until IgA and IFN- γ analysis. The jejunum, i.e., the portion of the small intestine between the opening of the pancreobiliary ducts and the yolk stalk, was excised from each bird. Ten centimeters of the middle portion of the jejunum was separated and stored at -20 C until prepared for analysis. At the time of analysis, the jejunal samples were thawed at room temperature, 2 g of jejunal sample was weighed, 20 mL of deionized water was added, and it was homogenized for 30 s with a mechanical homogenizer¹³ at a speed setting of 5. An aliquot (5 mL) of the sample was centrifuged at 20,000 x g for 30 min. The supernatant was obtained and stored at -20 C until analyzed for IgA and IFN- γ concentration.

Analysis of Ig in Serum, Bile, and Intestine

Serum samples for all treatments and age groups were analyzed for IgA, IgG, and IgM at the same time to avoid variation that may occur with analyses done at different times. Serum, bile, and jejunal IgA and serum IgG and IgM were determined by using a

¹² Image-Pro Plus Version 3.0, Media Cybernetics, Silver Spring, MD 20910.

¹³ VirTis, Gardiner, NY 12525.

double antibody technique ELISA kit¹⁴. Absorbance was measured at 450 nm. The absorbance of the control wells were adjusted to zero prior to measuring absorbance in the samples. Because absorbance units are linearly related to the logarithm of the Ig concentration (Piquer *et al.*, 1991), the absorbance measurements obtained could be used as estimates of Ig concentrations. Therefore, no standard curve was used to calculate Ig concentrations.

Analysis of IFN- γ in Serum and Intestine

The serum and intestine IFN- γ measurements were analyzed with a cytoset kit¹⁵, using the methodology described by Dalloul *et al.* (2003). Briefly, flat-bottom microplates³ were coated with 100 μ L of 0.2 M sodium carbonate buffer and covered for 18 h at 4°C and washed 3 times with PBS containing 0.05% Tween-20 (PBS-T). This step was followed by blocking with PBS-2% BSA¹⁶ for 2 h at room temperature, and then plates were washed 3 times with 400 μ L of PBS-T. To each well, 100 μ L of mouse anti-chicken IFN- γ was added and allowed to incubate for 2 h at room temperature on a plate shaker (700 rpm). The plates were washed 3 times with 100 μ L of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (dilutions were 1:1000) was added to each well and allowed to incubate for 30 min at room temperature with shaking. Then 100 μ L/well of 1.8 N H₂SO₄ was added to the plate and the optimal density was read at 450 nm by a microplate reader.

Lesion Scores

On d 25, 5 birds per pen (20 birds per treatment) were killed for duodenum (*E. aceruvlina*) and jejunum (*E. maxima*) samples to be evaluation for lesion scores. The

¹⁴ Bethyl Laboratories, Inc., Montgomery, TX 77356.

¹⁵ Biosource, Camarillo, CA 93012

lesion scoring method used in this study was described by Johnson and Reid (1970). It is a 4-point system, based on the small reddish lesions formed in the small intestine 6 d after a challenge with *Eimeria*. The higher the lesion score, the more severe the *Eimeria* infection (0 means no *Eimeria* infection). The intestines were also visually evaluated for the appearance for necrotic enteritis.

Statistical Analysis

All the data were subjected to ANOVA procedures for completely randomized designs and for factorial arrangements of treatments and their interactions using the GLM procedure of SAS® (SAS Institute, 1990). Statistical significances of differences among treatment group means were determined using Duncan's multiple range test (Duncan, 1955). A probability level of $P \leq 0.05$ was used to determine statistical significance.

RESULTS

The growth performance and feed efficiency results are presented in Table 5.3. Overall (d 0-40), birds that were fed diets supplemented with 1% Gln for the entire experimental period had significantly higher body weight (BW) gains and better feed efficiencies than the birds fed the control diets, regardless of vaccination or challenge ($P < 0.0001$). The addition of dietary Gln during the starter period (Trt 2, 5, 8, 11) increased BW as compared to its perspective control except for the birds that were vaccinated and challenged (Trt 10 and 11). Vaccination with *E. acervulina* and *E. maxima* sporulated oocysts immediately after placement decreased the growth performance of broiler chicks fed the control corn-SBM diet during the first 2 wk, whereas the birds fed diets supplemented with 1% Gln during the starter period performed the same as the non-vaccinated control birds. However, the vaccinated birds

¹⁶ Sigma Chemical Co., St. Louis, MO

fed the 1% Gln supplemented diets for the entire experimental period gained significantly more weight than the non-vaccinated birds fed the control corn-SBM diet. The vaccinated, challenged birds fed diets supplemented with 1% Gln for the entire experimental period (Trt 12) performed the same as the unchallenged, non-vaccinated birds fed the control diet (Trt 1). Overall (0-40 d), the non-challenged birds had better BW gains and feed efficiencies than the birds challenged with *Eimeria*. The challenged birds fed diets supplemented with Gln had significantly higher BW gains and feed efficiencies than the birds challenged with *Eimeria* and fed the control corn-SBM diet. The birds fed diets supplemented with 1% Gln for the entire experimental period and challenged with *Eimeria* (Trt 9) had the same BW as the birds fed the control diet (Trt 1). The carcass yield results are presented in Table 5.4. In general, the birds fed diets supplemented with 1% Gln for the entire experimental period had larger breast meat yields and front-half meat yields, regardless of vaccination or challenge than birds fed the control corn-SBM diet.

The d 25 (6 d post-challenge) lesion scores for *E. acervulina* and *E. maxima* are presented in Table 5.5. After the *E. acervulina* and *E. maxima* challenge on d 19, there were differences in lesion scores of the upper (duodenum) small intestine and in the mid (jejunum) small intestine as seen with *E. acervulina* and *E. maxima* respectively ($P < 0.0001$). Birds fed the control corn-SBM diet and challenged with *E. acervulina* and *E. maxima* (Trt 7) had the highest lesion scores in the upper small intestine and middle small intestine. Birds fed diets supplemented with 1% Gln had decreased incidences of lesions and secondary infections (positive for necrotic enteritis). The vaccinated, challenged birds fed diets supplemented with Gln (Trt 11, 12) had the lowest lesion scores and no

incidences of secondary infections. Birds fed diets supplemented with 1% Gln had significantly lower *E. acervulina* and *E. maxima* oocyte litter counts (data not shown) as compared to the birds fed the control corn-SBM diet, regardless of vaccination ($P < 0.01$). During wk 1, vaccinated birds fed supplemental Gln diets had higher *E. maxima* litter counts as compared to birds fed non-Gln supplemented diets; but by the end of the experiment, they had the lowest litter counts. The birds that were vaccinated, regardless of the diet that was fed, had almost no fecal *E. acervulina* and *E. maxima* oocyst output by wk 3.

Villi height in the duodenum of chicks d 25 and 40 post hatch is shown in Table 5.6. The birds fed diets supplemented with Gln had longer villi, regardless of vaccination or challenge, as compared to the birds fed the control diet in their respective treatment groups. The vaccinated, challenged birds fed diets supplemented with Gln for the entire experimental period had the longest villi as compared to all the other treatments ($P < 0.01$). The challenged birds had longer villi than the vaccinated birds, regardless of dietary treatment.

Ig concentrations of chicks d 25 and 40 post hatch are presented in Table 5.6. Overall, birds fed diets supplemented with 1% Gln for the entire experimental period, regardless of vaccination or challenge, had significantly higher intestinal IgA concentrations as compared to the birds fed the control corn-SBM diet ($P < 0.02$). At d 40 the birds fed Gln-supplemented diets for the entire experimental period that were vaccinated and challenged (Trt 12) or non-vaccinated and non-challenged (Trt 3) had the highest intestinal IgA concentrations and the challenged birds fed the control diet had the lowest intestinal IgA concentrations. Birds fed diets supplemented with 1% Gln,

regardless of vaccination or challenge, had higher IgA concentrations in the bile on d 40 (data not shown) as compared to the birds fed the control corn–SBM diet ($P < 0.03$). Birds fed diets supplemented with 1% Gln diets had higher serum IgA ($P < 0.02$), IgG ($P < 0.04$), and IgM ($P < 0.04$) concentrations, regardless of vaccinated or challenge, as compared to the birds fed the control diet.

The d 25 and 40 serum and intestinal cytokine IFN- γ concentrations are presented in Table 5.6. Birds fed diets supplemented with Gln had higher serum IFN- γ ($P < 0.03$) and intestinal IFN- γ ($P < 0.01$) concentrations, regardless of vaccinated or challenge, as compared to the birds fed the corn-SBM control diet.

DISCUSSION

Body weight gain, feed conversion, lesion scores, and oocyst shedding are commonly used to evaluate the resistance or susceptibility of poultry to a coccidiosis challenge (Zhu *et al.*, 2000). Poor weight gain and feed efficiency are characteristic of coccidiosis-infected broiler chicks, particularly through the 4- to 9-d period after infection, when the parasite is rapidly reproducing in the upper small intestine (Allen, 2000; Matthews and Southern, 2000; Zhu *et al.*, 2000). Birds challenged with *Eimeria* and fed diets supplemented with 1% Gln had improved growth and feed efficiency as compared to the challenged birds fed the control diet, which suggests that dietary Gln supplementation may have a beneficial effect on improving growth performance of broilers challenged with *Eimeria*. One preventive measure against the *Eimeria* infection is a vaccination program. The supplementation of Gln in the diet allowed the vaccinated birds to perform better than the vaccinated control birds and eliminated the weight depression that is often observed after vaccination. These results presented agree with

the study reported by Yi *et al.* (2005) in which the dietary addition of Gln allowed vaccinated and challenged birds to perform better than their perspective groups. The vaccination allowed birds to have better performance during an *Eimeria* challenge.

Some factors that may contribute to the increased growth performance observed when diets were supplemented with Gln may be the supply of enteral Gln as an energy substrate for rapidly proliferating enterocytes and a nitrogen source for nucleotide synthesis (Calder and Yaqoob, 1999). Another factor may be Gln role in maintaining mucosal structure, especially in the maintenance of the tight junction and permeability of intestinal mucosa (Panigrahi *et al.*, 1997), since IgA is a marker for gut barrier function (Sell, 1991). IgA functions primarily by preventing the attachment of bacterial to the mucosal cell (Burke *et al.*, 1989). The role of the gut as a barrier is to prevent the spread of intraluminal bacteria in systemic organs and tissues, which helps to reduce the incidences of infection. It may be concluded that birds fed diets supplemented with 1% Gln may have had better gut barrier function since the birds had a higher IgA concentration in the intestines. Because the birds had high IgA concentrations, the gut may have been better protected against the invading parasites and thus may be why the chicks appeared to be more resistant to the *Eimeria* infection and did not experience the same negative effects (i.e. reduced growth, higher lesion scores, and secondary infections) as the control birds did.

Glutamine may also act as a signal or regulator of metabolic processes, increasing protein synthesis and decreasing protein degradation in skeletal muscle of young broilers (Haussinger *et al.*, 1994). Chickens fed additional Gln in the diet had higher breast meat yields, regardless of vaccination or challenge, possibly indicating that Gln

supplementation could be used for protective roles against infection while allowing remaining nutrients to be used for muscle development.

Birds challenged with *Eimeria* had increased small intestine lesion scores compared with the vaccinated birds, indicating more severe intestinal infection of *E. acervulina* and *E. maxima* in the non-vaccinated birds and the success of the vaccination program in alleviating the deleterious effect of *Eimeria* infection. The increase in lesion scores and incidences of secondary infections in birds fed the control diet leads one to believe that a coccidian vaccination program along with Gln supplementation in the diet reduce the susceptibility of chickens to an *Eimeria* infection. This may indicate that Gln supplementation can stimulate intestinal integrity and immune system maturation and suggests that the birds fed diets supplemented with Gln had better gut barrier protection via the epithelial cells and intestinal lymphocytes due to the protective roles intestinal epithelial cells and intestinal lymphocytes play in maintaining gut barrier function (Adjei *et al.*, 1994; Bartell and Batal, 2004; Bartell and Batal, 2005; Naka, 1996; Yi *et al.*, 2005). Early modulation of immune elements in the intestinal epithelium because of the lack of available Gln may be one explanation since it has been suggested that Gln may be an essential amino acid during infections (Newsholme, 2001). Another reason may be explained by Gln role in maintaining the epithelial barrier (Adjei *et al.*, 1994; Naka 1996) and that the coccidiosis infection may have resulted in a higher demand for available Gln which may have resulted in a decrease in available Gln in the intestinal epithelium allowing *Eimeria* to more easily penetrate into the intestinal epithelium.

In the *Eimeria* challenged birds, histological examination revealed sloughing of the intestinal lining and reduced villous height. Villous atrophy is a well-known

histopathologic consequence of coccidiosis, and results in increased metabolic activity to compensate for epithelial destruction (Rose *et al.*, 1992). In the present experiment, significant increases of intestinal villous height during *Eimeria* infection in birds fed diets supplemented with Gln were observed and are in agreement with results reported by Yi *et al.* (2005). The increases in villus height in birds fed supplemental Gln diets appeared to correlate to better nutrient absorption and utilization as evidenced by the improvement in growth during *E. acervulina* and *E. maxima* challenge. New epithelial cells are produced in the intestinal mucosal crypts and migrate in an orderly fashion along the villi to the tips where cells desquamate (Schat and Myers, 1991). During infection, this metabolic process may be enhanced to protect the exposed lamina propria and to replace infected or damaged cells and thus may be how Gln is supplying energy needed for new epithelial cells and migration. However, in another study in our lab in which birds were fed diets supplemented with Gln (new source) there was an increase in villi height although there an improvement in growth performance was not observed (Bartell and Batal, 2005).

The principal defense mechanism against protozoa (*E. maxima* in this case) that survive within macrophages is cell-mediated immunity (Lillehoj and Trout, 1996). T-cells induce a positive immune response that limits oocyst production in primary and subsequent coccidial infections (Lillehoj, 1998; Lillehoj *et al.*, 1989; Martin and Lillehoj, 1993; Martin *et al.*, 1994). Cytokines have been used as measures of cellular immunity to coccidial infection (Lillehoj and Trout, 1996; Yun *et al.*, 2000); in particular, IFN- γ has been used as a common marker of T-cell responses to *Eimeria* (Martin *et al.*, 1994; Yun *et al.*, 2000). In the present study, serum IFN- γ concentrations were higher in the birds fed diets supplemented with 1% Gln upon challenge compared to the birds fed the control

corn-SBM diet. Chicken IFN- γ regulates acquired immunity by activating lymphocytes and enhancing expression of the major histocompatibility complex class II antigens (Kaspers *et al.*, 1994). IFN- γ is the macrophage-activating cytokine that provides the means by which TH1 CD⁺4 T-cells activate macrophages to kill intracellular parasites (Abbas *et al.*, 2000). The results of this study indicate that Gln supplementation may be an effective immunostimulant to increase IFN- γ production and activate macrophages to kill intracellular *Eimeria* parasites. The early cellular immune responses characterized by local IFN- γ production are critical for gut defense against coccidiosis (Dalloul *et al.*, 2000). Correlation of disease resistance with early local production of IFN- γ indicates an important role of this cytokine in protective immunity against avian coccidiosis (Yun *et al.*, 2000). Increased intestinal IFN- γ levels in *Eimeria*-infected chicks fed diets supplemented with Gln compared with chicks fed the control diet may be related to their enhanced disease resistance.

In conclusion, the addition of Gln to the diet eliminated the weight depression often observed with vaccination. Birds fed diets supplemented with Gln may have had better gut barrier function along with enhanced immune responses during the *Eimeria* challenge. Therefore, dietary Gln supplementation during *Eimeria* vaccination and challenge may improve growth performance due to the improvements observed in intestinal development, maintenance, and increases in humoral and cell-mediated immune responses.

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TABLE 5.1. Composition of Experimental diets¹ (as-fed basis)

Ingredients	Starter		Grower		Finisher	
	Control	1% Gln	Control	1% Gln	Control	1% Gln
	%					
Corn	50.00	51.30	60.60	61.90	67.30	68.50
Soybean meal	38.59	35.83	31.77	29.00	25.16	23.80
Poultry fat	5.00	5.00	3.50	3.50	3.00	3.00
Limestone	1.50	1.50	1.52	1.52	1.42	1.42
Dicalcium P	1.80	1.80	1.30	1.30	1.05	1.05
Vitamin premix ²	0.25	0.25	0.25	0.25	0.25	0.25
Mineral premix ³	0.08	0.08	0.08	0.08	0.08	0.08
Salt	0.30	0.30	0.30	0.30	0.30	0.30
DL-Met	0.18	0.21	0.05	0.09	---	0.02
L-Thr	---	---	---	---	0.90	---
Sand	2.30	2.73	0.63	1.06	0.54	0.58
L-Gln	---	1.00	---	1.00	---	1.00

¹ Calculated composition 22.5% CP, 3,100 kcal TME/kg, 0.9% total sulfur amino acids, 1.2% total Lys for starter period (0 to 19 d), 22.0% CP, 3,150 kcal TME/kg 0.72% total sulfur amino acids, 1.1% total Lys, for grower period (19 to 32 d), 18.0% CP, 3,200 kcal TME/kg 0.6% total sulfur amino acids, 0.85% total Lys, for finisher period (32 to 40 d).

² Vitamin premix supplied (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), 12.0 µg; 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, 25.5 µg; trans-retinyl acetate, 1,892 µg; all-racatoeopheryl acetate, 11 mg; ethoxyquin, 150 mg.

³ Mineral premix supplied (per kilogram of diet): manganese (MnSO₄·H₂O), 60 mg; iron (FeSO₄·7H₂O), 30 mg; zinc (ZnO), 50 mg; copper (CuSO₄·5H₂O), 5 mg; iodine (ethylene diamine dihydroiodide), 1.5 mg; selenium (Na₂SeO₃), 0.3mg.

TABLE 5.2. Experimental treatments

Treatment Group	Dietary Regiment	Vaccination ¹ (D 0)	Challenge ² (D 19)
1	Control Corn-SBM ³ (0 to 40 d)	No	No
2	1% Gln Starter ⁴ (0 to 19 d)	No	No
3	1% Gln ⁵ (0 to 40 d)	No	No
4	Corn-SBM	No	Yes
5	1% Gln Starter	No	Yes
6	1% Gln	No	Yes
7	Corn-SBM	Yes	No
8	1% Gln Starter	Yes	No
9	1% Gln	Yes	No
10	Corn-SBM	Yes	Yes
11	1% Gln Starter	Yes	Yes
12	1% Gln	Yes	Yes

¹Birds were vaccinated orally with 500 viable sporulated oocysts of *Eimeria acervulina* and *Eimeria maxima* post hatch (d 0).

²Birds were challenged orally with 250,000 viable sporulated oocysts of *Eimeria acervulina* and 100,000 viable sporulated oocysts of *Eimeria maxima* on d 19 post hatch.

³SBM = soybean meal.

⁴The 1% Gln diet was fed during the starter period (0 to 19 d), after which the corn-SBM diet was fed for the rest of the experimental period (19 to 40 d).

⁵The 1% Gln diet was fed for the entire experimental period (0 to 40 d).

TABLE 5.3. The effect of dietary supplemental glutamine and *Eimeria* vaccination and challenge on broiler performance

Treatment ¹	Days 0-19		Days 19-32		Days 19-32		Days 0-40	
	Weight (g/chick)	Gain (g:kg)	Weight (g/chick)	Gain (g:kg)	Weight (g/chick)	Gain (g:kg)	Weight (g/chick)	Gain:Feed (g:kg)
1. Corn-SBM	548 ^{bc}	649 ^{abcd}	762 ^{bc}	510 ^f	1896 ^d	503 ^{cde}		
2. 1% Gln (Starter) ²	627 ^a	660 ^{abc}	800 ^{ab}	528 ^{ef}	2038 ^b	530 ^{bcd}		
3. 1% Gln	636 ^a	691 ^a	855 ^a	713 ^a	2108 ^a	615 ^a		
4. Corn-SBM+Vac ³	519 ^{cd}	619 ^{cd}	688 ^d	507 ^f	1795 ^e	548 ^b		
5. 1% Gln (Starter)+Vac	534 ^{bcd}	635 ^{bcd}	759 ^{bc}	519 ^f	1911 ^d	518 ^{bcd}		
6. 1% Gln+Vac	543 ^{bcd}	601 ^d	838 ^a	566 ^{cde}	2014 ^{bc}	539 ^{bcd}		
7. Corn-SBM+Challenge ⁴	552 ^b	604 ^d	772 ^{bc}	609 ^{bc}	1655 ^f	473 ^e		
8. 1% Gln (Starter)+Challenge	621 ^a	692 ^a	817 ^{ab}	649 ^b	1770 ^e	521 ^{bcd}		
9. 1% Gln+Challenge	635 ^a	679 ^{ab}	857 ^a	653 ^b	1905 ^d	529 ^{bcd}		
10. Corn-SBM+Vac+Challenge	513 ^d	624 ^{cd}	702 ^{cd}	517 ^f	1751 ^e	502 ^{de}		
11. 1% Gln (Starter)+Vac+Challenge	531 ^{bcd}	630 ^{bcd}	721 ^{cd}	533 ^{def}	1815 ^e	515 ^{bcd}		
12. 1% Gln+Vac+Challenge	545 ^{bc}	638 ^{bcd}	814 ^{ab}	575 ^{cd}	1953 ^{cd}	541 ^{bc}		
Pooled SEM	10.0	20.0	20.0	10.0	20.0	10.0		
Interaction p-values								
Diet	0.0001	0.01	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Vaccination	0.0001	0.0001	0.0001	0.0001	0.15	0.87		
Challenge	----	----	0.02	0.0005	0.0001	0.0002		
Diet*Vaccination	0.0002	0.03	0.32	0.008	0.44	0.0007		
Diet*Challenge	----	----	0.74	0.0001	0.35	0.07		
Vaccination*Challenge	----	----	0.33	0.01	0.0001	0.06		
Diet*Vaccination*Challenge	----	----	0.50	0.0002	0.62	0.0008		

^{a-d}Means within columns having the same superscript do not differ significantly (P < 0.05).

¹Means represent 4 pens per treatment; 30 chicks per pen (120 chicks per treatment).

²The 1% Gln diet was fed during the starter period (0 to 19 d), after which the corn-SBM diet was fed for the rest of the experimental period (19 to 40 d).

³Birds were vaccinated orally with 500 viable sporulated oocysts of *Eimeria acervulina* and *Eimeria maxima* post hatch (d 0).

⁴Birds were challenged orally with 250,000 viable sporulated oocysts of *Eimeria acervulina* and 100,000 viable sporulated oocysts of *Eimeria maxima* on d 19 post hatch.

TABLE 5.4. The effect of dietary supplemental glutamine and *Eimeria* vaccination and challenge on Day 40 carcass yields (%) of broilers

Treatment ¹	Carcass ⁵				Wings ⁷				Front Half ⁸		Back Half ⁹	
	Breast ⁶	%			%			%		%		%
1. Corn-SBM	72.2 ^{abcd}	21.4 ^{bcd}	11.5 ^{cd}	33.0 ^{de}	43.6 ^c							
2. 1% Gln (Starter) ²	72.6 ^{ab}	22.3 ^{ab}	11.5 ^{bcd}	33.8 ^{abcd}	44.1 ^{bc}							
3. 1% Gln	72.8 ^a	23.0 ^a	11.7 ^{abcd}	34.7 ^a	44.6 ^{bc}							
4. Corn-SBM+Vac ³	71.1 ^{bcd}	21.3 ^{cd}	11.5 ^{bcd}	32.8 ^{de}	44.4 ^{bc}							
5. 1% Gln (Starter)+Vac	71.8 ^{abcd}	21.4 ^{bcd}	11.6 ^{bcd}	33.0 ^{cde}	44.4 ^{bc}							
6. 1% Gln+Vac	73.2 ^a	22.8 ^a	11.6 ^{bcd}	34.4 ^{ab}	45.0 ^{ab}							
7. Corn-SBM+Challenge ⁴	70.8 ^d	20.6 ^d	12.1 ^a	32.7 ^e	44.3 ^{bc}							
8. 1% Gln (Starter)+Challenge	72.4 ^{abc}	20.7 ^d	12.0 ^{ab}	32.6 ^e	45.8 ^a							
9. 1% Gln+Challenge	72.7 ^{ab}	21.4 ^{bcd}	11.9 ^{abc}	33.3 ^{cde}	44.8 ^b							
10. Corn-SBM+Vac+Challenge	70.8 ^d	21.1 ^{cd}	11.4 ^d	32.5 ^e	44.0 ^{bc}							
11. 1% Gln (Starter)+Vac+Challenge	70.9 ^{cd}	21.7 ^{bc}	11.9 ^{abcd}	33.5 ^{bcd}	44.1 ^{bc}							
12. 1% Gln+Vac+Challenge	71.8 ^{abcd}	22.1 ^{abc}	12.0 ^{ab}	34.1 ^{abc}	44.4 ^{bc}							
Pooled SEM	0.49	0.30	0.15	0.35	0.32							
Interaction p-values												
Diet	0.001	0.02	0.16	0.0001	0.0001							
Vaccination	0.03	0.48	0.12	0.85	0.0001							
Challenge	0.01	0.24	0.001	0.02	0.0001							
Diet*Vaccination	0.42	0.11	0.28	0.74	0.05							
Diet*Challenge	0.85	0.16	0.86	0.41	0.0001							
Vaccination*Challenge	0.53	0.002	0.11	0.03	0.0001							
Diet*Vaccination*Challenge	0.22	0.39	0.07	0.18	0.05							

^{a-e}Means within columns having the same superscript do not differ significantly ($P < 0.05$).

¹Means represent 4 pens per treatment; 10 chicks per pen (40 chicks per treatment).

²The 1% Gln diet was fed during the starter period (0 to 19 d), after which the corn-SBM diet was fed for the rest of the experimental period (19 to 40 d).

³Birds were vaccinated orally with 500 viable sporulated oocysts of *Eimeria acervulina* and *Eimeria maxima* post hatch (d 0).

⁴Birds were challenged orally with 250,000 viable sporulated oocysts of *Eimeria acervulina* and 100,000 viable sporulated oocysts of *Eimeria maxima* on d 19 post hatch.

⁵Carcass Yield= Chilled Carcass wt./Live wt.*100.

⁶Breast Yield= Breast wt./Chilled Carcass wt.*100.

⁷Wing Yield= Wing wt./Chilled Carcass wt.*100.

⁸Front Half Yield= Front Half wt. (all white meat plus bones)/Chilled Carcass wt.*100.

⁹Back Half Yield= Back Half wt. (thighs and drums)/Chilled Carcass wt.*100.

TABLE 5.5. The effect of dietary supplemental glutamine and *Eimeria* vaccination and challenge on Day 25 leison scores¹

Treatment ²	<i>E. acervulina</i> (duodenal score)	<i>E. maxima</i> (jejunal score)	Birds positive for necrotic enteritis / Total birds tested
1. Corn-SBM	0 ^a	0 ^a	0 / 20
2. 1% Gln (Starter) ³	0 ^a	0 ^a	0 / 20
3. 1% Gln	0 ^a	0 ^a	0 / 20
4. Corn-SBM+Vac ⁴	0 ^a	0 ^a	0 / 20
5. 1% Gln (Starter)+Vac	0 ^a	0 ^a	0 / 20
6. 1% Gln+Vac	0 ^a	0 ^a	0 / 20
7. Corn-SBM+Challenge ⁵	2.7 ^f	3.15 ^f	4 / 20
8. 1% Gln (Starter)+Challenge	2.2 ^e	2.3 ^e	2 / 20
9. 1% Gln+Challenge	1.55 ^d	1.95 ^d	0 / 20
10. Corn-SBM+Vac+Challenge	0.5 ^c	0.6 ^c	1 / 20
11. 1% Gln (Starter)+Vac+Challenge	0.3 ^{bc}	0.3 ^b	0 / 20
12. 1% Gln+Vac+Challenge	0.05 ^a	0.1 ^{ab}	0 / 20
Pooled SEM	0.06	0.05	
Interaction p-values			
Diet	0.0001	0.0001	
Vaccination	0.0001	0.0001	
Challenge	0.0001	0.0001	
Diet*Vaccination	0.05	0.009	
Diet*Challenge	0.0001	0.0001	
Vaccination*Challenge	0.0001	0.0001	
Diet*Vaccination*Challenge	0.05	0.009	

^{a-f}Means within columns having the same superscript do not differ significantly (P < 0.05).

¹Leison were scored according to the method described by Johnson and Reid (1970) on a 4 point system with 0 being no *Eimeria* infection and 4 being the most severe.

²Means represent 4 pens per treatment; 5 chicks per pen (20 chicks per treatment).

³The 1% Gln diet was fed during the starter period (0 to 19 d), after which the corn-SBM diet was fed for the rest of the experimental period (19 to 40 d).

⁴Birds were vaccinated orally with 500 viable sporulated oocysts of *Eimeria acervulina* and *Eimeria maxima* after hatch (d 0).

⁵Birds were challenged orally with 250,000 viable sporulated oocysts of *Eimeria acervulina* and 100,000 viable sporulated oocysts of *Eimeria maxima* on d 19 after hatch.

TABLE 5.6. The effect of dietary supplemental glutamine and *Eimeria* vaccination and challenge on villous height and immune responses

Treatment ¹	Duodenal Villous Height	Intestinal		Serum		Intestinal	
		IgA Concentration	IgA Concentration	IgA Concentration	IFN- γ Concentration	IFN- γ Concentration	IFN- γ Concentration
			<u>DAY 25</u>				
1. Corn-SBM	287.0 ^f	1.898 ^c	1.324 ^e	1.210 ^c	1.118 ^c		
2. 1% Gln (Starter) ²	438.0 ^{de}	2.083 ^c	1.965 ^d	1.847 ^b	1.729 ^b		
3. 1% Gln	605.7 ^{bc}	2.235 ^b	2.262 ^c	1.973 ^{ab}	1.981 ^{ab}		
4. Corn-SBM+Vac ³	378.0 ^e	1.619 ^d	1.444 ^e	1.020 ^c	1.314 ^c		
5. 1% Gln (Starter) ² +Vac	553.5 ^c	1.919 ^c	2.099 ^d	1.763 ^b	1.737 ^b		
6. 1% Gln+Vac	578.8 ^c	2.205 ^b	2.410 ^b	1.985 ^{ab}	2.097 ^{ab}		
7. Corn-SBM+Challenge ⁴	361.3 ^e	1.922 ^c	1.933 ^d	1.329 ^c	1.452 ^c		
8. 1% Gln (Starter)+Challenge	486.5 ^d	2.271 ^b	2.445 ^b	1.803 ^b	1.945 ^{ab}		
9. 1% Gln+Challenge	637.8 ^b	2.650 ^a	2.624 ^a	2.410 ^a	2.360 ^a		
10. Corn-SBM+Vac+Challenge	463.4 ^d	1.632 ^d	1.916 ^d	1.346 ^c	1.641 ^{bc}		
11. 1% Gln (Starter)+Vac+Challenge	561.6 ^c	1.871 ^c	2.145 ^c	1.764 ^b	2.256 ^a		
12. 1% Gln+Vac+Challenge	755.9 ^a	2.277 ^b	2.639 ^a	2.433 ^a	2.617 ^a		
Pooled SEM	17.14	0.05	0.20	0.06	0.11		
			<u>DAY 40</u>				
1. Corn-SBM	572.4 ^d	1.716 ^c	1.626 ^d	1.104 ^c	1.048 ^c		
2. 1% Gln (Starter) ²	669.2 ^c	1.984 ^b	1.855 ^c	1.748 ^b	1.629 ^b		
3. 1% Gln	807.3 ^{ab}	2.317 ^a	2.311 ^b	1.927 ^{ab}	1.811 ^{ab}		
4. Corn-SBM+Vac ³	513.7 ^d	1.451 ^e	1.285 ^e	1.092 ^c	1.149 ^c		
5. 1% Gln (Starter) ² +Vac	602.9 ^{cd}	1.809 ^{bc}	1.897 ^c	1.643 ^b	1.637 ^b		
6. 1% Gln+Vac	774.5 ^b	2.082 ^{ab}	2.099 ^{bc}	1.885 ^{ab}	1.979 ^{ab}		
7. Corn-SBM+Challenge ⁴	481.1 ^e	1.039 ^f	1.301 ^e	1.239 ^c	1.324 ^c		
8. 1% Gln (Starter)+Challenge	592.8 ^d	1.618 ^d	1.899 ^c	1.731 ^b	1.874 ^{ab}		
9. 1% Gln+Challenge	686.3 ^c	1.833 ^b	2.839 ^a	2.140 ^a	2.169 ^a		
10. Corn-SBM+Vac+Challenge	626.5 ^{cd}	1.626 ^d	1.236 ^e	1.436 ^c	1.547 ^{bc}		
11. 1% Gln (Starter)+Vac+Challenge	738.6 ^{bc}	1.855 ^b	1.276 ^e	1.647 ^b	2.156 ^a		
12. 1% Gln+Vac+Challenge	898.4 ^a	2.311 ^a	2.171 ^{bc}	2.314 ^a	2.417 ^a		
Pooled SEM	25.47	0.05	0.23	0.04	0.08		

^{a-f}Means within columns and age period having the same superscript do not differ significantly ($P < 0.05$).

¹Means represent 4 pens per treatment; 3 chicks per pen (12 chicks per treatment).

²The 1% Gln diet was fed during the starter period (0 to 19 d), after which the corn-SBM diet was fed for the rest of the experimental period (19 to 40 d).

³Birds were vaccinated orally with 500 viable sporulated oocysts of *Eimeria acervulina* and *Eimeria maxima* post hatch (d 0).

⁴Birds were challenged orally with 250,000 viable sporulated oocysts of *Eimeria acervulina* and 100,000 viable sporulated oocysts of *Eimeria maxima* on d 19 post hatch.

CHAPTER 6

CONCLUSION

Supplementing glutamine in broiler diets may be used as a stimulant for the development of the gastrointestinal tract and immune system and as a means to control enteric diseases, such as Coccidia. Some protective effects that have been demonstrated with glutamine supplementation in humans and rats include improved gut barrier function, increases in epithelial cell proliferation, proinflammatory cytokine expression, enhanced ability to mount an immune response, improved immune cell function, and decreased mortality. Four experiments were conducted to evaluate the use of supplemental glutamine in broiler diets and its effect on growth performance, development of the gastrointestinal tract, and immune response of broiler chicks.

Experiments 1 and 2 were conducted to evaluate the effects of supplemental glutamine on growth performance, development of the gastrointestinal tract, and humoral immune response of chicks. Growth performance, relative intestinal weights, villi height, lymphoid organ weights, and IgA and IgG concentrations increased significantly when 1% glutamine was supplemented in a broiler chicken diet. Based on the depressed body weight gains and IgG titers, 4% glutamine in the water, feed, or both may have been toxic. Dietary supplementation of glutamine was determined to be the best route of administration due to glutamine precipitating out of the water solutions and low water intake when glutamine was added to the water at either the 1 or 4% level. Supplementing the diet with glutamine for 21 days (entire length of the studies) was determined to be the best length of time for supplementation since it yielded the optimal growth response and immune trends in the chicks.

A third experiment was conducted to evaluate the effects of supplemental glutamine, glutamic acid, histidine, and asparagine on growth performance, development of the gastrointestinal tract, and immune response of chicks when challenged with sheep red blood cells (SRBC). An improvement in growth performance was observed in the birds supplemented with 1% glutamine from the old source (source used in Experiments 1 and 2). There was no improvement in performance in birds supplemented with glutamine from a new source, which indicates that where the glutamine is manufactured may affect the benefits of glutamine supplementation. A significant improvement in relative intestinal weights, villi height, lymphoid organ weights, IgA and IgG concentrations was observed in the non-challenged and SRBC-challenged chicks fed diets supplemented with 1% glutamine (both sources). The SRBC hemagglutination titers were increased significantly when 1% glutamine (both sources) was supplemented in the diet. The significant improvement in the anti-SRBC titers observed when birds were fed diets supplemented with glutamine may indicate better performance of chicks in response to an enteric disease challenge, such as Coccidia. The depressed body weight gain observed in the birds fed diets supplemented with 2% dietary glutamine suggests that a 2% level of glutamine supplementation may be toxic. The depressed growth and antibody titers in the birds fed diets supplemented with 1% histidine suggests that the addition of 1% histidine (total 1.61% histidine in the diet) to the diet is toxic and causes a decrease in performance. Although there was no benefit to supplementing 1% glutamic acid to the diet on performance, organ weights, or antibody responses, the addition of dietary 1% glutamic acid did improve villi height. There was no benefit in supplementing the diet with 1% asparagine on growth performance, intestinal relative weights and morphological development, lymphoid organ weights, or antibody titers. The supplementation of 1% glutamic acid, histidine, and asparagine to the diet did not

duplicate the benefits observed with glutamine supplementation and the effect appears not to be due to a deficiency in nitrogen.

The increases in anti-SRBC titers observed in birds challenged with SRBC and fed diets supplemented with 1% glutamine in the previous study indicate that during an enteric disease challenge the growth performance of broiler chicks may be improved when the diets are supplemented with 1% glutamine. Therefore, an experiment was conducted to evaluate the effects of supplemental glutamine on growth performance, development of the gastrointestinal tract, and immune response in chicks vaccinated and challenged with *Eimeria*. An improvement in growth performance, intestinal villi height, and IgA, IgG, and IgM concentrations was observed when 1% glutamine (old source) was supplemented in the diet for the entire experimental period (0 to 40 days) regardless of *Eimeria* vaccination or challenge. Glutamine supplementation in the diet eliminated the weight depression viewed with a vaccination and coccidial challenge. Glutamine supplementation in the diet has been shown to improve gut barrier function by protecting the gut against bacteria that triggers an inflammatory response, thereby impairing nutrient absorption which leads to malabsorption of nutrients and bacterial translocation that causes infection. The addition of dietary glutamine may have better protected the gut against the coccidial infection by not allowing inflammation of the gut wall and increasing nutrient utilization thereby enabling the birds to compensate for the challenge or vaccination thus a decrease in performance did not occur.

The results of these studies indicate that the use of dietary supplemental glutamine could be an alternative to the use of antibiotics along with other additives in poultry feeds due to the immunomodulatory effects of glutamine. Glutamine's role in the maintenance of the immune system and the high antibody responses of chicks to SRBC when chicks are fed diets

supplemented with glutamine, suggest that glutamine may be beneficial during vaccination or enteric disease challenges, such as Coccidia. Broiler chicks fed diets supplemented with 1% glutamine had enhanced development of the gastrointestinal tract and immune responses. Improvement in growth performance was observed when 1% glutamine (old source) was supplemented in the diet. However, the supplementation of glutamine's effect on growth performance appears to be source dependent.